# REVIEWS OF VETERINARY RESEARCH-WHAT NEXT?

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# PREFACE

We are extremely glad to present the book "Reviews of veterinary research-what next?" to our professional community of researchers across the world. It is our humble effort to throw some light on what happens in veterinary research.

Research is a critical component in advancement of our scientific knowledge and it is never ending. Research always builds upon future needs and with the strong foundation from past achievements. Veterinary research, most of the time, doesn't limit itself to veterinary medicine, care, treatment or conservation but transcends species boundaries. For example, study about wildlife diseases may be beneficial to conservation in the first hand, but will be helpful to figure out the genesis of zoonotic diseases and even pandemics that surfaces unexpectedly. In this context, at the end of the day, it even contributes the human wellbeing.

We have to set priorities for research on a need based expansion of our knowledge to tackle issues in a scientific way. There are so many questions a researcher needs to answer before planning a research study. What was our past? Till where we reached? How far we have to go? What we have with us? What is the scope? And so on. To get a clear picture of this scenario, we have to review the past research. Such reviews will become the directions to future.

In this book, we bring some meticulously done reviews about various emerging topics in veterinary field with universal relevance. The young researchers who contributed to this book keep these as base for their research and we can expect better outcome in the near future. This book intended not only to spread knowledge, but aims to develop a scientific temper, promote critical thinking and to help write in a scientific way. As editors, we were critical in our job, but the researchers' good intention, freedom of collection of information and expression in their own writing style in these reviews remains untouched. We

blindly believe the contributors for the originality of their effort with a friendly gesture and hence, we editors, wish to inform that, any opinions made in these chapters are the sole responsibility of the contributors.

We express sincere gratitude to our contributors, their mentors, guides and advisors for allowing the publication of their hard work. We extend our gratitude to the respective departments, institutions and universities who provide the best platform for these future researchers.

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# CONTENTS

# ADVANCEMENTS IN IMAGING FOR CUTANEOUS WOUND EVALUATION

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***ABSTRACT-***

*Skin is the largest multi-layered external defence system that protects the body from pathogenic invasion. A cutaneous wound means disruption in the continuity of skin. Wound assessment is the key in the care of patients with wounds, allowing us to reach an accurate diagnosis, raise the short-and long-term goals, and determine the appropriate interventions at each stage. A complete wound assessment must include the wound morphometry, attributes of the wound like duration, blood flow, infection, oedema, inflammation, host factors and environmental factors that impact on optimum wound management. It is essential that the measurement tool used is highly accurate and repeatable. Digital imaging and software (Digital planimetry) with smart phones integrating digital camera and software applications are emerging as inexpensive, easy-to-use, reliable and accurate tools for wound measurements. Optical features of skin components can be non-invasively assessed for estimating the severity of wounds, the healing potential and the healing rate. Hyper spectral imaging*

*is the imaging of Visual (VIS) and Near Infrared (NIR) spectrum which detects perfusion parameters of tissue and wounds which are useful to detect and monitor local deterioration of perfusion and oxygenation and their kinetics, thus helpful to predict the healing. Conventional method to detection of wound infection is by microbial swabbing but, it is a time consuming process and by that time, the bacterial bio-burden in the wound may develop beyond control. Detection of pathogenic auto fluorescence by a point-of-care handheld device is the latest entry in this regard which helps the real time detection and identification of bacterial bio burden in the wound and its severity. Infrared Thermography remotely measures skin temperature which is considered one of the most reliable indicators of cutaneous perfusion and inflammation. These emerging non-invasive point-of-care technologies are a quantum leap in diagnosis and treatment of cutaneous wounds.*

***Keywords**: Wound, Morphometry, Digital planimetry*

## 1. INTRODUCTION

The skin or integument is one of the largest body organs and provides a complex and important boundary between the animal and its environment. A skin wound involves injury to skin and disruption in its continuity. Wound assessment is the key in the wound care, allowing reaching an accurate diagnosis, raising short and long-term goals, and determining the appropriate interventions at each stage. Measurement of wound or wound morphometry will provide baseline information. The techniques can be contact or non-contact. Contact technique has the risk of contaminating wound bed, damaging tissue, or inflicting pain during measurement. Measurement of surface area using digital imaging and software (Digital planimetry) is preferred nowadays as the non-contact, accurate and reliable way to measure and document wound. As the prevalence of chronic wounds continues to rise, the need for point of care wound assessment has also increased. Smart phones integrating digital camera and software applications are emerging as inexpensive, easy-to-use, reliable and accurate tools. Optical features of skin components can be non-invasively assessed for estimating the severity of

wounds, the healing potential and the healing rate with accuracy and speed using imaging technologies. This imaging helps in evaluating the status of wound in terms of blood flow, collagen remodelling, hemoglobin content, inflammation, temperature, vascular structure and water content etc., when there is no clinical manifestation or the condition is undetected by the conventional methods of investigations. Point-of-care imaging using hyper spectral imaging is helpful in imaging the perfusion status to detect and monitor local deterioration of perfusion and oxygenation. Detection of infrared emission from skin and wounds using special thermal imaging cameras are useful in detecting minute thermal changes in a wound thus helpful in predicting the hot and cold spots in wound. This is helpful in detecting the perfusion and inflammation which is aiding to assess the healing process. Real-time auto fluorescence detector helps in detecting the bacterial bio burden in a wound and even identifying the bacteria by the fluorescence methods. It helps to eliminate the time required for detection of infection by microbial swabbing and helps to clinician to start treatment early. The other use of this Real-time auto fluorescence detector is in deciding the margin of mechanical debridement, helpful in image guided swabbing, deciding the good time to apply skin graft in chronic wounds etc. The post COVID-19 world is supposed to be a new normal world where social distancing will be the most followed culture. These point-of-care devices are helpful in avoiding frequent hospital visit and promote telemedicine for evaluation of healing process.

## 2. Anatomy of skin

The skin or integument is one of the largest body organs and provides a complex and important boundary between the animal and its environment. It serves as the outer barrier of the organism. Skin offers the largest multi-layered external defence system that protects the body from pathogenic invasion. The skin may reflect the state of health of the animal as well as indicate cutaneous manifestations of internal disease, such as icterus, cyanosis, and oedema.

The skin consists of an outermost non-vascularized epidermis and the more deeply situated dermis or corium. The skin is underlaid by a subcutis (*tela subcutanea* or hypodermis), which is not part of the skin. The subcutis functions as a moveable support for the skin allowing it to glide over underlying tissues. It connects the dermis with the fascia and the various forms of hair (pili) that compose the coat.

Epidermis is composed of four layer or strata including *stratum basale, stratum spinosum, stratum granulosum* and *stratum corneum.* The surface of the epidermis is rough in area where hairs emerge obliquely. The protective function of skin is largely a result of keratinization and this process involves epidermal cell differentiation.

Dermis derives from the mesoderm, consists primary of a meshwork of fibrils embedded in a gel like matrix (ground substance) composed mainly of proteoglycans such as hyaluronic acid, dermatin sulphate, chondroitin-4-sulphate and chondroitin-6-sulphate. The dermis makes up the greatest portion of the integument and is responsible for most of the structural strength of the skin. Hair follicles, nerve endings, glands, smooth muscle, blood vessels, and lymphatic channels are all found in the dermis as well.

## 3. Wound and its classification

A skin wound involves injury to skin and disruption in its continuity. Since intact skin is of vital importance to protect the organism against environment, regenerative mechanisms must be activated to resolve a defect. Boateng *et al.* (2008) classified wounds based on number of skin layers and area of skin affected as superficial wounds (that affects the epidermal skin surface alone), partial thickness wounds (injury involving both the epidermis and the deeper dermal layers, including the blood vessels, sweat glands and hair follicles) and full thickness wounds (underlying subcutaneous fat or deeper tissues are damaged in addition to the epidermis and dermal layer). Pavletic (2010) reported that, from the information gathered from the history and physical examination, a wound can be classified into

one of four basic categories according to its condition. These four categories, in their increasing order of severity, are clean, clean-contaminated, contaminated and dirty & infected. The classification of wounds according to potential risk of infection as class I or clean, class II or clean-contaminated, class III or contaminated and class IV or dirty/infected. Murawala *et al.* (2012) opined that every animal in existence has evolved mechanisms of repair with the goal to restore tissue homeostasis and architecture after insult. Ultimately, this repair should recapitulate the original in both form and function.

### 3.1. Wound examination

Lazarus *et al.* (1994) suggested that complete wound assessment must include the extent of the wound (non-invasive assessment like perimeter, maximum dimensions of length and width, surface area, volume, amount of undermining, and determination of tissue viability and Invasive methods to quantify the extent of a wound), associated attributes of the wound (duration, blood flow, oxygen, infection, oedema, inflammation, repetitive trauma and/or insult, innervation, wound metabolism, nutrition, prior wound manipulation, and systemic factors), host factors that influence wound status (wound burden and wound severity), and environmental factors (demographics, systemic agents that affect wound repair, and systemic disorders) that impact on optimum wound management. Lozano-Platonoff *et al.* (2015) opined that wound assessment is the key in the care of patients with wounds, allowing us to reach an accurate diagnosis, raise the short-and long-term goals, and determine the appropriate interventions at each stage. This assessment should always include the wound topography (location), morphology (shape and characteristics both of the wound bed and the edges which means size, shape, bed, and edge of the wound, as well as the skin around it), exudate characteristics (amount, colour, consistency, and odour), and signs of infection (superficial or deep infections). Wound measurement provides baseline information while continuous measurement helps to predict healing and aids monitoring of treatment efficacy and evaluation. One of the problems in

performing clinical trials on wound healing is the lack of objective evaluation methods. The evaluation method should be adapted to the wound type and the wound healing phase (debridement phase, granulation tissue formation, epithelialization phase, or remodelling phase).

### 3.2. Wound measurement

Paul *et al*. (2015) opined that, the ability to phenotype wounds for the purposes of assessing severity, healing potential and treatment is an important function of evidence-based medicine.Wound measurement is the only evidence-based predictor of healing. Many works in wound area measurements in human patients proved that planimetric wound measurements, focussed on wound area, were a predictor of healing or non-healing. Wound's boundary is determined by the subjective assessment of the human observer who performs the measurements and decides whether or not a particular part of the area in question belongs to the wound. The available common methods used for obtaining the wound measurements like length, breadth, perimeter and area are ruler method, Kundin device, acetate tracing and planimetry mainly. Even though inexpensive and easy, ruler methods lack precision, inaccuracy while representing area of wound having an irregular shape, overestimation of area during linear measurements (Khoo and Jansen, 2016) and kundin device needs additional assumptions and coefficients for accuracy (Nemeth *et al.,* 2010). These overestimations can be upto 40 per cent. All these methods are contact methods. To combat the inaccuracy of ruler-based measurements, other methods have been developed to measure wound surface area, namely manual planimetry using acetate film and digital planimetry using digital photography. Acetate tracing is the simplest and gold standard which gives precision in measurement, but that is also a contact method. Pereira *et al.* (2019) opined that, for wound inspection, touching the wound area can be useful, but should be avoided since it can cause pain for the animal and may be un-hygienic. Wendland and Taylor (2017) opined that every contact technique of wound measurement had the risk of contaminating the wound bed,

damaging the tissue, or inflicting pain because of the direct interaction with the wound during measurement. Contact wound techniques carry the risk of contamination and wound disturbance. Mukherjee *et al.* (2017) reported that researchers across the world were looking forward to non-touching and non-invasive wound monitoring techniques based on imaging methods.

It is essential that the measurement tool used is highly accurate and repeatable. Van Poucke *et al.* (2010) opined that, before a tool can be applied in clinical practice, a validation process that assesses the reliability and repeatability of its use by different health care professionals should be performed and in wound assessment, specification of the region of interest (wound bed) should also be defined.

Use of electronic devices was superior to manual techniques to get valid wound area measurements. Mayrovitz and Soontupe (2009), in their study, suggested that computerized planimetry of digitized wound photographs using software is an accurate and reliable way to measure and document wound areas and an associated wound closure parameter. Khoo and Jansen (2016) demonstrated the precision and reliability of digital planimetry over the more conventional methods of ruler measurements and acetate tracings by reviewing the literature in English published between 2000 and 2014.

### 3.3.1 Wound imaging for measurement

Salcido (2011) opined that wound care is a ‘‘visual specialty,’’ which not only requiring a qualitative description of the area of interest, but ample use of photographic techniques to supplement the medical record. Photogrammetry is the science of obtaining reliable information about the properties of surfaces and objects without physical contact with the objects, and of measuring and interpreting this information (Schenk, 2005). Sprigle *et al.* (2012) reported that advanced wound measurement technologies can be vision-based technologies or software-based systems. Vision-based technologies utilize either

stereophotogrammetry (SPG) or structured lighting to obtain wound images. In the contrary, even though software-based systems use digital photographs of a wound to measure its area, the clinician has to place a target on the body to provide the computer with a scale upon which area can be calculated. These digital images are loaded into the software and the clinician traces the border to obtain the area. These digital images are ideal for electronic transmission and can provide reliable monitoring of wounds over time.

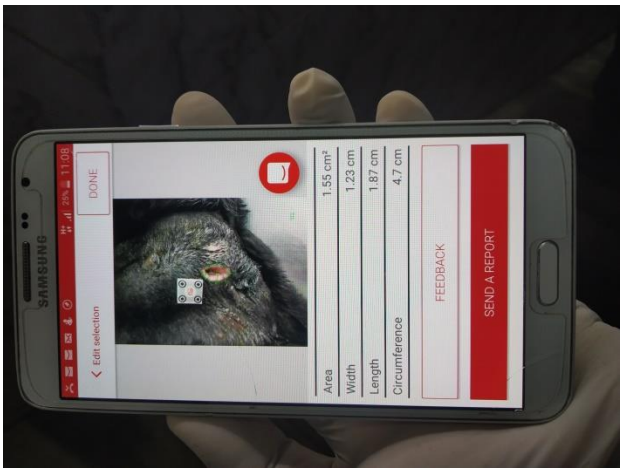
### 3.3.2. Point-of-care wound imaging for measurement

The transformation in the use of handheld communication devices like mobile phones from a mere communication device to a multi-use handy system happened and is still happening in an amazing speed, thanks to technology. With high-definition integrated cameras, user-friendly applications, fast processors, embedded software and high-speed internet availability mobile phones are no more just phones, but a handy, point-of-care assistants in medical field. Pires and Garcia (2015) reported that the calculation of the wounded area using mobile phone application consists of three phases, these are: image acquisition, image processing, surface reconstruction and calculations. Many workers decided to take advantage of advances in mobile technology by developing applications that is an inexpensive, easy-to-use, reliable and accurate tool for wound measurement. There are smartphone applications that enabled non-contact wound surface area and temperature measurements. In the recent years, Khong *et al.* (2017) reported that many smart phone apps (both android and iOS) have been designed to support wound care. Dastjerdi *et al.* (2019) reported that there are commercial applications for wound assessment using a single photo such as “imito” (http://imito.io) and “Lesion Meter” (http://lesionmeter.com) based on a specific scale descriptor or calibrated marker which has to be placed near the wound (Figure 1). These methods required manual delineation of the wound. Richli (2020) reported that “ImitoMeasure” app calculates the exact surface of the polygon using digital planimetry. Measurement quality depends on errors such as

measuring environment like distance to calibrated marker or small tilt of the marker that makes it not exactly parallel to the picture, inter-operator variations when tracing the wound boundaries on screen and systematic errors like device camera optics distortion.

**Figure 1**

**Figure 2**



### 3.3.3. Wound imaging for diagnosis

Basically, imaging is the visual representation of an object or its part or its form. Images can be the visual documents of event of that particular moment. The biggest advantage of imaging is that it is non-invasive and less time consuming. Optical features of skin components can be non-invasively assessed for estimating the severity of wounds, the healing potential and the healing rate with accuracy and speed using imaging technologies. This sort of "seeing the unseen" methods are getting popular in imaging for diagnosis rather than imaging for visualization or documentation. The imaging for diagnosis depends upon the optical technologies that have potential to supplement traditional clinical wound evaluation and research, by providing detailed information regarding skin components imperceptible to visual inspection (Paul *et al*., 2015). This imaging which sees the unseen helps in evaluating the status of wound in terms of blood flow, collagen remodeling, hemoglobin content, inflammation, temperature, vascular structure and water content etc., when there is no clinical manifestation or the condition is undetected by the conventional methods of investigations. This early diagnosis is of greater clinical importance as the medical or surgical intervention will be more and more difficult and prognosis will be poor as time and disease process advances. Some of the technologies using for wound assessment are Near Infrared (NIR) imaging, Laser Doppler Imaging (LDI), Hyper Spectral Imaging (HSI), Fluorescence Imaging and Thermal Imaging. Technologies like optical coherence tomography, orthogonal polarization spectral imaging, microscopy, spatial frequency domain imaging, photo-acoustic detection are also there.

### 3.3.4. Point-of-care wound imaging for diagnosis

International consensus document on wound management, introduced concept called TIME (Tissue viability, Infection/Inflammation, Moisture balance, Edge of wound) frame work. The consensus panel later recommended updating TIME to recognise the factors with the integration of repair/

regeneration (R) and social factors (S). The new framework provided structured guidance on approaches to managing wound parameters and it identified where advanced adjunctive therapies should be considered alongside standard care. TIME becomes TIMERS. These sort of advancements warranted more patient-centric approach to wound management. There are limit less opportunities to expand on the role of remote sensing technology in patient-centred care.

An infection is defined as the overwhelming multiplication of bacteria, which bombards the host's defences and invades the surrounding tissues, thus impairing wound healing (Blumenthal and Jeffery, 2017). Traditionally, detection of wound infection is done in two ways. One is based on clinical symptoms and by visual inspection under white light. Other one is the microbial swabbing for culture to identify the pathogen. Diagnosing microbial infection based on traditional clinical signs and symptoms in wounds of asymptomatic patients is especially challenging at the bedside. Bacteria are invisible to the unaided eye. Subsurface bacterial burden can be missed during standard wound examination protocols and this can be influenced by the level of clinician experience, possibly contributing to wound chronicity, if left unmanaged (Wu *et al.*, 2015). Microbiologic swabbing is the most common method for bacteria detection and identification. It helps to identify the organism and thus to decide the appropriate antibiotic treatment. But, it is a time consuming process which takes 2 to 5 days and by that time, the bacterial bio-burden in the wound may become more and even to an extend which affect the quality of outcome of treatment. The urgent need to eliminate unnecessary use of antibiotics in wounds has been hampered by diagnostic uncertainty and the time required for obtaining culture results.

#### 3.3.4.1. Hyperspectral imaging

Hyper spectral imaging is the imaging of Visual (VIS) and Near Infrared (NIR) spectrum. The perfusion parameters of tissue and wounds can be detected with high spectral and spatial resolution. A spectrometer and scanning unit, integrated into the

camera, acquires the hyperspectral data of the object, tissue or sample. Integrated pushbroom-imaging-spectrometer helps in separation of detected light into spectral bands from 76 500–1000 nm, which are finally integrated on a camera sensor probe (Daeschelin *et al.*, 2017). The data is transferred to analysing software where it is processed visually for the user by creation of pseudo-color images (Figure 3) The parameters provided by hyper spectral imaging are tissue hemoglobin oxygen saturation ($StO_2$), tissue hemoglobin index (THI), near infrared perfusion (NIR) and tissue water index (TWI). These parameters are useful to detect and monitor local deterioration of perfusion and oxygenation and their kinetics especially in wounds, thus helpful to predict the healing (Daeschelin *et al.*, 2017). With the easy to use compact camera system (Figure 4), hyperspectral measurements of patients in the normal clinical environment are possible and in comparison to the laser Doppler (or laser-speckle) imaging the determination of hemoglobin oxygenation is an important advantage (Kulcke *et al.*, 2018).

**Figure 3**


(www.diaspective-vision.com)

**Figure 4**

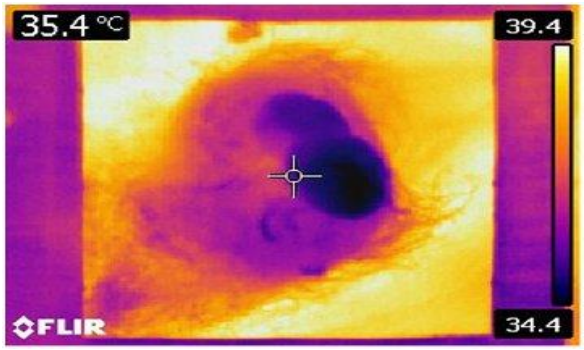
(www.diaspective-vision.com)

### 3.3.4.2. Infrared thermography (IRT)

All objects, including skin, emit infrared radiation (IR). Infrared radiation emission can be measured using infrared detectors and has been found to correlate with temperature, as a product of emissivity. Infrared Thermography works on the principle that, all objects with a temperature above absolute zero emits radiation and recording of that makes it possible to view one's environment with or without illumination. This allows thermographic measurements from skin to be analyzed and processed to create color-coded images express the relative skin

temperature over a defined area. Measuring skin temperature is considered one of the most reliable indicators of cutaneous perfusion, and evidence suggests that infrared thermographic monitoring may be an effective method of predicting tissue viability complication (Clockie, 2017). It is non-contact in nature and can be used for imaging from a distance. Amount of infrared energy emitted, transmitted, and reflected by an object is processed with dedicated software to visually represent by images called Thermograms. Special cameras called thermal cameras use sensing device consisting of an array (typically rectangular) of light-sensing pixels (that respond to mid and long wavelength infrared) at the focal plane of a lens called Focal Plane Array (FPA). Modern computerized thermography produces an accurate and reproducible high-resolution image that can be analysed both qualitatively and quantitatively for minute changes in skin surface heat emissions. Diagnostic Infrared Thermography helps to detect temperature associated with inflammatory process in wounds (Figure 5) and identify the hot and cold spots in a wound indicating the perfusion and inflammation. These parameters are helpful in assessing a wound which appears to be normal in normal visualization. Infrared imaging is designed to be used in addition to other tests to provide physiological information that cannot be obtained from any other examination procedure. Smartphone integrated thermal cameras and applications are already in the market and gaining popularity (Figure 6).

**Figure 5**




(Van der Saag *et al.*, 2018)

**Figure 6**


(www.flir.com)

### 3.3.4.3. Real-time auto fluorescence detector

Florescence Imaging is detection of fluorescence and imaging it for various purposes. It can be done by illumination of skin by a laser light at specific excitation wavelengths, detection of emission wavelengths and filtration to collect desired wavelength to form images. These sorts of detection systems already proved sensitive and discriminatory. It is widely used in flow cytometry in industrial microbiology, to detect pollutants and microbes in environment, to identify potential pathogenic contaminants like bacteria in foods and to analyse the composition of aerosol to detect aerosolized bacteria in biological warfare (Dartnell *et al.*, 2013).

Fluorescent response of a target is by detailing the fluorescence intensity. It is provided by an Excitation-Emission Matrix (EEM) and generated by recording of emission spectra in response to incremented exciting across a broad range. This is visually represented by two-dimensional map of colour-coded emission intensity (Dartnell *et al.*, 2013).

This fluorescence can be either exogenous fluorescence or endogenous fluorescence. An example of an exogenous process is Indocyanine Green (ICG) Fluorescence Imaging, which requires injecting indocyanine green dye into the systemic circulation (Paul *et al.*, 2015). This helps in visualizing wound perfusion. Indocyanine Green (ICG) is an FDA approved agent which can be injected interstitially, intravenously or applied topically. The major disadvantage with this etchnology is that, it needs injection of this dye, which makes it an invasive technique.

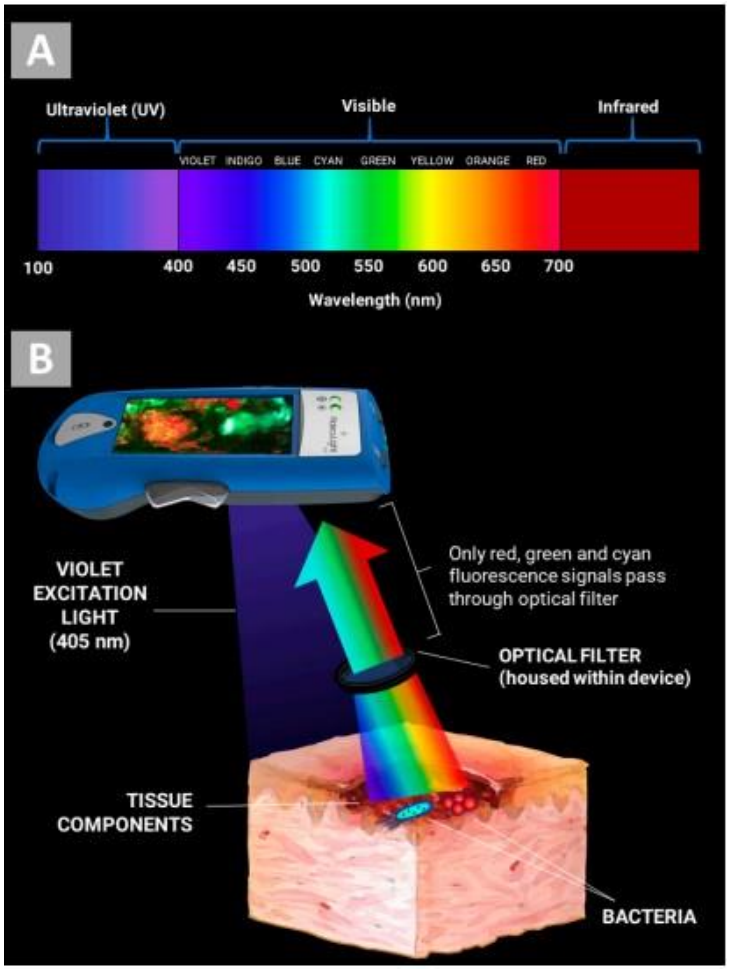
The endogenous fluorescence can be either tissue auto fluorescence or pathologic auto fluorescence. Tissue auto fluorescence is due to endogenous fluorophores. These are fluorescent components native to tissues. Among the, predominant ones are red blood cells and extracellular matrix proteins like collagen, elastin or fibrin. The fluorescence of tissue components are green and yellow and the shades will vary with tissue type according to the relative density of tissues. For example, slough tissue appears bright green due to its high fibrin content. Fluorescence of Haemoglobin (Hb) in blood will appear as dark black or maroon (Paul *et al*, 2015).

In the case of pathologic auto fluorescence, red fluorescence is caused by a by-product of bacterial haem production called porphyrin. These are produced by a vast majority of pathogenic bacteria. Pyoverdines, specifically produced by Pseudomonads, will produce cyan fluorescence. This is mostly due to a common wound pathogen called *Pseudomonas aeruginosa* (Rennie *et al.*, 2019).

It is well known that excitation with ultraviolet or violet light produce fluorescence within the visible spectrum. The point-of-care auto fluorescence detector device emits violet light causing the tissues and bacterial organisms to produce fluorescence. This auto fluorescence is collected, filtered and displayed on the device screen real-time. The images not only display tissue and pathogenic auto fluorescence, but show spatial patterns of bacterial burden, if any (figure 7). The device requires a dark environment for fluorescence imaging. A sensor detects

and a green light indicates the ambient light (sufficient darkness) for image capture. Contamination with ambient light can cause artefacts and misinterpretation (Rennie *et al.*, 2019).

**Figure 7**

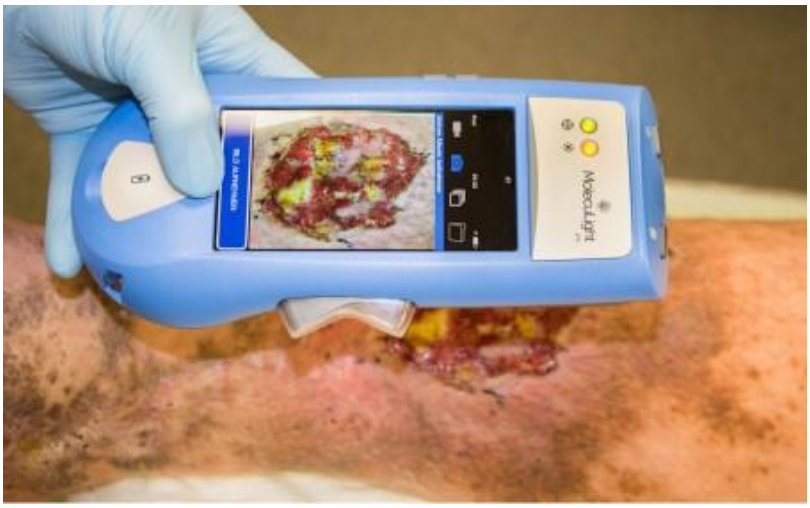



(Rennie *et al.*, 2019)

The auto fluorescence detector device, currently marketed as Moleculex, was helpful to identify the bio-burden of wounds, especially chronic wounds where clinical signs are not so prominent. Otolino-Perry *et al*. (2017) used real-time auto fluorescence to visualise bacteria in and around the wound bed (Figure 8) and to guide swabbing during the clinical assessment. The bacterial load in wounds was sampled more accurately using

autofluorescence image-guided swabbing compared with the Levine technique. The variety of its use includes finding efficacy of mechanical debridement (Moelleken *et al.*, 2020), to determine most appropriate time to apply skin grafts in a chronic wound, to guide the timing of dressing changes in wounds (Raizman, 2019) and many more.

**Figure 8**




(Moelleken *et al.*, 2020)

**Conclusion**

Skin, being the largest organ and the first line defence against environmental insults, poses an important role in animal body. Cutaneous wound management is always a challenge for clinicians as the restoration of a skin defect to its near normalcy at the least possible time without compilation is the primary and most important aim of each and every attempt in this regard. Time is precious in all aspects of healing. Time is precious in wound management. Many ways are there to assess the progression of healing process, to find out inflammation and perfusion and bacterial bio burden. The emerging technologies in imaging are taking this to a new height where point-of-care handheld devices revolutionize the bedside management and assessment which in turn reduces the hospitalization and cost of treatment. Other than improving the quality of diagnosis and

intervention strategies, the non-invasive and non-contact measures are promising in veterinary wound management as it is viewed in terms of animal welfare also.

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# IMMUNOTOXINS IN CANCER THERAPY

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***ABSTRACT-***

*Cancer is one of the major reasons of death in the most countries. Recently, immunotherapy has become one of the best methods of cancer treatment, along with chemotherapy and radiation. "Immunotoxin therapy" is a promising way of cancer therapy in this field. Immunotoxins are proteins that consist of a protein toxin conjugated to a specific targeting moiety. The targeting moiety usually is an antibody or ligand, such as monoclonal antibody, antibody fragment, a cytokine or a growth factor. The toxins usually are plant toxins, bacterial toxins or human-origin cytotoxic elements. Almost all toxins enzymatically inhibit protein synthesis resulting in cell death. To date only two immunotoxins have been approved by U.S. Food and Drug Administration for the treatment of hematologicaltumors: Lumoxiti and Ontak. Many other molecules are either under development or under clinical trials for different forms of cancer. Although immunotoxins exhibit great potency in early clinical trials, there are obstacles that limit successful treatments, including immunogenicity, nonspecific toxicity, and poor penetration. However, efforts are underway to address these*

*problems. With further improvements, it is anticipated that immunotoxins will play an increasing role in cancer therapy.*

***Key words**: Cancer, Immunotherapy, Immunotoxins*

## INTRODUCTION

Cancer is one of the major reasons of death in most countries and is the second leading cause of mortality worldwide. There are several procedures involved in cancer therapy; the most important one being surgery, chemotherapy and radiotherapy. Even though they have provided favourable response in some cases the mortality rate has still remained higher on most cases. Even after removal of solid tumours by surgery, the remaining residual cells may lead to tumour relapse. Treatment by radiotherapy has non-specific effect thereby affecting the normal cells. Although chemotherapy generates good response initially, after a while the cancer cells gain resistant to chemical agents used. These factors led to the development of new strategies for cancer therapy to target the cancer cells specifically.

Monoclonal antibodies (mAbs) are new classes of cancer therapeutics as they can recognize and specifically bind to target cells. Various methods have been tried to increase the efficiency of antibodiesas they are rarely able to completely eliminate the cancer cells. One among them is combining the chemical agents or toxins to antibodies and these are known as immunotoxins (ITs) (Akbari *et al.,* 2017).

Immunotherapyhas rapidly become one of the best methods of cancer treatment, along with chemotherapy and radiation. The first immunotoxin approved by the Food and Drug Administration (FDA) was DAB389IL2. Presently research projects are going on in finding proteins that in combination with immunotoxins have minimal immunogenicity and maximum potency for target cell killing (Allahyari *et al.,* 2017).

### Immunotoxins

Immunotoxins (ITs) are bi-functional chimeric molecules composed of an antibody fragment attached to a toxin

component. They attain their toxic potency from the toxin part and their specificity from the antibody.

The linkage of the antibody to toxin can be accomplished by two general methods, chemical or genetic. Chemical construction of ITs utilizes different reagents to crosslink antibody and toxin. Genetic construction uses hybrid genes to produce antibody-toxin fusion proteins in *Escherichia coli.*

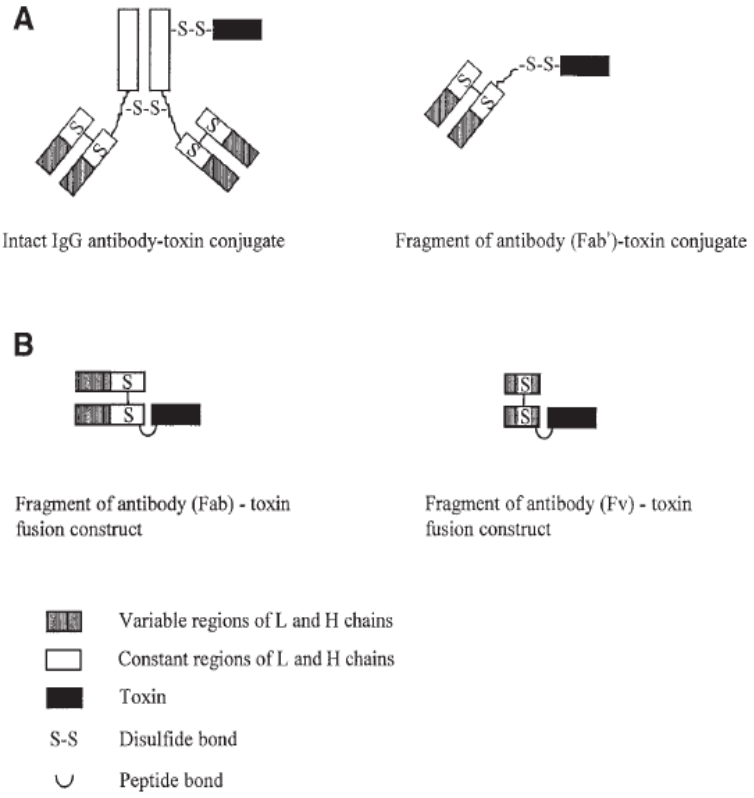


Fig 1. The structure of antibody–toxin constructs obtained by (A) chemical and (B) genetic engineering procedures.

(Ghetie and Vitetta, 2001)

Till date, four different generations of ITs have been generated and tested for cancer therapy (Akbari *et al.,* 2017).

**First generation**: The full length mAbs connected chemically to the natural toxin. Here the antibody portion and toxin portion were prepared separately and chemically conjugated *in vitro* and

were linked by disulphide bond or a non reducible thioether bond. The major drawbacks of first generation ITs was their low specificity to cancer cells, low stability of bonds between toxin moiety and Ab fragment there by releasing the unconjugated mAbs into circulation in turn decreasing the antitumour efficacy, and the larger size of whole Ab and toxin used which hindered their penetrability.

**Second generation**: In order to overcome the drawbacks of first generation ITs the second generation ITs were developed. The cell binding domain of toxin moiety which was of no use was eliminated. This modified toxin was chemically connected to full length mAbmoiety. Similar to first generation ITs the linkage was not stable and had highly immunogenic effect in human patients.

**Third-generation:** Unlike first and second generation where disulphide and thioester bonds were used, here the toxin and Ab were connected by peptide bond by using rDNA technology. Compared to first and second generation the size here was significantly reduced which in turn reduced immunogenicity and increased penetrability.

**Fourth-generation:** The major drawback of IT is its immunogenicity. So, in order to overcome this drawback the human or humanized antibody formats are used for construction of ITs. In the fourth generation ITs, to reduce the immunogenicity of toxic moiety, the human/humanized antibodies are fused to endogenous cytotoxic proteins of human.

**Types of cytotoxic moiety**

Toxins from bacterial, plant and human origin are used for construction of ITs. Bacterial toxins such as Diphtheria toxin (DT) (Potala *et al.,* 2008), Pseudomonas exotoxin A (PE); plant toxins, including ricin, gelonin, saporin, and poke weed antiviral protein; and endogenous proteins of human origin such as Granzymes and RNases are the most common toxins used in construction of ITs (Mathew and Verma, 2009).

**Table 1: Classification of toxins**

| Toxins | Source | Mechanism | Modifications |
|---|---|---|---|
| **ADP ribosylating toxins:** | | | |
| Diphtheria toxin | *Corynebacterium diphtheria* | ADP ribosylation of EF2 | a) DT486<br>b) DT388 or DT389 (deletion of cell-binding domain)<br>c) CRM107 (point mutation in cell-binding domain of DT) |
| Pseudomonas exotoxin | *Pseudomonas aeroginosa* | ADP ribosylation of EF2 | a) PE40 and PE40KDEL<br>b) PE38 and PE38KDEL<br>c) PE38QQR<br>d) PE35 |
| **Pore-forming toxins:** | | | |
| Cholera toxin | *Vibrio cholera* | ADP ribosylation of Gs-a subunit of G protein | CET40 (domains II and III) |
| **Ribosome inactivating toxins:** | | | |
| Holotoxins – ricin | *Ricinus communis* | N-glycosylation of 28S rRNA | a) Ricin<br>b) Ricin chain A (RTA)<br>c) bR (blocked ricin)<br>d) dgA (deglycosylated ricin A-chain) |
| Hemitoxins – saporin (SAP), pokeweed antiviral protein (PAP) | *Saponaria officinalis, Phytolacca americana* | N-glycosylation of 28S Rrna | |
| **Ribonucleases:** | | | |
| Fungal toxins: α-sarcin, restrictocin<br>HPR, ECP, EDN | *Aspergillus* sp.<br><br>Human | Cleavage of 28S rRNA<br>Degradation of RNA | |

**Abbreviations:** DT: diphtheria toxin; CRM107 or cross-reacting material – mutant of DT; PE: Pseudomonas exotoxin A; CET40: cholera exotoxin A; RTA: ricin toxin A; HPR: human pancreatic ribonuclease A; ECP: eosinophilic cationic protein; EDN: eosinophil-derived neurotoxin. (Choudhary *et al.,* 2011)

**Bacterial Toxins**

**Diphtheria toxin (DT):** Diphtheria toxin (DT) is a secretory toxin excreted by the bacterium *Corynebacterium diphtheria.* It kills the target cells by inhibiting protein synthesis via ADP-ribosylation of elongation factor 2 (EF2) (Holmes, 2000). The toxin has 535 amino acids arranged in two fragments known as A and B.The DT before binding to the cell surface receptors gets broken into two fragments. Then the toxin is uptaken into the target cells by endosomal vesicle via clathrin-coated pathway. The acidic conditions of endosomal vesicles lead to dissociation of DT fragments via reducing of disulfide bond and then the enzymatic part of the DT (A fragment) is released into the cytoplasm. Then, the A fragment inactivates EF2 via ADP-ribosylation that results in inhibition of protein synthesis and cell death by apoptosis (Dosio *et al.,* 2014). Based on this knowledge, DT toxin are used in cancer targeted therapy by fusing the first 388 amino acid of DT to the targeted moiety which known as DT-based immunotoxins (Potala *et al.,* 2008).

**Pseudomonas exotoxin A (PE or ETA):** The single polypeptide chain of natural PE composed of 613-residues organized in three domains. The N-terminal domain I (amino acids 1–252) recognized as Ia, is cell-binding domain and residues 365–404 recognized as Ib, with unidentified function, domain II (amino acids 253–364) that is required for translocation into the cytoplasm and domain III (residues 405–613) which is catalytic domain of toxin and responsible for inhibition of protein synthesis and cell death (Weldon *et al.,* 2009). Various forms of PE including PE40, PE38, and PE25 derivatives have been used in IT generation.

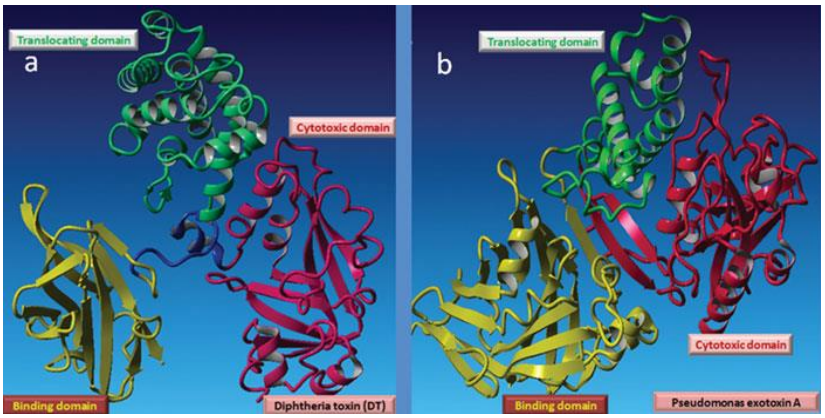


Fig 2: Three-dimensional models of two bacterial toxins. (a) Diphtheria toxin, (b)*Pseudomonas aeruginosa*exotoxin A have three successive domains, including receptor binding domain (yellow), translocation domain (green) and the adenosine diphosphate (ADP)-ribosylation domain (red). The model produced by YASARA software.

## Plant Toxins

**Ricin:** It is a very potent toxic protein derived from the castor plant, *Ricinus communis*. Natural ricin is a 64 kDa protein which comprises oftwo domains, joined together via a disulfide bond, the A-domain inhibits protein synthesis through permanent inactivation of eukaryotic ribosomes and the B-domain, a lectin, which acts as binding domain to mammalian cell surface glycoproteins and glycolipids (Weidle *et al.,* 2014). In ricin-based ITs, A-domain is genetically fused to targeted moiety by replacing B-domain. In endosomal compartments, A-domain is released from targeted moiety by proteolysis.

**Gelonin:** Gelonin is an approximately 30 kDa consisting of 258 residues. It is a single-chain soluble glycoprotein toxinderived from the seeds of the Himalayan plant *Gelonium multiflorum.* The toxin has potent N-glycosidase activity which inactivates the 60S eukaryotic ribosome subunit by cleaving the adenine in the 28S rRNA. Gelonin inhibits the binding of the 60S ribosome subunit to EF2; as a result, the protein synthesis is inhibited resulting in the cell death. A recombinant de-glycosylated form of gelonin (rGel) has been constructed which consists of 251 residues (Li *et al.,*

2017). The c-terminus of rGel is the active site of toxin and has cytotoxic and catalytic activities.

**Endogenous proteins of Human origin**

Due to non-immunogenic and high toxic potencies of endogenous proteins of human origin when compared to those of bacterial-or plant-derived toxins, they could be very potent candidates for the production of less or non-immunogenic ITs known as humanized IT for cancer-targeted therapy.

**Granzymes:** Granzymes are serine proteases that produced by cytotoxic T lymphocytes and natural killer (NK) cells (Kurschus and Jenne, 2010). Five granzymes have been recognized in human including A, B, H, M, and K; among which granzyme B is the most potent enzyme which cuts substrates at vital aspartic acid residues. After joining of the cytotoxic T lymphocytes to a tumour cell or virus infected cells, the perforin from cytotoxic T lymphocytes makes channel for granzyme B uptake. The accumulation of granzyme B in the cytosol activates the caspase cascade resulting in induction of apoptosis and cell death. Even in mitochondria, the granzyme B cleaves the Bid protein, leading to the change in mitochondrial membrane permeability and induction of apoptosis in target cell. Various granzyme B-based ITs have been generated and evaluated which exhibited high anti-tumour activity on tumour cells (Dalken *et al.,* 2006).

**RNases:** Due to RNA degradation property, ribonucleases can be utilized as a toxic agent to promote cell death through prevention of protein synthesis (Weidle *et al.,* 2014). Human-derived RNases because of their non-immunogenic properties are good candidates for therapeutic applications. All immuno-RNases after internalization into the cancer cells release into the cytoplasm and then degrade RNA ultimately resulting in cell death by induction of apoptosis (Makarov and Ilinskaya, 2003).

**Mechanism of action of immunotoxins**

Immunotoxins enter the cell through receptor-mediated endocytosis. Upon binding to a specific receptor, the toxin receptor complex is internalized by the process of clathrin mediated endocytosis to form an endosome. For PE and DT, the

low pH brings about unfolding of the protein, proteolytic cleavage and the release of the activity domain in the cytosol. In the cytoplasm the toxin inhibits protein synthesis by ADP ribosylation of the diphthamide residue of EF2. Release of ricin in the cytosol leads to N-glycosylation of residues in 28S rRNA there by preventing association of EF1 and EF2 with 60S ribosome, while restrictocin cleaves the 28S rRNA leading to protein synthesis inhibition. Cholera toxin acts by ADP ribosylation ofGs, a subunit of G proteins leading to an increased cAMP level and pore formation in the membrane resulting in cell death (Choudhary *et al.,* 2011).

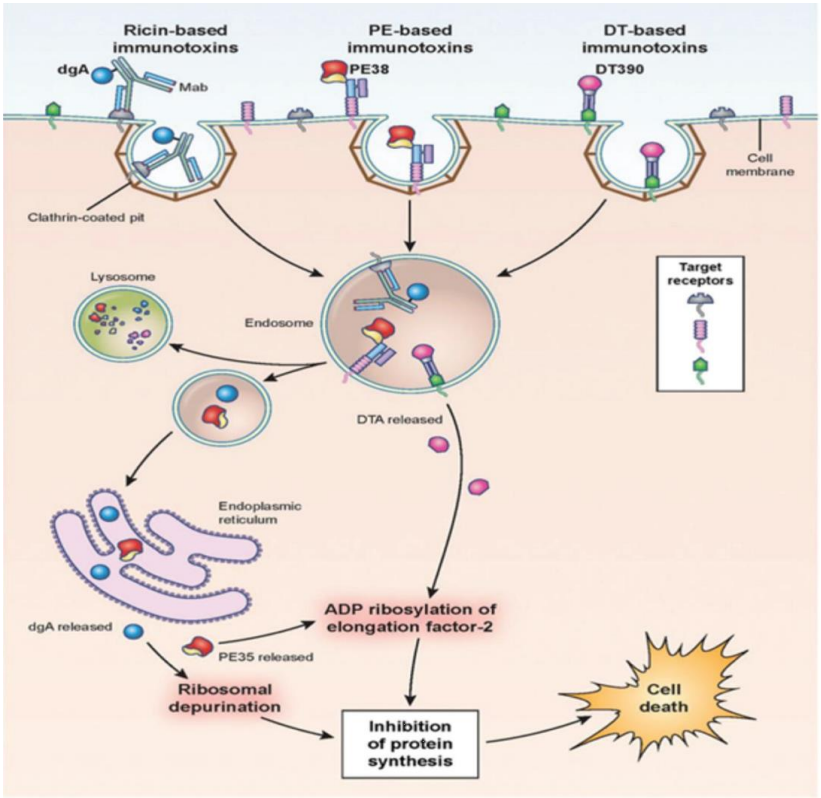


Fig 3: Mechanism of action of immunotoxins (Mei *et al.,* 2019)

**Immunotoxin against different types of tumours**

Targeted therapy is an emerging technique in cancer therapy and goes to update the conventional therapies in future. For effective treatment of cancer, it is necessary to specifically

direct the killing agent toward the surface molecules of tumour cells. Immunotoxin molecules are highly potent agents in cancer therapy as they contain selective binding domains (Madhumathi and Verma, 2012)

**Targeting haematological tumors**:

A wide range of haematological cancers, from leukemia to multiple myeloma, have been challenged with different immunotoxins.

***DD (Ontak™)* or DAB389IL2 (Ontak™):** It is the first IT that FDA approved for clinical usages in the treatment of recurrent cutaneous T-cell lymphoma (CTCL). It is the chimeric protein of human IL-2 and a condensed form of DT (DAB389). Ontak targets the high-affinity IL-2R, highly expressed in various tumours such as CTCL, Hodgkin's disease (HD), and other B- and T-cell leukemias and lymphomas. Ontak is being currently evaluated in combination with other treatments for various cancers despite ofsignificant side effects and retreatment limitation (Choudhary *et al.,* 2011).

**Anti-CD25 immunotoxins:** CD25 (low affinity IL2 receptor) greatly outnumbers CD122 and CD132 (high affinity receptors) on most malignancies. To target IL2R+ disorders expressing CD25, anti-Tac antibody, which binds with a higher affinity to CD25 alone, was used instead of IL2. An IT with a single-chain Fv fragment of the anti-CD25 monoclonal antibody (MAb) anti-Tac fused to truncated PE, PE40, was also constructed (Chaudhary *et al.,* 1989). It showed promising results in preclinical trials on CD25+ cells and malignant cells from ATL patients and in mice bearing CD25+ human xenografts (Kreitman *et al.,* 2000).

**BL22 (CAT-3888):** BL22 is composed of a disulfide stabilized anti-CD22 MAbRFB4(dsFv) fused to PE38, which aims CD22 molecules present on the surface of certain B-cell malignancies (Mansfield *et al.,* 1997). Although CD22 isalso present on normal B cells, the usual B-cells range can be replenished even after BL22 treatment, because the CD-22 molecule is absent on B-cell stem cells. Preclinical testing of BL22 in several lymphoma cell linesand leukemic patient samples (Kreitman *et al.,* 2000)

delivered promising results. Mice bearing human CD22+ CA46 Burkitt's lymphoma xenografts demonstrated complete regressions.

**Combotox (RFB4-dgA plus HD37-dgA):** CD22 and CD19 are surface antigens highly expressed on malignant B cells. To target B-cell lymphoma cells, two separate ITs – RFB4-dgA [deglycosylated ricin A-chain (dgA) conjugated to RFB4 (anti-CD22 MAb)] and HD37-dgA (dgA conjugated to anti-CD19 MAb) have been constructed. Separate Phase I trials using continuous infusion (CI) of RFB4-dgA and HD37-dgA showed evidence of antitumour activity (Schnell *et al.,* 2003).

**Targeting solid tumours**

Application of ITs in solid tumour treatment encountered some obstacles. Ineffective penetration capacity of ITs into tumour tissues and neutralizing activity of perfect immunity of patients has affected the development of immunotoxins. Emerging new technologies would help us to refine IT designing to defeat such problems. Among the IT therapies against solid tumours, treatment of brain tumours has had a promising result because of emerging new drug delivery methods, thereby facilitating IT penetration (Kioi *et al.,* 2006).

**Targeting mesothelin (SS1P):** Mesothelin is a differentiation surface antigen highly expressed invarious tumours such as malignant mesothelioma, ovarian cancer and pancreatic cancers, and nominally expressed in normal tissues, rendering it an attractive candidate for ligand-targeted therapies. To target such cancers, PE38 was fused with SS1 (an antimesothelindsFv) to construct SS1P [SS1(dsFv)-PE38], a recombinant IT with high affinity for mesothelin. Encouraging results were obtained from SS1P treatment of athymic nude mice bearing A431/K5 (human epidermoid carcinoma cell line) xenografts and tumour cells obtained directly from patients with mesothelioma and ovarian cancer (Li *et al.,* 2004).

**Anti-Lewis Y immunotoxins:** Lewis Y (LeY) antigen is an oncofetal carbohydrate antigen overexpressed on many epithelial carcinomas. Many generations of ITs using MAb B1 and

MAb B3 have been produced for targeting the LeY antigen (Pastan*et al.,* 1991). The first generation immunotoxin was a chemical conjugate of truncated PE toxin PE38 and anti- Lewis Y MAb B3 – called LMB-1.

**Anti-c-erb2/Her2/neu-erb38:** Owing to high surface expression of gp185 on breast tumours, and its relatively restricted expression in normal adult tissues, this protein has been targeted in a subset of breast and ovarian carcinomas. erb38 is an IT with a dsFv fragment of the erbB2-specific monoclonal antibody e23 linked to PE38 (Azemar *et al.,* 2003).

**IL13-PE38:** Enhanced expression of the IL13 receptor in tumour cells of glioblastoma multiforme (GBMs) and limited presence in normal brain allows IL13 receptor to be used as a promising GBM target (Kioi *et al.,* 2006).

**Immunogenicity, Nonspecific Toxicity, and Other Side Effects**

In the development of ITs immunogenicity and nonspecific toxicity are the major obstacles. The plant or bacterial-derived toxins are foreign proteins, and may induce neutralizing antibodies in patients, thus causing rapid removal of the IT from the bloodstream, resulting in a decrease in therapeutic effect. The clinical success of recombinant immunotoxins (RITs) in patients with a normal immune system is limited by their immunogenicity (Kaplan *et al.,* 2018). When treating patients with haematological malignancies, the immune system is suppressed by either the disease or chemotherapy. However, with solid tumours, the immune system is intact and the ITs are neutralized after one cycle thus preventing effective retreatments (Mazor *et al.,* 2014).

Although newer versions of ITs are constructed with improved safety and exhibit sound potency in treating various cancers, the dose administrated to achieve efficient therapeutic outcomes is limited, mainly due to IT-related side effects (Bokori-Brown *et al.,* 2018). The commonly reported toxicities against normal cells and tissues are vascular leak syndrome (VLS), hepatotoxicity, and renal toxicity. Several studies have been reported and indicated that VLS may be resulted from the weak

binding of ITs, especially the Ricin Toxin A (RTA)-based ones to normal endothelial cells when exiting blood vessels to targeting cells and tissues (Moss *et al.,* 2019).

**Future prospects**

Immunotoxins are proteins that combine the advantages of the specificity of the targeting portion of antibody and the potent cytotoxicity of protein toxins. The area of IT development is in rapid expansion. To date, two ITs, Ontak and anti-CD22 with PE38, have been approved by the U.S. FDA to treat CTCL and HCL. Clinical trials of other immunotoxins have been performed in hematologic malignancies and solid tumours. Immunotoxins could be used as a single agent or in combination with other agents. At present, the truncated forms of bacterial-derived protein toxin PE and DT are the most frequently used toxins for IT development. This foreign-protein–related immunogenicity will affect the therapeutic outcome by inducing the formation of neutralizing antibodies, especially in solid tumour treatment. Thus future research need to be performed to reduce the immunogenicity or humanizing the toxic moiety or even utilizing full human-origin protein cytotoxins to construct immunotoxins.

Further, the identification of novel cancer-related antigens or receptors with high specificity and selectivity as well as development of mAbs ready to be internalized once bound to the targets are other aspects that should be extensively worked on. Efforts also should be devoted to optimize the constructs' orientation, molecular weight and the linkage strategies to reduce the nonspecific toxicity to normal tissues and achieve a higher max tolerance dosage to enhance the therapeutic outcome. Co-administration with other agents such as chemotherapies and immune suppressors should also be considered to augment the therapeutic outcomes either by increasing cytotoxic activity or reducing immunogenicity. With further improvements, it is envisaged that in the near future ITs will expand the repertoire for treating cancers.

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# GLAUCOMA IN SMALL ANIMALS

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***ABSTRACT-***

*Glaucoma in small animals is an insidious blinding disease owing to the optic neuropathies, vascular dysregulation, damage of retinal ganglion cells and optic nerve head leading to an irreversible loss of vision. The manifestation of glaucoma is classified based on either on aetiopathogenesis or its duration of onset in animals. Varied clinical signs are evident in dogs and cats depending on stage of the disease with afflictions occurring in the cornea and anterior chamber like ciliary flush or keratitis or keratic precipitates, aqueous flare & uveitis, hypopyon or hyphema, or lens abnormalities. The diagnosis of glaucoma can be made by measuring and monitoring the intra-ocular pressure, visualization of iridocorneal angle by a gonioscope, assessment of allied clinical signs with an ophthalmoscope and slit lamp biomicroscope and in detail with imaging equipments (like ultrasound biomicroscope, Doppler, optical coherence tomography or OCT, magnetic resonance imaging). The treatment and prognosis go hand -in hand in therapeutic decision making based on progression of clinical signs and stage of disease at presentation in small animals.*

*The insidious nature of glaucoma challenges the pet owner in identifying visual problems with their pets and subsequently the animals are seldom presented in the initial onset phases of the disease. Hence, alleviation of pain, clinical signs like uveitis or intraocular inflammation and ocular hypertension and treatment of underlying aetiology are primarily done to stabilize the patient. Treatment methodologies vary for a potentially visual eye presented hopefully earlier at onset rather than a late presentation with a blind eye. The condition can be unilateral or bilateral in small animals though the former is commonly observed in dogs which over time do have chances of becoming a bilateral affliction. The need of the hour calls for more evidence-based studies in small animals regarding the aetiopathogenesis, diagnosis and treatment modalities to preserve vision in the affected eye and conserve vision in the other normal eye as well.*

***Keywords****: glaucoma, ocular hypertension, dogs and cats, tonometry, intra ocular pressure, optic nerve head, ophthalmic artery*

**INTRODUCTION**

Glaucoma is often described as a painful, complex group of blinding optic neuropathies often associated with elevated intraocular pressure (IOP), progressive deterioration and loss of retinal ganglion cells and their axons which finally entails to degeneration of the optic nerve head and the retina (Komaromy *et al*., 2019).

Glaucoma is often confused with ocular hypertension. Ocular hypertension is a clinical condition wherein the intraocular pressure is detectable and perseverant (acute or chronic), at a higher-than-normal level. Sometimes it can occur with no detectable changes in vision or damage to any ocular structure. It is attributed to the poor drainage of aqueous humour in the eye. Increased IOP, assessed with tonometry, without any optic nerve/retinal abnormality or visual loss is termed ocular hypertension.

The most important risk factor for the development of glaucoma in humans and animals is elevated intraocular pressure

(IOP). It is recommended that caution be exercised in diagnosing glaucoma based merely on a single, elevated IOP reading. Elevated IOP in the absence of clinical evidence of glaucomatous optic nerve and retinal damage is termed “ocular hypertension”, to distinguish it from overt glaucoma. Both ocular hypertension and glaucoma should be therefore distinguished from falsely elevated IOP measurements attributed to improper tonometric technique, inappropriate restraint, patient stress and corneal factors that may render the tonometer reading inaccurate. Also, a diagnosis of glaucoma cannot be excluded based on a single low or normal IOP reading. In glaucomatous cats, as much as in other species including glaucomatous dogs and humans, the IOP may fluctuate considerably, both within and between days. Cats with glaucoma are typically presented late in the course of disease. It is estimated that glaucoma in cats is under-diagnosed due to its insidious onset and gradual progression, as well as limitations of some commonly used tonometers in this species (McLellan and Miller, 2011). Treatment of glaucoma in feline patients is often clinically challenging, especially as it is often secondary in occurrence to other diseases.

**Aetiopathogenesis**

The balance in the formation and drainage of aqueous humour results in intraocular pressure (IOP). This balance between inflow and outflow of aqueous helps to maintain the IOP between 15- and 25-mm Hg in the dog and cat. Aqueous humour is produced in the ciliary body by energy-dependent ionic transport as well as energy-independent hydrostatic and colloid osmotic gradients. The enzyme carbonic anhydrase forms an important component of the energy dependent phase of aqueous humour production. The aqueous humour from the ciliary body passes into the small posterior chamber between the base of the iris, lens, and zonules. It moves by bulk flow through the pupil into the anterior chamber to the iridocorneal angle or ciliary cleft. Aqueous then passes between the large pectinate ligaments to the large extratrabecular spaces of Fontana in the uveal trabecular meshwork (UTM). The UTM is formed by collagen beams covered by phagocytic trabecular cells. Aqueous humour

then flows externally to the finer extratrabecular spaces of the corneoscleral trabecular meshwork (CSTM). The inner CSTM is attached to the outer fibers of the ciliary muscle to form the cribriform ligament. Parasympathomimetic drugs may act here to increase drainage of aqueous by opening up the extratrabecular spaces of the CSTM. The CSTM is separated from the more external angular aqueous plexus (AAP) by an endothelial cell lining. Aqueous humour movement then occurs via pressure-dependent transcellular channels that connect the CSTM to the trabecular veins of the AAP. The aqueous in the trabecular veins drains into the intrascleral plexus, which subsequently flows into the ciliary, conjunctival, arid vortex veins. A small percentage of the aqueous humour also (15% in the dog, 3% in the cat at normal IOP) drains from the eye through unconventional routes (uveoscleral flow). Aqueous can pass through the iris stroma and/or between the fibers of the ciliary muscle from the trabecular meshworks to reach the supraciliary and suprachoroidal spaces and then be absorbed by the blood vessels of the iris, ciliary body and choroid. Disorders like iridocyclitis which cause enlargement of the intramuscular spaces of the ciliary musculature and interendothelial cell junctions of iris capillaries remarkably increase uveoscleral outflow. The flow of aqueous humour via the trabecular meshworks is often driven by the pressure gradient, IOP-EVP, where EVP is the episcleral venous pressure. The EVP is normal in primary glaucoma in the Beagle breed of dog, but is elevated in some secondary glaucomas associated with congestion of the retrobulbar blood vessels (Peiffer and Gum, 1976).

The aetiopathogenesis can be attributed to a primary cause/ initial insult or a secondary cause. In primary cause, an initially elevated IOP leads to vascular dysregulation that is affecting ocular/orbital arterial hemodynamics, reduced blood flow to eye, poor oxygen delivery and perfusion. The subsequent obstruction to the axoplasmic flow occurring within retinal ganglion cells (RGCs) axons at lamina cribrosa leads ultimate to altered optic nerve microcirculation at lamina. A secondary etiopathogenesis is attributed through the excitotoxic damage by

glutamate or glycine- injured neurons leading to oxidative damage via nitric oxide and reactive oxygen species. This in turn causes a characteristic change in optic nerve head (ONH) wherein cupping of optic disc occurs by loss of ganglion cell loss at axons. This ultimately leads to dysfunction and death of retinal ganglion cells (RGC) which results in an irreversible loss of vision (Agarwal *et al*., 2009).

The current concept in the aetiopathogenesis of glaucoma is attributed to a multifactorial pathogenesis of glaucoma due to pathophysiology of glaucomatous optic neuropathy. The apoptotic neuronal loss occurs initially through vascular insufficiency (disruption of autoregulation of ocular hemodynamics, including ischemic hypoxia, oxidative stress, free radical accumulation), leading to compromised cerebral and ocular perfusion which results in direct damage to retinal ganglion cells.

**Classification of glaucoma**

Based on aetiology, glaucoma is divided into congenital, primary and secondary forms. Based on duration of onset as acute or chronic type.The congenital form can be due to any malformation or genetic abnormality. Primary form comprises abnormalities of iridocorneal angle like narrow or closed angle, open or normotensive form or goniodysgenesis. Secondary forms are attributed to following phacomorphic, intraocular tumor, systemic diseases, steroid- drug induced, uveitis induced, trauma or developmental factors induced condition (Shields *et al*., 1989).

**Clinical signs of glaucoma**

In cases with high intraocular pressure presence of a few of the following signs can be variably evident like; pain (squinting, tearing, enophthalmos, elevated nictitans), vision loss, mydriasis and a negative pupillary light reflex maybe elicited. A dazzle reflex is usually present in acute cases but absent in chronic cases. Other signs include injected episcleral vessels, diffuse corneal edema and a cupped optic nerve head (ONH). In cases of secondary glaucoma various signs like anterior uveitis, corneal inflammation (ciliary flush, keratic precipitates), anterior

chamber inflammation (characterized by aqueous flare/ hypopyon/hyphema) and lens abnormalities (cataract, capsular pigmentation, synechia) can be observed (Brooks *et al.,* 1989).

**Diagnosis of glaucoma**

It can be diagnosed through a thorough ophthalmic examination including eliciting clinical history-persistence of an acute or chronic condition, detailed ophthalmic examination with ophthalmoscope and slit-lamp, documentation of clinical signs, tonometry to measure intra ocular pressure (IOP), gonioscopy to assess iridocorneal angle and finally imaging techniques like ultrasonography(high resolution ultrasound or ultrasound biomicroscopy), Optical coherence tomography (OCT) or MRI or magnetic resonance imaging (Tai and Seymour, 2019).

Tonometry involves different devices which operate on various principles to measure and monitor the IOP. The indentation technique involves the Schiotz tonometer which is gravity based essentially and requires technical skill and proper restraint of the animal patient (Pickett *et al*., 1988). An applanation tonometer includes the TonoPen vet, Golman and Perkins, non-contact tonometers as well as an ocular surface response analyser. The rebound technique employs a TonoVet which makes no or minimal contact and helps elicit multiple readings in a hassle free manner. The indentation and applanation techniques necessitate desensitization of cornea with local anesthetics like proparacaine (0.5%) prior use of the tonometer on the ocular surface to elicit a reading. Latest versions of tonometer are contactless with ocular surface response analysers or Pascal dynamic contour tonometer.

Gonioscopy involves the use of a prismatic lens to assess the structure of the iridocorneal angle and visually appreciate any abnormalities in form and function (Palmberg, 1989). The vascular theory of glaucoma etiopathology holds it as a consequence of insufficient blood supply due to either increased IOP or other risk factors. Blood flow abnormalities in orbital arteries in Beagles with primary open angle glaucoma was documented by Gelatt *et al*., 2003. Therefore, hemodynamic

evaluation of the orbital circulation is significant in understanding the etiopathogenesis, course and sequelae of this disorder. Hemodynamics in orbital vasculature can be quantified using non-invasive, pain-free and much reproducible technique of colour -Doppler imaging (CDI); a feasible tool which allows to estimate blood flow velocity and resistance to blood flow in orbital vessels including ophthalmic artery in humans and animals. An association between orbital hemodynamics, autoregulation, vascular ischemia, ocular hypertension and glaucoma in animals can be thus deduced based on ocular blood flow, resistance parameters and IOP (Yang *et al*., 2011).

Early disease recognition or diagnosis is expected to be facilitated by the increased accessibility and affordability of powerful diagnostic technologies like high-resolution imaging tools (high-resolution ultrasonography [HRUS]/ultrasound biomicroscopy [UBM], optical coherence tomography [OCT] and anterior segment angiography) and more frequent IOP measurements by telemetric devices or home monitoring. Furthermore, as evidenced through recent advances in canine glaucoma genetics, improving molecular laboratory tools, such as next-generation sequencing and proteomics, will positively facilitate the detailed investigation of genetic risk factors as well as molecular and cellular mechanisms of the disease (Komaromy *et al*., 2019).

**Therapy and management of glaucoma**

The primary goal of glaucoma therapy is maintenance of vision by preserving optic nerve function. This is accomplished by lowering the IOP to levels that help prevent further optic nerve damage. One of the problems is that no one knows what a "safe IOP" is for the dog and cat (Brooks *et al*., 1989). A general rule is the more advanced the disease, the lower the IOP should be, given that the remaining axons in severely damaged optic nerves are more susceptible to further pressure-induced damage (Brooks, 1990). Success or failure of therapy should be assessed in terms of the effect of the therapy on the patient's sight and comfort. Current treatment regimens are directed toward

lowering the IOP by decreasing production of aqueous humour or reducing the resistance to outflow of aqueous humour. Pragmatism, rather than physiology, has guided therapeutic strategies in maintaining vision by reducing IOP (Brooks *et al*., 1983). Decreasing aqueous humour outflow from a physiological point of view is deemed not ideal, because the flow of aqueous is essential to the nutrition of the lens and cornea but it could render these tissues more vulnerable to decompensation in course of an elevation in IOP. Reducing the flow of aqueous humour could also decrease removal of retinal by-products through the vitreous and might harm the very optic nerve ganglion cells that the decreasing IOP were supposed to protect. The most physiologic approach to glaucoma therapy is decreasing the resistance to the outflow of aqueous humour through conventional outflow pathways. Future therapies will utilize the unconventional pathways as well. The ideal therapy for glaucoma should prevent IOP-induced pathological alterations of the optic nerve. Knowledge is lacking at the present time as to how this can be accomplished other than by reducing the IOP (Cook, 1997). Evidence in humans indicates that the earlier in the disease therapy begins, the better the response. Hence, early detection is essential (Corcoran *et al*., 1994). The lower the IOP, the slower the optic nerve and vision deteriorate. Once severe optic nerve damage is present, pressure may be too high to maintain vision (Gelatt *et al*., 1989). A potential problem is that some of the drugs used to reduce IOP can be deleterious to vision. Carbonic anhydrase receptors are also found in the cornea, retina, and choroid. The use of carbonic anhydrase inhibitors may adversely affect corneal, retinal, and choroidal metabolism.

**Medical Management**

The medical treatment of glaucoma, traditionally, is based on the use of several classes of drugs which are considered efficient and safe. It is prudent to use a minimum number and strength of medications to reduce the IOP to a level sufficient to preserve sight. There may only be a small safety margin of tissue left in those patients with severe optic nerve damage. There are

no methods currently available in veterinary ophthalmology to determine the pressure level required to prevent further optic nerve damage. "Maximum medical therapy" is reached when the IOP reaches unacceptable levels and no further escalation of medical treatment is available or appropriate (Komaromy *et al*., 2019).

**Cholinergic Agents**: Cholinergic drugs act to increase the conventional outflow of aqueous humour by causing contraction of the ciliary muscle. Ciliary muscle fibers are attached to the CSTM. Contraction of the ciliary muscle results in the opening of the intertrabecular spaces and increased transcellular vacuolation in the cells between the CSTM and trabecular veins. Pilocarpine is a direct-acting parasympathomimetic. Demecarium bromide and phospholine iodide are long-acting anti-cholinesterase agents that cause ciliary muscle contraction by allowing acetyl choline to build up at the ciliary muscle muscarinic receptor sites. These drugs are contraindicated in the presence of glaucoma associated with uveitis. These drugs can increase pupillary block and will decrease uveoscleral outflow. They should be used carefully in narrow angle glaucoma because they may exacerbate angle closure. Some clients will complain of pilocarpine causing canine and feline patients' eyes to be more red or irritated. Pilocarpine is also associated with retinal detachments in humans, although no reports exist for such a complication in dogs and cats (Brooks, 1990).

**Adrenergic Agonist Drugs**: These drugs can affect aqueous humour flow through the trabecular meshwork, uveoscleral outflow, aqueous humour production, and the ocular microcirculation. Sympathomimetic agonists reduce IOP by reducing production and increasing outflow of aqueous humour. These agonists are often combined with parasympathomimetics (e.g., epinephrine with pilocarpine).

**Beta-adrenergic Antagonists:** Timolol (non- selective β-blocker) has become the most commonly prescribed medication for humans with glaucoma. I find this to be very effective in lowering the IOP in the dog and cat. Betaxol (a cardioselective β-

blocker) is less efficacious in my experience in the dog and cat. The ocular hypotensive effects of timolol are additive to those of carbonic anhydrase inhibitors, cholinergic agents, and sympathomimetics. Timolol increases retinal blood flow in humans, which may be protective of retinal and optic nerve function (Brooks, 1990).

**Carbonic Anhydrase Inhibitors**: Carbonic anhydrase-inhibiting diuretics reduce aqueous humour production by the ciliary body up to 50%. This effect is independent of diuresis. They can cause metabolic acidosis at toxic levels, to manifest panting, nausea, and vomiting. Methazolamide is associated with development of nephrolithiasis in dogs and humans. Outflow resistance caused by trabecular compression and meshwork collapse at high IOP can improve when the lOP is reduced by carbonic anhydrase inhibitors (CAI). The reduced IOP can reduce ciliary muscle ischemia and allow the cholinergics to work. This may explain the synergistic action of CAis, cholinergics, and beta-blockers. Carbonic anhydrase inhibitors can also be effective when the meshworks are obstructed in secondary glaucomas and are unresponsive to medications that reduce outflow resistance (McLellan and Miller, 2011).

**Hyperosmotic Drugs**: Hyperosmotic drugs can be used in acute attacks of glaucoma to rapidly lower IOP. Mannitol (1g/kg) is given slowly intravenously over a 15-minute period. Glycerol (50%, 1-2 ml/kg) is administered orally. These medications can be repeated if necessary. Both drugs reduce the water content of the vitreous to lower IOP. The osmotic effect of oral glycerol is more variable than intravenous mannitol, but may be more convenient because of the route of administration. Water should be withheld for 1 hour after administration of these drugs. Hyperosmotic drugs may not lower IOP in cases of secondary glaucoma produced by inflammation from surgery or other causes (Cook, 1997).

**Maintenance and Prophylactic Therapy**: The use of CAI dichlorphenamide (10 mg/kg divided two times per day), the β-blocker timolol, and the acetylcholinesterase. Inhibitor

demacarium bromide as the initial maintenance therapy for most types of primary glaucoma with IOP greater than 25 mm Hg is a popular protocol. All of the drugs are administered twice a day. Mannitol is added to this regimen in cases of acute glaucoma. If the mannitol does not lower IOP, surgery is recommended. The demecarium bromide is not used if anterior uveitis is present. Changes in dosage or frequency of administration are made as the disease progresses. In secondary glaucomas, the inciting cause is identified and removed or suppressed. Animals with uveitis and glaucoma, for example, are also treated with topical corticosteroids following a diagnostic work-up for the anterior uveitis. Atropine, although indicated in uveitis therapy, is contraindicated in glaucoma associated with uveitis. It is a recommended prophylactic therapy for the unaffected eye, in cases of primary glaucoma that present with one eye affected. Prophylactic therapy significantly extended the interval between diagnosis of glaucoma in some dog breeds with primary glaucoma and the development of glaucoma in the second eye. The long-term or prophylactic use of antiglaucoma drugs is not without risk, given that these medications are known to cause ultrastructural changes in the conjunctiva, Tenon's capsule, and trabecular meshwork in humans. Systemic side effects may also occur from the use of any of these drugs in selected canine and feline patients. Frequent ocular and systemic examination of affected dogs and cats is extremely important (Komaromy *et al*., 2019).

**Future Medical Therapy for Glaucoma:** Future therapy will be more specific and directed at very basic biochemical levels as a more clear understanding of trabecular meshwork physiology, optic nerve axonal transport, ocular hemodynamics, and the nature of aqueous humour production and turnover control mechanisms is attained. Apraclonidine, an alpha-2 adrenergic agonist, has been used to lower IOP following laser glaucoma surgery in humans. It reduces production of aqueous humour. Prostaglandins increase uveoscleral outflow in cats and reduce IOP in dogs, but present formulations are too irritating topically to use therapeutically. Topical CAis will become available in the

near future. For skolin, an adenylatecyclase activator, increases cyclic adenosine monophosphate levels when used topically, and lowers IOP in rabbits. Angiotensin converting enzyme inhibitors are more potent than timolol when used topically in rabbits and hold great promise for use in humans, dogs, and cats (Willis *et al*., 2002).

**Surgical Glaucoma Therapies:** Alternative therapeutic modalities may become necessary to prevent further optic nerve damage when maximum medical therapy is attained. The decision to intervene in the management of glaucoma with fistulizing surgery or cyclodestructive procedures is based on the drugs used, owner compliance, side effects of the medication, and the surgical expectations. In glaucoma associated with lens luxations, lens removal alone or in combination with other procedures may restore normal IOP through surgery consistently below a threshold to prevent further optic nerve damage (Brooks, 1990 and Bedford, 1989). Surgical procedures may be divided into those that reduce the resistance to aqueous humour outflow (fistulizing procedures) and those that decrease the production of aqueous humour by the ciliary body (cyclodestructive procedures).

**Fistulizing Procedures:** Fistulizing procedures or glaucoma filtration surgeries: procedures involving iridencleisis, cyclodialysis, and posterior sclerectomy to allow aqueous humour to bypass the iridocorneal angle and filter into the subconjunctival space, when treating glaucoma in the dog and cat. Results were good in the short-term but scarring of the bleb and a resurgence of elevated lOP occurred in 3-6 months. A more promising technique for bypassing the iridocorneal angle and thereby increasing aqueous outflow involves subconjunctival placement of silicone, silastic, or nylon implants with anterior chamber tubes. Aqueous humour passes to the subconjunctival space via the valved or nonvalved connecting tube. Complications include expense of the implant, difficult surgical technique, and obstruction of the tubing by inflammatory cells and fibrin. Molteno, Joseph, and Krupin-Denver implants have been used in the dog with some success. The rechanneling of aqueous flow to a

single region may adversely affect trabecular meshwork, lens, and corneal metabolism opposite the artificial site of aqueous humour outflow (Cook, 1997).

**Cyclodestructive Procedures:** Cyclodestructive procedures are used to reduce aqueous humour production by the ciliary body. Cyclocryotherapy using nitrous oxide or liquid nitrogen to freeze the ciliary epithelium has been proven satisfactory in reducing IOP in the glaucomatous dog and cat, but the postoperative inflammation can be quite severe, and transient IOP elevations and retinal detachments are common. A 2.5- 4 mm cryoprobe is placed on the conjunctiva 5 mm from the limbus. Eight to ten sites are frozen to - 80°C for 1-2 minutes each. The long posterior ciliary arteries enter the globe at the three and nine o'clock positions, and these areas should be avoided to prevent anterior segment infarction and ischemia. The results of the freezing will be known in 2-4 weeks. Cyclocryotherapy can be repeated if the IOP remains high. It was reported that transcleral cyclophotocoagulation with the Nd:YAG laser was a more efficacious and less traumatic method of reducing aqueous humour in the dog and cat. The laser is applied to 30 spots 5 mm posterior to the limbus for 0.5 seconds each at an energy level of 16 watts (8 J per site). More or less energy can be delivered to the globe depending on the case, but hyphema and phthisis bulbi are risks of this procedure if more than 250 J are delivered to a canine or feline globe. Transcleral cyclophotocoagulation will rapidly lower IOP with minimal postoperative inflammation. It is the surgical procedure of choice in the early glaucomatous eye that still retains vision. The expense of the equipment' is the only limitation to its use in veterinary ophthalmology. Intravitreal injection of gentamicin has been used to reduce IOP in permanently blind glaucomatous eyes without intraocular infection or neoplasia. I believe this rather simple and inexpensive technique should only be used when glaucoma has been confirmed tonometrically, all other medical options have been explored, and the blindness is definitive. Twenty milligrams of gentamicin combined with 0.4 mg of dexamthasone injected intravitreally will severely damage the ciliary body and reduce

IOP. Corneal opacity, cataracts, severe uveitis, pain, and phthisis bulbi are complications of this procedure (Brightman *et al*., 1982).

**Salvage Procedures:** Enucleation or a placement of a cosmetically acceptable intrascleral silicone implant following evisceration is indicated when vision is lost and the IOP cannot be controlled medically or surgically in the dog and cat. The source of pain is removed and no further expensive medication is necessary. Prosthetic implants are contraindicated when glaucoma is associated with intraocular infection or neoplasia (Brightman *et al*., 1977). Eviscerated tissue and enucleated globes should be submitted for histopathologic analysis because glaucoma secondary to neoplasia is common in the cat and noted in the dog.

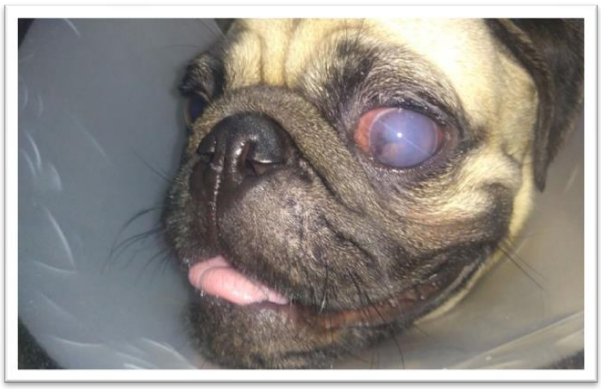
Early recognition of clinical signs, timely diagnosis and selection of the most effective method of surgery warranted based on the patient's stage of glaucoma and the underlying cause of the same are important factors when going for a glaucoma surgery. Several surgical procedures exist for dogs with primary glaucoma, like a cyclodestructive technique or an aqueous outflow bypass procedure or a combination of both procedures. Salvage procedures such as enucleation, evisceration with an intrascleral prosthesis, or chemical intravitreal injection may be advised for chronically blind eyes with absolutely no hope of vision restoration. Client compliance and expectations, feasible therapeutic goals, status of vision (visual versus blind) at presentation of animal patient, financial constraints of clients, surgical expertise or facilities at hand and underlying systemic disorders/co-morbidities should be considered in the selection of the most appropriate and feasible surgical option, specifically for each patient (Sapienza, 2008).

**Conclusion**

Conjunctivitis, mydriasis, corneal edema, lens luxation, buphthalmos, and blindness are caused by elevated IOP in the glaucomas. Primary glaucomas with a bilateral potential for development are noted in two cats and several dog breeds, with

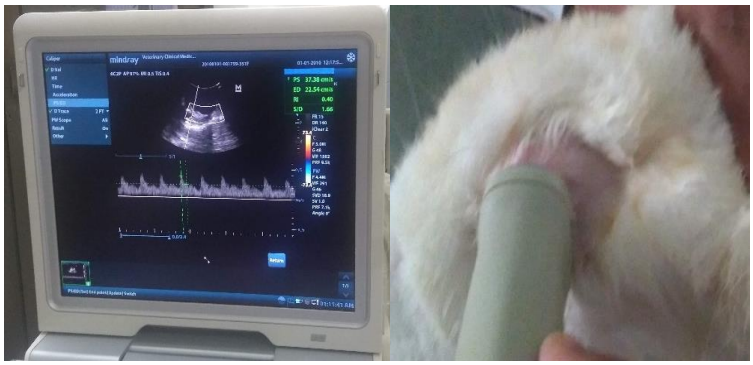
secondary glaucomas caused by uveitis and neoplasia common in the cat and dog. Tonometric evaluation is essential for the early diagnosis and management of glaucoma. Medical management or surgical intervention will have to be chosen appropriately. While much progress has been made in the understanding and treatment of canine glaucoma, there is still no cure and many affected dogs go blind. The improved knowledge of disease mechanisms and the development of reliable biomarkers are critical so that animals at risk or in early stages of disease can be identified more readily. Early diagnosis facilitates effective, mechanism-based treatment before the occurrence of any clinically appreciable optic nerve damage and vision loss.

It is essential to conduct more evidence based medical research on animals and detailed studies to understand the neurogenic and vascular aspects of aetiopathogenesis, course and sequelae of glaucoma in various species. Secondary glaucomatous manifestations are clinically better understood and literature documented than primary or normotensive glaucoma in veterinary literature, inciting an urgent need for the same to better understand the disease and work to improve on diagnostic and efficient treatment modalities to preserve vision at the earliest course of disease recognition and diagnosis in all species. We also require the evidential support of holistic glaucoma animal models to assess the efficacy of various therapeutic methods based on etiopathogenesis and type of glaucoma. The evolution of a more structured protocol for systematic imaging diagnosis of lesions, vascular studies and genetic predisposition research is the need of the hour and will make a sea change in the approach to treatment.



Unilateral secondary glaucoma in a 2 year old male Pug dog (Dept. of Vet. Surgery and Radiology, TVCC, CVAS, Mannuthy)

Schiotz tonometry: measuring IOP in a dog



Colour Doppler imaging for ocular hemodynamics and blood velocity, resistance parameters assessment in a clinically normal 3year old NewZealand white rabbit (7.5MHz linear probe,Mindray® with sterile ophthalmic coupling gel(D-panthenol gel 5%, Optholifesciences ltd.) at TVCC,CVAS, Mannuthy.

Anterior chamber paracentesis to reduce IOP in a case of secondary glaucoma in a 2 year old female Doberman dog

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# WASTE MANAGEMENT USING BLACK SOLDIER FLY –AN EMERGING TECHNOLOGY

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***ABSTRACT-***

*The smart concept of converting organic waste in to high protein, high energy resources, has its traces from ancient times. Producing maggots from the organic waste is one such concept which finds its recognition in the animal husbandry, especially in poultry sector. This concept can be utilized by the farmers as a new venture to reduce the cost on feed. Maggot is a general term denoting the larval stages of flies. Although there are many identified maggots which can be used in this sector now, the black soldier fly larvae are the very common ones prevailing and flourishing in our tropical climatic condition. It has voracious feeding nature and thrives easily on different organic wastes like abattoir waste, food waste, human faeces, mixed fruits and vegetable wastes and manure of different species of birds and animals. Just like one answer for two questions, this concept helps*

*to manage the household waste and at the same time the produced larvae can be supplemented as energy rich, protein rich diet in the feed, replacing other costlier feed components like soya bean, fish meal, maize, broken rice etc.*

***Keywords**: Organic waste, Black soldier fly larvae, Feed*

## INTRODUCTION

As per the reports of the World Bank in 2019, cities of the world generated 2.01 billion tonnes of solid waste in 2016, amounting to a footprint of 0.74 kilograms per person per day. The more than 1.3 billion people in India generate the highest amount of waste in the world. Currently, based on calculations by the World Bank, India is the largest producer of waste, owing largely to the size of population. India produces 277 million tonnes of municipal solid waste every year, according to a 2016 estimate. Operating this essential municipal service requires integrated systems that are efficient, sustainable, and socially supported. Aerobic and anaerobic decomposition like composting, vermicomposting and biogas plants are the common methods adopted. Nowadays waste decomposition using black soldier fly is getting more acceptance among farmers.

The black soldier fly, *Hermetia illucens* is a an insect This fly is highly reputed in converting low quality biomasses in to high protein, high energy maggot meal and more than 80% survival obtained when they reared on food waste and BSF larvae on organic wastes helps to minimize the emission of greenhouse gases. The residue after decomposition can be utilised as fertilizer, the larvae act as a source for the production of various products like feed, chitin, biodiesel etc. Use of black soldier fly (BSF) larvae has a great potential in organic waste management (Diener *et al.,* 2011). This paper focuses on the peculiarities and advantages of black soldier fly and how we can treat food waste using this fly.

### 1. Black soldier fly (BSF)

The black soldier fly (*Hermetia illucens Linnaeus*) is a member of the Stratiomyidae family. The adult fly is wasp-like and 15-20 mm long. Primarily black, the female's abdomen is

reddish at the apex and has two translucent spots on the second abdominal segment. The male's abdomen is somewhat bronze in colour. The black soldier fly or BSF originates in the south of the United States, but during World War II they spread into Europe, Asia, including India, and even to Australia. The development of international transportation since the 1940s has resulted in its naturalisation in many regions of the world, nowadays this insect present in the rest of the world, between latitude 40° south and 45° north (Rindhe *et al*., 2019).

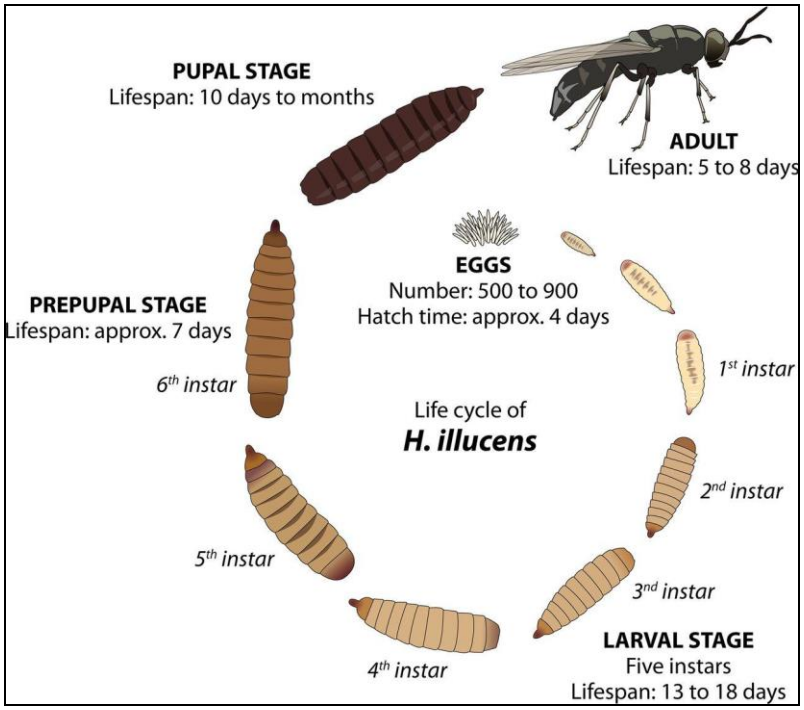
The adult is a black fly with white legs. The flies have no stinging, chewing or sucking mouthparts. Because of this they don't transmit diseases, contrary to other fly species. They are therefore not considered as a vector in the countries where they occur naturally. The larvae however are always hungry. They can feed on organic materials such as plant residues, manure and even carcasses. The fast growth of the larvae suppresses the development of other insects that enjoy the same substrate (Carl *et al*., 2019).

This insect, considered as a non-pest. In nature it is commonly found in habitats suitable for larval development such as marshlands and generally damp places with animal waste, rotten fruit or any decaying organic matter (Li *et al*., 2011).The adult fly does not have mouthparts and doesn't even feed during its short lifespan. They feed only as larvae, and so must accumulate a large fat body for larval development and adult survival(Nguyen *et al.*, 2015).Unlike many pests that consume waste, black soldier fly larvae do not carry bacteria or diseases and are capable of inactivating Escherichia coli and Salmonella (Erickson *et al*., 2004).

Kitchen waste had the greatest mean rate of reduction (consumption by black soldier fly) per day and produced the longest and heaviest black soldier fly (Nguyen *et al.*, 2015).There is a good opportunity to utilise these flies for bioconversion considering the fact that approximately 1.3 billion tonnes of food is wasted from the food produced each year in world (Gustavsson *et al.,* 2011). The larvae convert organic waste

material faster than worms used in vermicomposting. A colony of 2,000 larvae can consume about a kg of house hold food waste per day. They have large and powerful chewing mouthparts and hence are able to consume organic compounds before they have time to decompose, thereby immediately eliminating odour (Rindhe *et al*., 2019). Additionally, the larvae modify the microflora of manure, potentially reducing harmful bacteria such as *Escherichia coli* 0157:H7 and *Salmonella enteric* (Van Huis *et al.,* 2013). It has been reported that the larvae contain natural antibiotics which act on growth promoter in the animal feed (Newton *et al.,* 2008).

**2. Life cycle of BSF**




Source: St-Hilaire *et al.,* 2007

The female fly lays a package of 400 to 800eggs close to decomposing organic matter, into small, dry, sheltered cavities. Shortly after having laid the eggs, the female dies. The closeness of the eggs to the decomposing organic matter ensures that the larvae have their first food source nearby after hatching. The

sheltered cavities protect the eggs from predators and prevent dehydration of the egg packages by direct sunlight. On average, the eggs hatch after four days and the emerged larvae, which are barely a few millimetres in size, will search for food and start feeding on the organic waste nearby. The larvae feed voraciously on the decomposing organic matter and grow from a few millimetres size to around 2.5 cm length and 0.5 cm width, and are of cream-like colour (Dortmans *et al*., 2017).

Under optimal conditions with ideal food quality and quantity, the growth of the larvae will require a period of 14-16 days. However, the BSF larva is a very resilient organism and has the ability to extend its life cycle under unfavourable conditions. The larval stage is the only stage during which the Black Soldier Fly feeds and, therefore, it is during this time of larval development that enough fat reserves and protein are stored that allow the larvae to undergo pupation, emerge as flies, find mates, copulate and (as a female) lay eggs before dying.

After having gone through five larval stages, the larvae reach the final larval stage, the prepupa. When transforming into a prepupa, the larva replaces its mouthpart with a hook-shaped structure and becomes dark brown to charcoal grey in colour. It uses this hook to easily move out and away from the food source towards a nearby dry, humus-like, shaded and protected environment that it deems safe from predators and is where the imago emerge from the pupa and fly off without significant hindrance.

The process of pupation is the transformation from a pupa into a fly. The pupation stage is initiated when the prepupa finds a suitable location and becomes immobile and stiff. For a successful pupation, it is best if the environmental conditions do not change too much or, in other words, that they remain warm, dry and shaded. Pupation takes around two to three weeks and ends when the fly emerges from its pupa shell. The emerging process is a very short procedure. It takes less than five minutes for the fly to break open the part of the pupa that used to be the head section, crawl out, dry and then spread its wings and fly off.

After emerging, the fly lives for about one week. During this short life, it will search for a partner, copulate and (for the female) lay eggs. As a fly, BSF do not feed. Only a source of water or a humid surface is required to stay hydrated. What is important in this life stage is an abundant amount of natural light and a warm temperature (25-32°C). A humid environment may prolong the life span and, thus, enhance the chance for successful reproduction. It has been observed that the flies prefer to copulate in the light of the morning. After copulation, the females then search for an ideal location to lay their eggs (Dortmans *et al*., 2017).

**3.Production of black soldier fly larvae**

Waste reduction and insect biomass produced were assessed with different types of wastes (Diener *et al*., 2011). It was found that 68 per cent waste reduction and a higher insect biomass yield was obtained with municipal organic waste followed by chicken manure (50per cent reduction) and pig manure (39 per cent reduction).

A native colony of black soldier flies can be started in the warm months by attracting the female black soldier flies to lay their eggs near a source of food with a strong odour. The females ready to lay eggs can detect in the air the chemical signal of a future larval-food source. A method to attract black soldier flies in urban areas is to ferment dried corn kernels by soaking them in the water. Once the corn kernels ferment, the mixture releases a strong odour that can be useful for attracting black soldier flies. Sour milk may be another good option (Bullock *et al*., 2013).

Insect biomass is highly nutritious, dried fly larva contains 42.1 per cent crude protein, 34.81per cent ether extract (lipids), 14.61 per cent ash, 7.91 per cent moisture, 7.01 per cent crude fiber, 5.01 per cent calcium, 1.51 per cent phosphorus, 1.41 per cent nitrogen free extract (Park, 2015).

Body composition of the larvae depends on the quality and quantity of ingested food (Nguyen *et al.*, 2015). Larvae fed on different substrates had varying body protein content (ranging from 37.0 to 62.7 per cent DM) and fat content, which

showed more variation (ranging from 6.6 to 39.2per cent DM) than protein content (Barragan-Fonseca *et al.*, 2017).

### 4.1. Small to medium scale production

Medium to small-scale operations include free-range animal husbandry, farms that have a mix of animals, or residential homes that have pet waste and/or compost. For a medium to small-scale operation, a modular approach will be more effective, allowing for the Black Soldier Fly Larvae operation to be easily scalable. Each modular unit is referred to as a grub tub.

Materials for a single grub tub will include a plastic tote bin (>20 gallon, depends on the quantity of waste available), 2"x10' PVC pipe, 2x 90-degree PVC elbow joints, and a 5-gallon collection bucket.

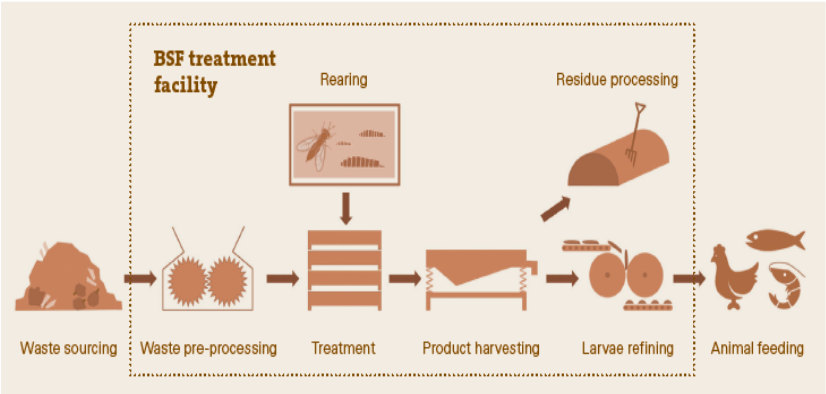
To build a grub tub, cut two equal sections of PVC pipe so that the grubs can travel from the center of the tub, out the side of the tub (through a hole cut in the plastic), and into a collection bucket. The PVC pipe should protrude approximately six inches from the tub. At the protruding end, place the elbow joint and a small section of PVC to reach the lip of the collection bucket. Cut the bottom 6 inches of the PVC in half along the length of the pipe to create a larger opening for the larvae to enter. The goal is to have the migrating Black Soldier Fly Larvae drop in the 5 gallon collection bucket, from which they cannot escape.

The grub tub must be filled with a food source manually. When filling, be sure that the opened end of the PVC lies above the level of the manure so that Black Soldier Fly Larvae have easy access to the point of exit (Bullock *et al*., 2013).

Small to medium scale production

Source: Bullock *et al.*, 2013

## 4.2. Large scale Production




Source: Dortmans *et al.,* 2017

### 4.2.1. BSF rearing unit

To ensure the treatment of a defined amount of waste on a regular basis, the rearing unit needs to provide a defined number of five day old larvae, so-called 5-DOL, everyday. It is, therefore, important to control the single production steps during rearing and to monitor the performance of each step. In a well-engineered BSF nursery, it is possible and easy to control the number of prepupae that are allowed to pupate. This helps estimate the number of flies that shall emerge, which in turn

provides an indication of how many egg packages will be deposited, how many larvae will hatch and how many of these larvae are available for bio waste treatment. Monitoring of the survival rates at every step in this cycle keeps track of the colony's overall performance and indicates problems at any particular step. Survival rates may differ from one nursery to another.

Egg deposition and egg harvest from a management perspective, it is important that all egg packages are concentrated in one specific location. This will significantly facilitate harvesting of the eggs. For this, we supply the cages with a suitable medium (called "eggies") that satisfies the flies' requirements regarding a safe location (i.e. sheltered cavities) for egg deposition, as well as an "attractant" which mimics decomposing organic matter that attracts the female to lay eggs close by. Once the egg packages are deposited into the eggies, they are harvested before any larvae hatch.

Egg harvest is measured by the difference in weight between empty and full eggies. A standardized type (and weight) of the empty eggie is, therefore, advisable. The number of eggs is the total egg mass divided by the average weight of an individual egg, which is 25µg.

**4.2.2. Egg hatching and larvae feeding**

The harvested eggies are placed together with eggies harvested on previous days over an open "hatchling container" with a high quality food source. We call this the "hatchling shower". The larvae will hatch over a period of several days. Placing recently harvested eggies together with the older eggies guarantees a constant "shower" of hatchlings into the nursery container. After hatching, larvae fall from the eggies into the hatchling container below where they will start feeding immediately. The high quality food source in the hatchling container consists of chicken feed for starter chicks, mixed with water. This mixture has a water content of around 70per cent.

The hatchling container below the hatchling shower is replaced with a new hatchling container at regular intervals

(every one to three days). The frequency determines the uniformity of the batch of larvae. As counting all these small larvae is too much work, the number of 5-DOL is estimated by counting the number of larvae in a small sample (approximately 2g), which then is extrapolated based on the total weight of all 5-DOL.

### 4.2.3 Waste receiving and pre-processing unit

Larvae are generally very tolerant when it comes to feeding substrates. The larvae strongly depend on symbiotic microorganisms which degrade cell structures and make nutrients available for the larvae to take up. With suboptimal feed, however, development time will be extended and the final larval weight will be lower.

A first step upon arrival of the waste involves a waste quality control to ensure that it contains no hazardous materials and no inorganic substances. A few plastic bags in the waste may not pose a significant problem and can be sorted out and removed manually. However, hazardous contaminants are critical to keep out as they may affect all the living organisms: the larvae, associated bacteria and, of course, the workers. Acids, solvents, pesticides, detergents and heavy metals fall into this category and it is especially critical to keep them out when they are in a liquid or dissolved form, as this can easily contaminate the whole batch of waste material. If such contamination is suspected, the waste should be refused.

With the waste quality ensured, the next required step then involves a reduction of the waste particle size. This can be achieved by using a shredder or hammer mill. Whatever type of technology is used, the equipment should shred the waste to particles of smaller than one to two cm in diameter. This helps to speedup Black Soldier Fly processing, as Black Soldier Fly larvae do not have appropriate mouthparts to break apart large chunks of waste, and increasing the surface area fosters the growth of the associated bacteria.

If the shredded waste has water content above 80 per cent, then the waste will need to be dewatered or mixed with

another, drier waste source to obtain moisture content below 80 per cent. If the water content is below 70 per cent, then water needs to be added. This can be determined by squeezing a handful of waste, and if less than a few drops of water emerge between your fingers, then the waste is too dry. If dry waste is moistened using water, the water has to be safe to use, meaning that it does not contain pathogens, heavy metals or other anti-nutritional elements.

There are different ways to dewater the waste. The simplest way is passive dewatering (by gravity), where the waste is filled into a cloth bag that acts as a filter and the water drains through the cloth into a bucket below. Other technologies to dewater might include a horizontal screw press or a cider press.

At the moment the organic waste is accepted at the site, a measurement of weight should be performed to know the daily waste intake of the facility. The best time to measure the total incoming waste is after it has been shredded as then it will probably temporarily be stored in containers. If dewatering of waste is required, it is best to obtain a weight measurement before and after the dewatering process.

**4.2.4. BSF treatment unit**

A specific amount of 5-DOL are transferred daily from the BSF rearing unit to the BSF treatment units containing the waste (we call these as “larveros”). The number of 5-DOL added will depend on the amount of bio waste that is contained in a specified volume and surface area.

As a rule of thumb work with the following numbers: 10,000 5-DOL in a larvero (40x60x17cm) feeding on 15kg of wet waste (75% water) for 12 days. While the 5-DOL feed and grow, more waste is added to the same larvero on day five and again on day eight, until the larvae have developed large enough to be harvested after 12 days of feeding. The amount of waste is also limited by the layer thickness of waste in the larvero. If the depth of the waste in the larvero is more than 5cm, larvae will have difficulties to process it entirely and the waste on the bottom will remain unprocessed.

Larveros can be stacked upon each other to optimize surface area requirements. However, it is necessary that the larveros are well ventilated to allow the moisture saturated air to be replaced. Also, provision of oxygen is crucial for the well-being of the larvae. For these purposes, we suggest to keep enough open space between the stacked larveros to allow for free flowing aeration.

It is further recommended to ventilate the stacks with fans during the last few days. This creates an active air flow over the surface of the larveros to increase evaporation. A crumbly waste residue will be the result, which can be easily sieved from the larvae. However, the intensity of active ventilation depends on the air humidity and the moisture content of the starting material and has, therefore, to be assessed individually in each context.

### 4.2.5. Product Harvesting

After 12 days of waste treatment by BSF larvae, each larvero is harvested. At this stage, the larvae have reached their maximum weight, but have not yet transformed into prepupae. Their nutritional value is, therefore, at its maximum. Harvesting is the process in which the larvae are separated from the residue. This can be done by using a manual or automated shaking sieve by which the larvae are easily separated from the residue. With a higher shaking frequency, the mesh size of the sieve can be bigger.

A sieve mesh size of around 3 mm for manual sieving and 5 mm for automated sieving is considered suitable. The sieve is placed at an angle and the content of the larvero is emptied onto the sieve. During the shaking, the larvae remain on the top of the sieve while the residue falls through the sieve into recipients. Given the angle of the sieve, the larvae are guided to the lower angle, which is connected to a bucket where the larvae drop into (Dortmans *et al.,* 2017).

## 5. Optimal conditions

Optimal environmental conditions and food sources for the larvae can be summarized as

### 5.1. Warm climate

The ideal temperature is between 24 and 30°C. If too hot, the larvae will crawl away from the food in search of a cooler location. If too cold, the larvae will slow down their metabolism, eat less and develop slower.

### 5.2. Shaded environment

Black Soldier Fly Larvae do not survive well in direct light or in extreme dry or wet conditions. If they are too far below the surface, they will perform little bioconversion. Female flies avoid any sites that are anaerobic when trying to lay eggs (Bullock *et al*., 2013). If their food source is exposed to light, they will move deeper into the layer of food to escape the light. The amount of waste is also limited by the layer thickness of waste in the larvero. If the depth of the waste in the larvero is more than 5cm, larvae will have difficulties to process it entirely and the waste on the bottom will remain unprocessed (Dortmans *et al*., 2017).

### 5.3. Particle size of the food

As the larvae have no chewing mouthparts, access to nutrients is easier if the substrate comes in small pieces or even in a liquid or pasty form. Reduction of the waste particle size can be achieved by using a shredder or hammer mill. Whatever type of technology is used, the equipment should shred the waste to particles of smaller than one to two cm in diameter. This helps to speedup Black Soldier Fly processing as Black Soldier Fly larvae do not have appropriate mouthparts to break apart large chunks of waste, and increasing the surface area fosters the growth of the associated bacteria (Dortmans *et al*., 2017).

### 5.4. Water Content of the food

Black soldier fly larvae develop most rapidly at 70 percent humidity. It is especially important to keep the grubs' feeding medium at a proper moisture level—not so dry that it cements the grubs into the feed, and not so wet that they cannot breathe through the pores in their exoskeleton (Bullock *et al*., 2013). The food source has to be quite moist with water content between 60 per cent and 90 per cent so that the larvae can ingest the substance**.**

**Advantages**

There are many advantages in using Black soldier fly for waste management, some of these include:

Waste reduction of up to 80 per cent on wet weight basis. Waste biomass can be converted into larvae and residue. Barragan-Fonseca *et al.* (2017) reported that larvae fed on different substrates had varying body protein content (ranging from 37.0 to 62.7% DM) and fat content, which showed more variation (ranging from 6.6 to 39.2% DM) than protein content. Feeding waste to larvae has been shown to inactivate disease transmitting bacteria. Prevents housefly breeding. The residue can be used in agriculture. Reduction in greenhouse gas emissions. High waste-to-biomass conversion rate. No need for sophisticated high-end technology to operate such a facility so it is suitable for low-income settings.

Soldier fly larvae provide another significant benefit i.e. house fly control in animal manures (Sheppard, 1983). The larvae repel ovipositing female houseflies (Bradley and Sheppard, 1984) and house fly larvae that do attempt to compete with dense populations of soldier fly larvae usually die. Houseflies can transmit more than 100 human and animal disease-causing organisms, flies mechanically carry ascarid and other nematode eggs on their feet from manure to pens, feed, and water, avian influenza virus has also been isolated from adult house flies. Housefly is the major pest species associated with poultry manure, especially in caged-layer operations and this could be prevented if manure is treated with Black soldier fly larvae.

BSF bio waste treatment offers an environmentally relevant alternative with very low direct greenhouse gas emissions and potentially high global warming potential reduction (Mertenat *et al.*, 2019).

**Conclusion**

Black soldier fly is a magical insect since it can solve a various problems like waste management, food and feed production and energy production through bio diesel, production

of chitin, and production of compost. It considerably reduces the global warming potential of various organic wastes by reducing the methane and carbon dioxide production from them. Insects can be farmed in high densities with small space requirements and they have a high bioconversion ratio (Oonincx and de Boer, 2012). This is a boon to the farmers to convert their farm waste into value added products and new venture for the reduction of various kinds of organic waste. Developing country like India should utilize this insect to make a solution for ever-growing problem "the waste management".

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# APPLICATION OF HIDDEN MARKOV MODELIN VETERINARY AND BIO-MEDICAL RESEARCH

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***ABSTRACT-***

*Hidden Markov model is a doubly stochastic process with an underlying stochastic process that is not observable (it is hidden), but can only be observed through another set of stochastic processes that produce the sequence of observed symbols. Hidden Markov model has got wide application in veterinary field such as medicine, biochemistry, genetics, movement pattern of animals, behavioural aspects etc. The present chapter discusses the application of hidden Markov model and hidden semi Markov model in veterinary and other bio-medical researches with the review of relevant researches conducted in various important aspects. Hidden Markov model and hidden semi Markov model is widely used in predicting the progress of diseases like cancer, Alzheimer's disease etc. and modelling of various disease states. Hidden Markov model and hidden semi Markov model has got wide applications in predicting protein coding genes of both prokaryotes and eukaryotes, predicting the location and orientation of alpha helices in membrane-spanning proteins, identification of heterogeneous regions of a DNA sequence and so on. Biologists should join together with biostatisticians for applying the hidden Markov model at the suitable places. Hidden Markov model and hidden Semi Markov model should be exploited by veterinary and biomedical researchers as these models have excellence in solving many research questions*

***Keywords****: Hidden Markov model, Hidden semi Markov model, research, application*

## INTRODUCTION

A Markov Model is a stochastic model which models sequential data. It provides a way to model the dependencies of current information with previous information. Markov model could be used to examine the probability of transition from one state to another state. Hidden Markov model is a doubly stochastic process with an underlying stochastic process that is not observable (it is hidden), but can only be observed through another set of stochastic processes that produce the sequence of observed symbols. The difference between Hidden Markov model apart from Markov Model or a Markov Chain is the fact that in a Markov Chain the states are observable while for Hidden Markov models, the states are statistical, having associated probability distributions called the observation probability density functions.

One limitation of hidden Markov model (HMM) is that dwell times (time spent in a specific state) are assumed to follow a geometric distribution; this assumption may not always hold. The hidden semi-Markov model (HSMM), an extension of HMM, relaxes this assumption and permits explicit modelling of dwell times using alternative statistical distributions. HSMM (Hidden semi Markov model) were similar to a classic hidden Markov model (HMM), but the main difference was that the unobserved process is semi-Markov in the sense that a change to a future hidden state depends on both the current state and the time spent on this state.

Hidden Markov model has got wide application in veterinary field such as medicine, biochemistry, genetics, movement pattern of animals, behavioural aspects etc. They are important tools in estimation and analysis of biological sequences and many other systems. This chapter discusses the application of hidden Markov model and hidden semi Markov model in veterinary and other bio-medical researches with the review of relevant researches conducted in various important aspects.

**Application in identifying disease progression and its modelling**

Hidden Markov model and hidden semi Markov model is widely used in predicting the progress of diseases like cancer, Alzheimer's disease and modelling of various disease states. A novel approach was developed for predicting body trajectories for cancer progression, where conditional probabilities of clinical data were modelled using Hidden Markov Model techniques. For the Hidden Markov Models a Bayesian approach was taken using the Hybrid Monte Carlo method, producing an ensemble of models rather than a single one. Bureau et al. (2003) presented an approach where the disease states are modelled as the hidden states of a continuous time hidden Markov model using the imperfect measurements of the disease state as observations. Co-variate effects on transitions between disease states were incorporated using a generalized regression framework. Parameter estimation and inference were based on maximum likelihood methods and rely on an expectation-maximization (EM) algorithm.

To improve the assessment of disease progression, Sukkar *et al.* (2012) proposed using Hidden Markov Models (HMM's) to model, in a more granular fashion, disease progression as compared to the clinical stages of the disease. Unlike many other applications of Hidden Markov Models, they trained their HMM in an unsupervised way and then evaluated how effective the model was at uncovering underlying statistical patterns in disease progression by considering HMM states as disease stages. In their study, they focused on Alzheimer's disease (AD) and show that their model, when evaluated on the cross validation data, could identify more granular disease stages than the three currently accepted clinical stages of "Normal", "MCI" (Mild Cognitive Impairment), and "AD". The Continuous-Time Hidden Markov Model (CT-HMM) was an attractive approach to modelling disease progression due to its ability to describe noisy observations arriving irregularly in time. They presented the first complete characterization of efficient expectation-maximisation (EM) based learning methods for CT-

HMM models. They demonstrated that the learning problem consists of two challenges: the estimation of posterior state probabilities and the computation of end-state conditioned statistics.

Wang *et al.* (2014) proposed a probabilistic disease progression model that addressed the challenge of modelling disease progression based on real-world evidence. As compared to existing disease progression models, the advantage of their model was three-fold:1) it learned a continuous-time progression model from discrete-time observations with non-equal intervals; 2) it learned the full progression trajectory from a set of incomplete records that only cover short segments of the progression; 3) it learned a compact set of medical concepts as the bridge between the hidden progression process and the observed medical evidence, which were usually extremely sparse and noisy. An approach was developed for estimating chronic kidney disease stage transition rates using hidden Markov models (HMMs), when the level of information and observation time varied among individuals.

**Applications to improve cardio-vascular health**

Cai *et al.* (2008) used a longitudinal data set covering 13 years from the Cardiovascular health study and evaluated the properties of a recently developed approach to deal with left censoring that fits a semi-Markov process (SMP) model by using an analogue to the stochastic EM (expectation-maximization) algorithm-the SMP-EM approach. It appeared that the SMP-EM approach gave estimates of duration-dependent probabilities of health changes similar to those obtained by using SMP models that have the advantage of actual duration data. SMP-EM estimates of duration-dependent transition probabilities appeared more accurate and less variable than multi-state life table estimates. Frenay (2009) used two methods to improve the transition modelling in hidden Markov models for ECG segmentation: a hidden Markov model state scission scheme which prevents ingoing and outgoing transitions in the middle of the waves and a bayesian network where the transitions were

emission-dependent. A hidden semi-Markov model was proposed in apnea-bradycardia detection to represent and characterize the temporal evolution of observed RR series and different pre-processing methods of these series were investigated. Evaluation was performed on a database of 233 apnea-bradycardia episodes manually annotated. The acquired ECG signals were processed to obtain RR series. The proposed detectors, applied to these RR series, were composed of two HMM or HSMM models, each one represented two distinct physiopathological states: absence and presence of apnea-bradycardia.

**Application in bio-medical research and disease surveillance**

Biomedical diagnosis system was developed by researchers for pattern recognition with normal and abnormal classes. Feature extraction processing was made by using the Doppler Ultrasound. During feature extraction stage, wavelet transforms and short-time Fourier transform were used. As next step, wavelet entropy was applied to these features. In the classification stage, hidden Markov model (HMM) were used. Detilleux (2008) developed a mixed hidden Markov model (HMM) for predicting breeding values of a biomarker (here, somatic cell score) and the individual probabilities of health and disease (here, mastitis) based upon the measurements of the biomarker. Hidden Markov model determined the transition probabilities between two states, and of misclassification. They concluded that the hidden Markov model allowing for misclassification was well suited to analyses of health service databases, since it was able to capture bias due to the fact that the quality and accuracy of the available information were not always optimal. Robertson *et al.* (2011) reported on the development of a hidden Markov model for analysis of frontline veterinary sentinel surveillance data from Sri Lanka. Visualization of state probabilities was used to indicate areas and times of unusual disease prevalence. Their analysis suggested that hidden Markov modelling was a useful approach for surveillance data sets from novel populations and having little historical baselines. Researchers developed a variety of

estimators based on multi-event or hidden Markov models for use under different experimental conditions. They applied the estimators to two case studies of avian mortality, one from pesticide exposure and another at wind turbines. Simple flexible hidden Markov model (HMM) for disease surveillance was developed by scientific community which was suitable for use with sparse small area count data and required little baseline data. Their findings suggested that the HMM provided an effective method for the surveillance of sparse small area notifiable disease data at low false alarm rates.

**Applications in molecular genetics and biochemistry**

Hidden Markov model and hidden semi Markov model has got wide applications in predicting protein coding genes of both prokaryotes and eukaryotes, predicting the location and orientation of alpha helices in membrane- spanning proteins, identification of heterogeneous regions of a DNA sequence and so on. Krogh *et al.* (1994) developed a hidden Markov model (HMM) to find protein coding genes in *E.coli* DNA using *E.coli* genome DNA sequence from the EcoSeq6 database maintained by Kenn Rudd. Generalized Hidden Markov Model (GHMM) can provide the framework for describing the grammar of a legal parse of a DNA sequence. Probabilities were assigned to transitions between states in the Generalized HMM and to the generation of each nucleotide base given a particular state.

A novel method to model and predict the location and orientation of alpha helices in membrane- spanning proteins was presented in 1998 based on a hidden Markov model (HMM) with an architecture that corresponded closely to the biological system. The model was cyclic with 7 types of states for helix core, helix caps on either side, loop on the cytoplasmic side, two loops for the non-cytoplasmic side, and a globular domain state in the middle of each loop. Hidden Markov models were more realistic than Markov models since they allow for the identification of heterogeneous regions of a DNA sequence. Research works were done showing application of hidden Markov models to a subsequence of the *Xylella fastidiosa* DNA data. The hidden

Markov model achieved valuable prediction results using only a limited number of parameters. An interpretable framework for protein secondary structure architecture was provided by hidden Markov model. Furthermore, it could be used as a tool for generating protein sequences with a given secondary structure content.

The combining relative solvent accessibility information with various dependency features in hidden Markov models could be used in various hidden Markov models to improve the accuracy of predicting the protein secondary structure. Based on the evaluation and application of their model, Schliehe-Diecks *et al*. (2012) highlighted the usefulness and advantages of hidden Markov models (HMMs), in general, and mixed HMMs in particular, for statistical analyses of (multiple) behavioural sequences and the generation of further testable hypotheses, in this case about the feeding behaviour of mouse lemurs and their determinants. CpG islands (CGIs) are very important and useful, as they carry functionally relevant epigenetic loci for whole genome studies. Analysis of CGIs at the DNA sequence level in cattle genomes was done in 2016. Researchers used hidden Markov model algorithm to detect CGIs. The hidden Markov model framework were well suited to deal with the main features commonly observed in accelerometer data, and could easily be extended to suit a wide range of types of animal activity data.

**Application in behavioural aspects and movement pattern of animals**

Scientists developed classification method of calculation of characteristics of the main sleep rhythms using hidden Markov models. The method was highly accurate and it provided reliable identification of the main stages of sleep. Another study concluded that combining relative solvent accessibility information with various dependency features in hidden Markov models could be used in various hidden Markov models to improve the accuracy of predicting the protein secondary structure. Based on the evaluation and application of their model, Schliehe-Diecks *et al*. (2012) highlighted the usefulness and

advantages of hidden Markov models (HMMs), in general, and mixed HMMs in particular, for statistical analyses of (multiple) behavioural sequences and the generation of further testable hypotheses, in this case about the feeding behaviour of mouse lemurs and their determinants. Joo *et al*. (2013) proposed two sets of alternative and state of the art modelling approaches. First, they considered hidden semi-Markov models (HSMMs). They compared the efficiency of hidden Markov, hidden semi-Markov, and three discriminative models (random forests, artificial neural networks and support vector machines) for inferring the fishermen behavioural modes, using a cross-validation procedure. Scientists applied hidden semi-Markov models (HSMM) to hourly geographic positioning system (GPS) location data to understand movement patterns of the endangered Florida panther and to discern factors influencing these patterns. Using the Viterbi algorithm they showed that differences in movement patterns of male and female Florida panthers. Their study demonstrated the use of HSMM methodology to precisely describe movement and to dissect differences in movement patterns according to sex and reproductive status.

**Conclusion**

Hidden Markov Model (HMM) and Hidden Semi-Markov Model (HSMM) might be explored in predicting the progress of diseases like cancer, Alzheimer's disease and modelling of various disease states. It could be used for finding the most probable stages of other relevant diseases especially the chronic diseases. Hidden Markov model and hidden semi Markov model has got wide applications in predicting protein coding genes of both prokaryotes and eukaryotes, predicting the location and orientation of alpha helices in membrane-spanning proteins, identification of heterogeneous regions of a DNA sequence and so on. Application in behavioural sciences, for analysing movement pattern of animals etc. shows the importance of the HMM model.

As we discussed, similar studies might be conducted in respective institutions after gaining sufficient knowledge about

the algorithm for training the HMM model. In this regard biologists should join together with biostatisticians for applying the HMM model at the suitable places. Hidden Markov model and hidden semi Markov model should be exploited by veterinary and biomedical researchers as these models have excellence in solving many research questions.

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# SELECTION OF SPERMATOZOA IN ASSISTED REPRODUCTION

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***ABSTRACT-***

*Sperm selection techniques in assisted reproductive technologies (ART) is biomimetic as it separates better spermatozoa from the rest of the ejaculates and removes seminal plasma as in the female reproductive tract. Thus, it becomes possible to render low quality semen sample suitable for ART. There are different sperm selection methods based on different parameters such as motility, apoptosis, zeta potential, interaction properties, morphology and membrane integrity of spermatozoa. Motility based sperm selection techniques such as swim up and colloid centrifugation are commonly used in animal practices. Single layer centrifugation is reported to be removing bacteria and certain viruses from the porcine spermatozoa and this point is practically important in avoiding the unwanted non-therapeutic usage of antibiotics in semen. Microchips are more advantageous that they can offer the sperm sorting in an optimized automated procedure and reduce sperm loss due to complex protocols and multiple transfers. Selection of best spermatozoa from the ejaculates of genetically valuable animals of rare breeds or endangered species may improve fertility in ART and thus facilitates the success of conservation programmes. Each technique has its own pros and cons. Hence, it is better to couple more than one technique to improve the quality and quantity of selected*

*spermatozoa. Research should be focussed to reduce the complexity of the latest techniques and make them cost effective so that they can be practically implemented in the normal animal ART settings also.*
***Keywords**: Assisted Reproduction, Sperm selection*

## 1. Introduction

Selection of spermatozoa within the female reproductive tract permits only those spermatozoa which are morphologically normal with intact membrane and good chromatin integrity to reach up to oviduct and fertilise the oocytes. Thus, it is self-evident that good embryos come from good gametes. As the presence of dead or abnormal sperms compromises the overall fertilising potential of the ejaculate, it is must to remove them prior to *in vitro* fertilisation. Sperm selection techniques in assisted reproductive technologies (ART) do the separation of better spermatozoa from the rest of the ejaculates as in the female reproductive tract and also remove spermatozoa from seminal plasma which mimic the effect of spermatozoa migrating away from the site of semen deposition under physiological conditions. Thus, it becomes possible to render low quality semen sample suitable for ART.

There are different sperm selection methods based on different parameters which are summarised here. Advantages and disadvantages of various selection methods under different categories are also briefly reviewed.

## 2. Selection of spermatozoa

There are different sperm selection methods based on various criteria such as,

a. Sperm motility
b. Apoptosis of spermatozoa
c. Sperm zeta potential
d. Sperm interaction properties
e. Sperm morphology
f. Sperm membrane integrity

### 2.1. Selection of spermatozoa based on sperm motility

Motile sperms are selected based on their natural ability to migrate into a defined medium.

#### 2.1.1. Swim up technique

Swim up technique is one of the classical sperm separation techniques used routinely as it is simple and cost effective with which highly motile and morphologically normal spermatozoa can be separated from other cells and seminal plasma. In this procedure, light low-density media is layered over the semen (or the centrifuged pellet) and the motile sperms are allowed to migrate up against gravity leaving all other non-dynamic factors within the sample behind (Fig.1). In 2019, Magdanz *et al.* confirmed that swim up separates bull spermatozoa with high motility, metabolic rate and tail length. Swim up was found to be an effective sperm sorting method in rams (Olivares *et al.,* 2017) and pigs (Morales *et al.,* 2012). Lowrate of sperm retrieval and chance of Reactive Oxygen Species (ROS) production (in conventional method with centrifugation) are some of the disadvantages of this method.

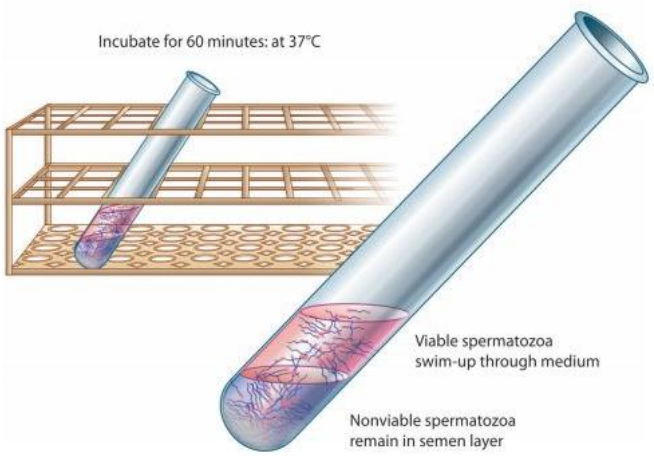



Fig.1. Sperm selection by swim up technique

#### 2.1.2. Swim-Down method

This technique relies on the natural movement of spermatozoa. A discontinuous bovine serum albumin is taken in a tube which becomes progressively less concentrated moving

from top to bottom. The semen sample is placed into the top of the medium and is incubated for one hour at 37°C. The most motile sperms will migrate to the gradient (Ing*et al.*, 1991).

**2.1.3. Density gradient centrifugation (DGC)**

DGC is the conventional gold standard technique for sperm selection. This method consists of centrifugation of semen over continuous or discontinuous gradients which separate the cells by their density, motility and centrifugation speed. The colloids used for DGC are Percoll (Polyvinyl-pyrrolidone (PVP) coated silica) and various types of silane coated silica. Bovipure is a commercial preparation for DGC which contains colloidal silica particles coated with silane specifically formulated for use with bull sperm. Tyrodes with lactate, albumin, pyruvate etc. are mixed with the colloid to achieve different densities for density gradient.

There are two gradients: a lower phase (high density) and an upper phase (low density). A gradient is prepared by carefully layering 2 mL of lower phase at the bottom of the 15 mL graduated centrifuge tube and a 2 mL of upper layer is layered on top without mixing the two gradients. Up to 2 mL of semen is layered on top and centrifuged. Centrifugal force and time should be kept at the lowest possible values (<300 g) to minimise the production of ROS. Motile, mature and morphologically normal spermatozoa have higher density than immotile or immature spermatozoa. Hence, after centrifugation, intact sperms form a pellet at the bottom of the tube (Fig.2) whereas leukocytes and cell debris are concentrated in the interface between seminal plasma and upper layer. Abnormal spermatozoa are collected in the interface between the upper and lower layer.

Efficacy of density gradient centrifugation in selection of spermatozoa was reported in ram (Alvarez *et al.*, 2010), buck (Batista *et al.*, 2011), boar (Noguchi *et al.,* 2013) and bull (Arias *et al.*, 2016). DGC was also successfully used to sort good spermatozoa from oligozoospermic and sub-fertile stallion for AI (Mari *et al.,* 2011). Marzano *et al.* (2020) reviewed that DGC is advantageous in prolonging sperm survival, maintaining chromatin integrity and selecting sperms with higher motility

with longer telomere length and without abnormal morphology. Discontinuous DGC is reported to be eliminating decapacitation factors, prostaglandin and reactive oxygen species (Jeyendran *et al.,* 2019). DGC is also found to be effective in eliminating the sperms with single stranded and double stranded DNA damage.

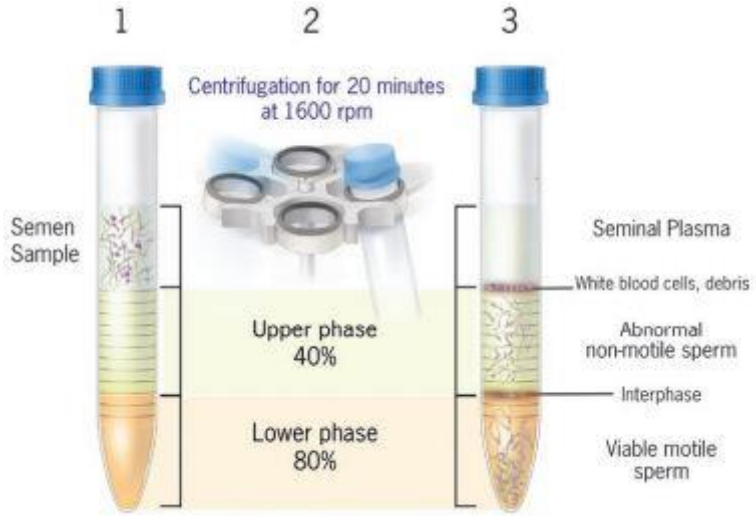



Fig.2. Density gradient centrifugation-based sperm selection

But, close cell to cell contact between healthy and defective sperm or leukocytes will result in generation of reactive oxygen species and there by oxidative stress. And also, DGC is not recommended for extremely low sperm content semen samples, highly viscous semen samples or samples containing high content of cellular debris.

### 2.1.4. Single layer centrifugation (SLC)

SLC is more convenient and less time consuming than DGC. In SLC, single layer of colloid is used and the semen is added to the top of the colloid. After centrifugation, seminal plasma is retained on the top of the colloid whereas the motile viable spermatozoa move down through the colloid and form pellet.

It is important that the semen should be added to the top of the colloid rather than injecting the colloid below the semen because placing the semen in the bottom of the tube may allow unselected spermatozoa, bacteria etc. which may coat in the bottom and contaminate the selected sperms. And also, loss of

integrity of sharp interface between colloid and semen may happen which affect efficient selection. The spermatozoa appearing in the pellet should be resuspended in an extender containing protein to prevent the spermatozoa from clumping together or adhering to slides or tubes. Androcoll-E and Androcoll-P are species-specific colloids used for SLC in equine and porcine respectively (Morrell, 2012 &2019).

The quality of cryopreserved spermatozoa of buck (Rabadan *et al.,* 2012) and ram (Sterbenc *et al.,* 2019) was found to be improving after selection using SLC from thawed semen. The SLC selected boar sperms had better linear motility, normal morphology, viability and cryopreservability than unselected sperms (Morrell*,* 2019). Morrell (2012 &2019) reported the use of SLC in separating the spermatozoa from bacteria but, this will be less effective if performed after several hours of semen collection. SLC combined with swim up could remove more than 99 percent of porcine circo virus 2 from spermatozoa (Blomqvist *et al.*, 2011). If SLC could be guaranteed to remove the pathogens, it would be adopted as a routine procedure prior to insemination for enhancing biosecurity and minimising the usage of antibiotics in semen.

But, the demerit expected in this method is that metal contamination of colloidal particles may induce oxidative stress and sperm DNA fragmentation.

**2.1.5. Migration-Sedimentation (MS) technique**

MS procedure uses the swim up technique and also relies on the natural settling of sperms due to gravity. Special tubes called as Tea-Jondet tubes are used for this in which the sperm cells migrate from a ring shaped well (Fig.3) into the medium above and then settle through the central hole of the ring. In 1996, Risopatron *et al,* observed this method as very promising for obtaining bull spermatozoa with optimal fertilisation capacity.

This is considered as a gentler and beneficial technique as it avoids centrifugation-based damages in spermatozoa. And this is usually used for samples with low motility. But, the tubes used are quite expensive.

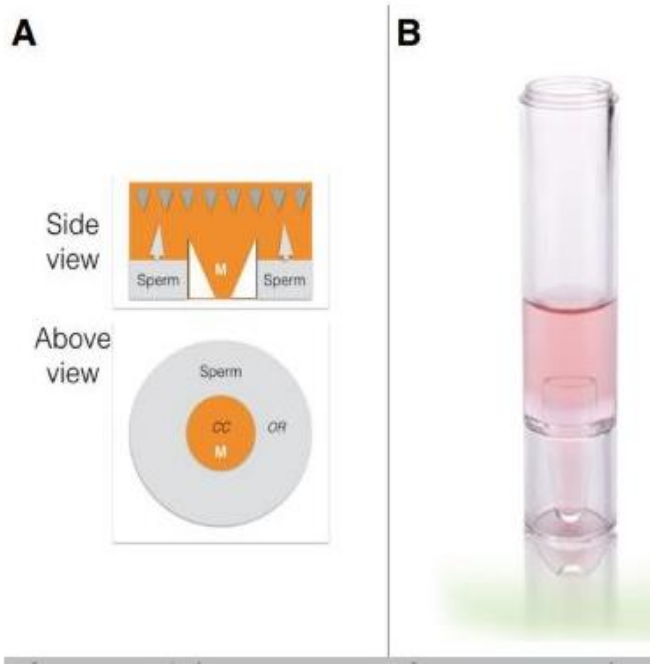
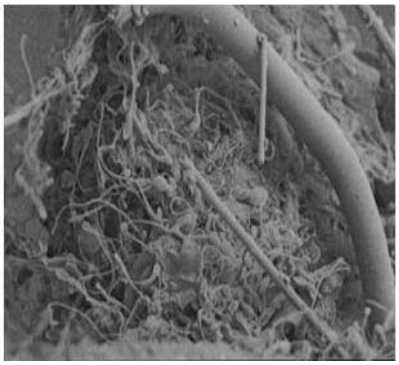



Fig. 3. A. Schematic representation of MS technique for sperm sorting; B. Special tube used for this

### 2.1.6. Glass wool filtration (GWF)

Here, the motile sperms are separated from their immobile counterpart with the densely packed glass wool fibres. The self-propelled movement of the spermatozoa and the filtering behaviour of the glass wool help in sperm separation. Non-viable sperms, leukocytes and debri are sticky and adhere to the glass wool column. In 2012, Arzondo *et al*. reported that glass wool filtration was an effective method for selecting bull spermatozoa for IVF. But, the technique is relatively expensive and may induce damage in the sperm membrane and acrosome probably due to the pore size of the glass wool fibres (Marzano *et al.,* 2020).

A



B

Fig.4. Scanning electron microscope images showing the arrangement of glass fibres in glass wool and the interaction of spermatozoa with them. A. Before filtration: large amounts of spermatozoa and noncellular material. B.Typical appearance of the lower part (bottom) of the filter: only a few spermatozoa, all of their tails in direct interaction with glass fibres (Engel *et al.,* 2001).

### 2.1.7. Sephadex filtration

Sephadex is a dextran gel available in different pore sizes (G-10 to G-20). The non-viable sperms tend to adhere to the Sephadex matrix to greater extent than motile and functional sperms and the viable sperms are filtered out. Satorre *et al.* (2012) observed that cryopreserved boar sperms after Sephadex filtration had better post-thaw quality and functionality. Cryopreserved buffalo sperms selected through Sephadex G-15 filtration yielded better fertilisation rate *in vitro* (Husna *et al.*, 2017).

### 2.1.8. Microfluidic devices

Microfluidic devices use microchannels to sort sperm based on motility and morphology (Jeyendran *et al.,* 2019). Based on the principle of positive rheotaxis of motile sperm *ie*. the ability to orient themselves and swim against the flow, Zhang *et al*. (2015) and Wu *et al.* (2017) developed microfluidic devices to sort spermatozoa with improved motility, morphology and DNA integrity for IVF and IUI protocols.

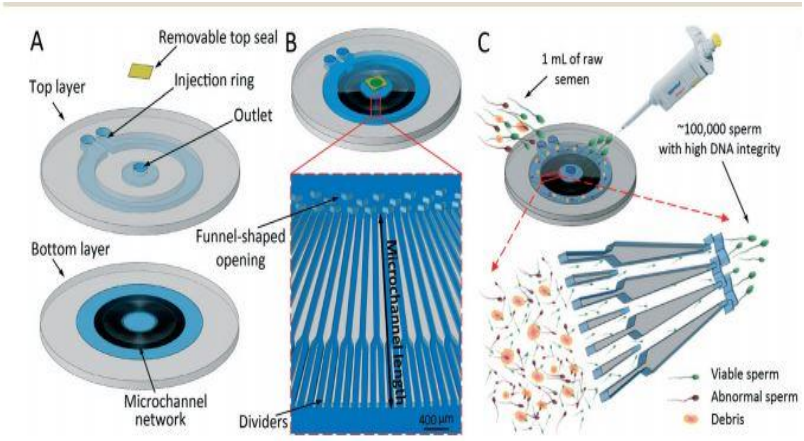



Fig.5. Sperm sorting with microfluidic system

DMSS (Diffuser type microfluidic sperm sorter, Nagata *et al.,* 2018), PRED (Positive rheotaxis extended drop, De Martin, *et al.,* 2017) are the modified systems of microfluidic devices. Another version, macro micro fluidic sperm sorter (MSS) does the sperm separation in which the healthiest and most motile sperms swim through a Nucleopore track- etched polycarbonate

membrane filter, leaving dead and immotile sperms in the bottom chamber.

The same physiological property was used by Chinnasamy *et al.* (2018) to develop Simple Periodic Array for Trapping And Isolation (SPARTAN) in which pillar arrays are present that sort highly motile and morphologically normal spermatozoa within 10 minutes with minimum DNA damage.

### 2.2. Selection of spermatozoa based on apoptosis

#### 2.2.1. Magnetic activated cell sorting (MACS)

MACS is one of the methods used in ART which is able to select non-apoptotic sperms in a non-invasive manner (Said *et al.,* 2015). The exposure of phosphatidyl serine residues on the plasma membrane indicates early apoptosis. Phosphatidyl serine is a phospholipid that is present in the inner leaflet of the plasma membrane. And it moves to the outer surface when the membrane gets damaged. Annexin-V is a phospholipid binding protein which has strong affinity for phosphatidyl serine.

In MACS, Annexin-V conjugated paramagnetic beads bind to sperms with externalised phosphatidyl serine. The sperm cell suspension is incubated with annexin-V conjugated microbeads and then allowed to run through the MACS column which is placed inside a magnet. The sperm cells that are not bound to the beads elute through the column, leaving the apoptotic sperms behind (Fig.5). Faezah *et al*. (2014) used this technique in sorting of bovine spermatozoa and found improved sperm survival rates after cryopreservation. One demerit of MACS observed is that it can't remove white blood cells, immature germ cells and debris of semen (Said *et al*., 2015). So, it can be used in conjunction with other sorting methods.

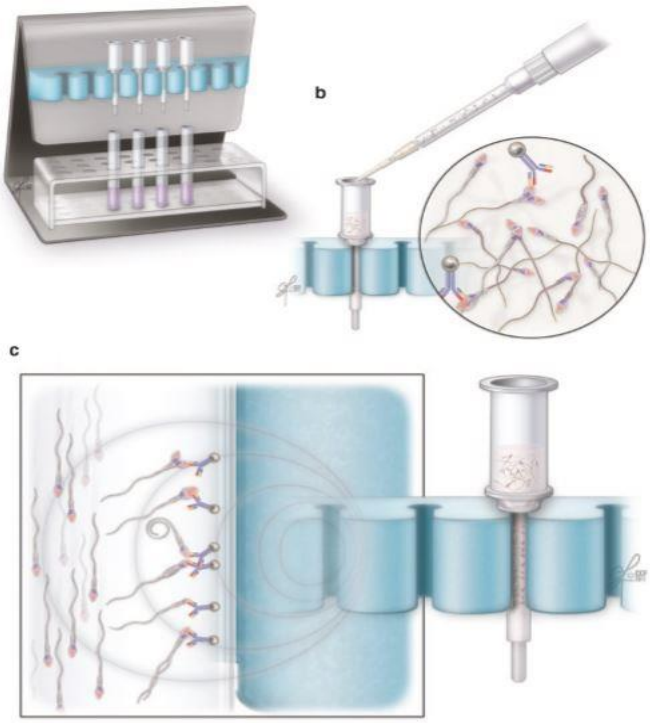



Fig.5. Sperm selection using MACS

PS binding properties of Annexin V can also be used along with glass wool filtration to improve the efficacy of sperm sorting.

**2.2.2. Fluorescence activated cell sorting (FACS)**

This technique is mainly employed in sperm sex sorting by flow cytometry in which the stained or labelled sperms are suspended in a laminar fluid flow. Afterwards each cell is hit by a laser beam and according to the emitted signal; deflection plates intervene to sort different sperm subpopulations. The X chromosome bearing spermatozoa have more DNA content and hence, adsorb more DNA specific stain such as Hoechst 33342.

But, the chemical and mechanical stress during sorting may increase the number of dead and damaged sperms. The dye and the laser exposure were found to be resulting in reduction of mitochondrial activity and thus motility of bovine spermatozoa (Carvalho *et al.,* 2010). In 2015, Quan *et al*. observed the detrimental effect of Hoechst 33342 staining on the viability of

ram spermatozoa. FACS based sperm sorting was reported to be inducing oxidative stress on mitochondria and DNA of stallion spermatozoa (Balao da Silva *et al.,* 2016).

Whereas, FACS using YO- PRO staining identifies changes in membrane permeability due to apoptosis (Ribeiro *et al.,* 2013) and this decreases the number of sperms with DNA fragmentation. Another fluorescent organic cation named proprietary fluorochrome (PF-1) which is able to detect the early changes in cell membrane damage can also be used in FACS (Funaro *et al*., 2013).

### 2.3. Selection of spermatozoa based on sperm zeta potential

Zeta potential is the electrical potential difference between the sperm membrane that is negatively charged and its surroundings. The negative charge is due to the presence of sialic acid containing glycoproteins of plasma membrane. The sperm zeta potential usually prevents intracellular interaction, self-agglutination and non-specific binding with the genital tract epithelium. Zeta potential is lower in sperms with more DNA damage. This property can also be used for selection of spermatozoa as follows.

#### 2.3.1. Electrophoresis

This can be done in a device with two inner chambers, two outer chambers and two external electrodes on their sides. The outer chambers are separated from the inner chambers by two polyacrylamide membranes with a 15 kDa pore size that allows the movement of small molecules, water and ions and retains the cell suspension within the inner chambers. The inner chambers actually consist of inoculation chamber and collection chamber which are separated by polycarbonate membrane with a 5 micro meter pore size to permit the transit of sperms and exclude the larger cells such as leukocytes and germ cells. Due to the potential applied to the two external platinum electrodes and to the circulation of the buffer solution through the chambers, sperms with negatively charged membrane (functionally intact) move from the inoculation chamber towards the collection chamber.

As the sialic acid content in the membrane of X and Y bearing spermatozoa is different, X bearing sperm is more negatively charged compared to Y bearing sperm. Hence, dielectrophoresis can be used for sex sorting of spermatozoa also.

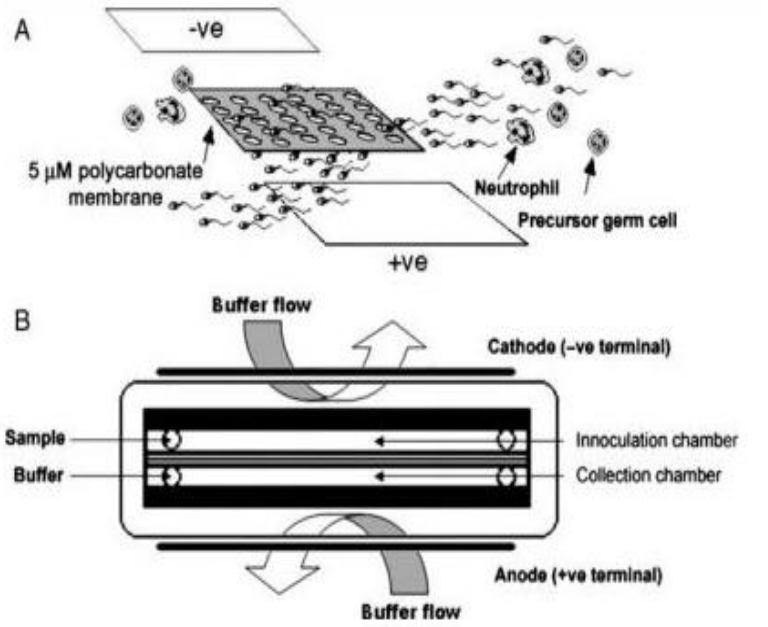



Fig. 6. Sperm sorting using electrophoresis

Electrophoresis is advantageous in such a way that it is fast and simple and also does not require centrifugation steps responsible for ROS production and chromatin damage. It is also able to remove immature germ cells and leukocytes. But, it requires expensive equipment and the separation should be carried out before capacitation and acrosome reaction as the zeta potential is disturbed after these events.

### 2.3.2. Zeta method

Here, the sperm sample is suspended in a protein free medium and submitted to centrifugation. Again, the sperm cells are transferred into fresh protein free medium and transferred to a tube. Then, the tube kept in a latex glove can be rotated a few times and removed from the gloves. The tube surface attains positive charge and the mature sperms adhere to the wall within

a minute (Fig.7) whereas the medium containing non-adhering sperms can be discarded. Then, the serum containing medium is added to neutralize the positive charge and to resuspend the adhering sperm cells.

Zeta method is advantageous with its simplicity, quickness, inexpensiveness and the lack of high voltage requirement. But, the lower sperm recovery rate limits its use. This may also result in reduction of sperm progressive motility and hence not preferred in cryopreserved semen.

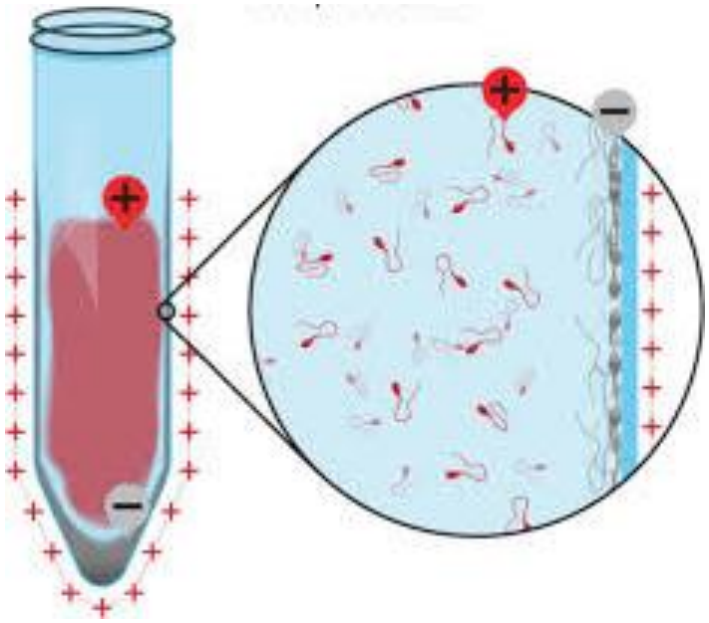


Fig.7. Zeta method of sperm selection

### 2.3.3. Microfluidic devices

Insulator based di-electrophoresis is such a technique to sort mature spermatozoa from immature cells. With a simple micro channel and cylindrical insulating structures, iDEP allows mature sperm cell immobilisation and enrichment. But, this technique also requires to improve purity and recovery rate.

## 2.4. Selection of spermatozoa based on sperm interaction properties

### 2.4.1. Hyaluronic acid binding assay (HBA)

Hyaluronic acid binding proteins (hyaladhesins) are the proteins involved in sperm-oocyte binding occurring during fertilisation. Only mature sperms that express receptors specific to hyaluronic acid can bind to the hyaluronic acid present in the extracellular matrix of cumulus oophorus and reach the oocyte for fertilisation. This physiological property is the basis of selecting mature spermatozoa with hyaluronic acid binding assay (Witt *et al.,* 2016).

Physiological ICSI (PICSI) sperm selection device is a dish with HA hydrogel micro spots on bottom. After a brief incubation, mature sperms bind their head to HA (Fig.8) and shake their tails around whereas freely moved sperms can be removed by gentle rinsing. The bound sperms can be picked up with an ICSI injection pipette.

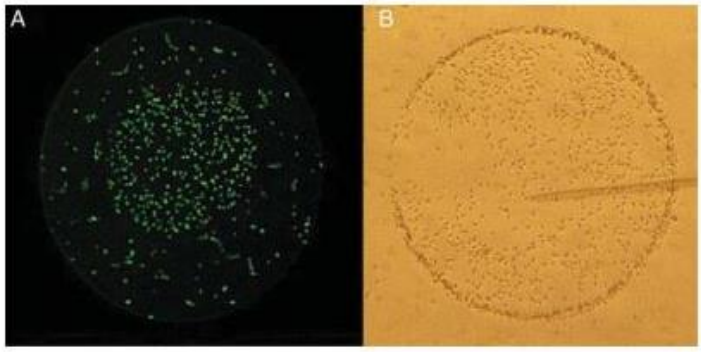



Fig.8. Sperm selection with Hyaluronic acid binding assay

In the sperm slow device, a micro drop of HA containing medium is connected to a micro drop of the sperm suspension and after a brief incubation, HA- bound sperms at the interface of the two micro drops are selected with an ICSI injection pipette. PICSI is less toxic than conventional ICSI in which PVP is employed to slow down motile sperms.

### 2.4.2. Zona pellucida binding assay (ZPBA)

The ZPBA consists in co-incubating supernumerary mature oocytes with sperm. The sperm cells attached to ZP are aspirated and microinjected. Here, the ZP sperm binding event occurring during fertilisation is reproduced *in vitro.*

### 2.4.3. Microfluidic devices

Chemotactic properties of spermatozoa are used here. Chemotaxis is the movement of cells in the direction of chemoattractant gradient that guides sperm cells towards the fertilisation site. The microfluidic device could be developed with acetylcholine gradient (Ko *et al*., 2012) or progesterone gradient (Zhang *et al.,* 2015) for sperm sorting. Advanced microfluidic devices with simultaneous chemical and temperature gradients are more effective than individual (Ko *et al.,* 2018).

## 2.5. Selection of spermatozoa based on sperm morphology

### 2.5.1. Motile sperm organelle morphology examination (MSOME) for Intracytoplasmic morphologically selected sperm injection (IMSI)

In MSOME, differential interferential contrast microscopy with ultra-high magnification (more than 6000 times) is used to select high quality sperm cells among sperm population within a PVP microdroplet covered with sterile paraffin oil in a sterile glass bottomed dish. Sperm head vacuoles and sperm organelle alterations such as acrosome, post acrosome, neck, mitochondria and tail abnormalities (Franco, 2015) can be evaluated with this (Fig.9).

The technique has the limitations of requirement of expensive equipment and skilled embryologist who should be able to select sperms rapidly as the prolonged incubation at 37°C may result in significant decrease of DNA integrity of spermatozoa.

### 2.5.2. Birefringence pattern examination

Sperm microtubules exhibit anisotropic properties which cause birefringence or double refraction if visualised under polarised light. Normal sperm nuclei, acrosome and motile flagella are characterized by an organized and highly compact texture which causes birefringence under polarised light

(Marzano *et al.,* 2020). The sub acrosomal protein filaments are longitudinally arranged in mature sperms and their nucleus exhibits higher birefringence. The sperm head with intact acrosome shows uniform refringence (Fig.9) whereas the acrosome reacted sperm head has birefringence localised in the post acrosomal region. But, the glass dish used for birefringence visualisation is more expensive than plastic dish.

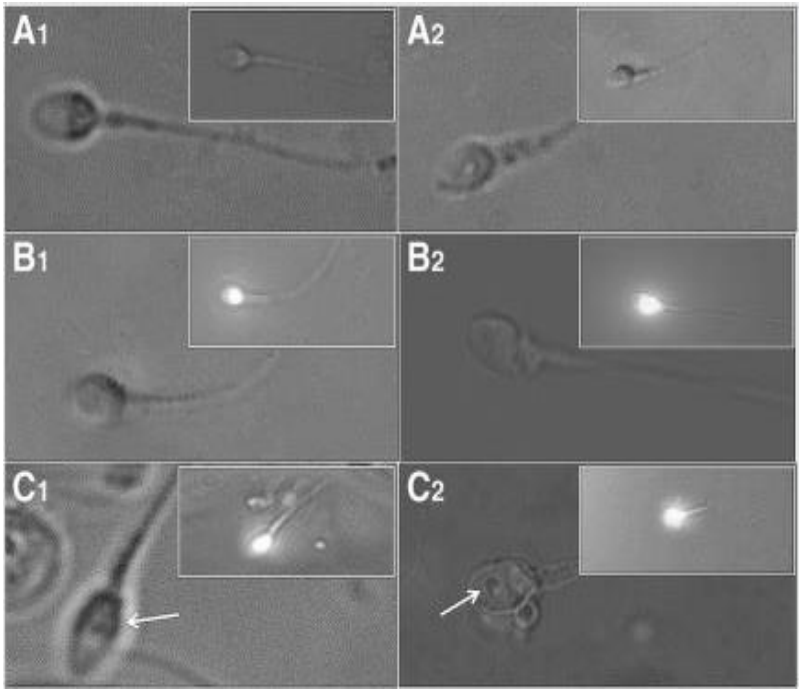



Fig.9. MSOME and Birefringence based sperm selection: A1-Non-birefringent, normal MSOME; A2-Non-birefringent, altered MSOME; B1-Birefringent, normal MSOME; B2-Birefringent, altered MSOME; C1-Birefringent, with small nuclear vacuoles; C2- Birefringent, altered MSOME due to large nuclear vacuoles

### 2.5.3. Microfluidic devices

De Wagenaar *et al.* (2016) developed an impedence based microchip for non-invasive identification of abnormal porcine spermatozoa in which sperms were put inside a 20 µm high and 20 µm wide microfluidic channel. Two electrode pairs were used to measure the impedence in a differential mode.

Cytoplasmic droplet containing sperm cells have a different shape detectable as a bump in the impedence plot.

In another novel microchip, a pump system maintains constant flow through the microchannel in which the sperm 3D morphology can be assessed with interferometric phase microscopy (IPM) on the basis of optical path delay (OPD) maps.

### 2.6. Selection of spermatozoa based on sperm membrane integrity

#### 2.6.1. Hypo osmotic sperm swelling test

The spermatozoa with intact membrane incubated in hypo-osmotic solution for a particular period will show coiled tail due to the influx of water and expansion of membrane. As the axonemal complex of sperm tail are closely surrounded by the plasma membrane, ballooning of plasma membrane results in curling or bending of tail. This change can be assessed with a phase contrast microscope. Thus, HOST is used to identify viable spermatozoa for ICSI.

#### 2.6.2. Motility activating substances

In case of viable sperms from immotile sperm population, certain chemical substances can be used to activate sperm motility. Eg. Pentoxifylline, Theophylline and Papaverine. They are phosphodiesterase inhibitors causing an increase in intracellular cAMP levels which induce sperm motility. Pentoxifylline is found to be increasing creatine kinase activity and nitric oxide production in spermatozoa which are involved in sperm motility.

This method is advantageous in such a way that it has immediate effect in enhancing sperm motility and thus easier and shorter selection. But, the effect of these substances on subsequent embryo development is not well studied.

#### 2.6.3. Laser Assisted Immotile Sperm Selection (LAISS)

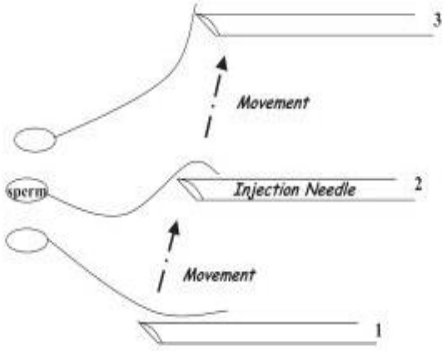
In LAISS, application of a single laser beam to the far end of tail of an immotile sperm causes curling of the tail in viable sperms only. This allows discrimination between viable and dead immotile spermatozoa. The laser beam causes an instantaneous

opening of the membrane pores in viable sperms through which influx of medium into the tail occurs and curling of tail happens. This method is found to be fast and safe but it needs expensive equipment and experienced embryologist.

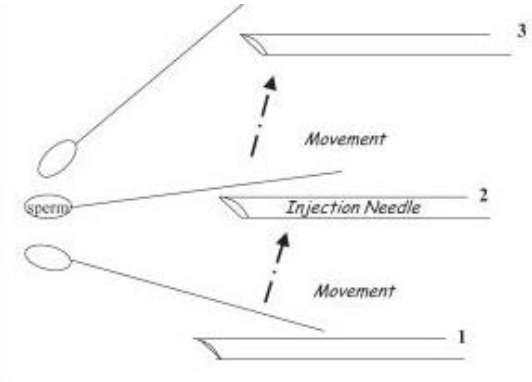
**2.6.4. Sperm tail flexibility test (STFT)**

Here, the sperm tail is mechanically touched with injection needle. The flexible sperm (viable) tail moves up and down independently of the head movement whereas an inflexible sperm (dead) tail moves together with the sperm head.

This is an easy and cost-effective method for selection of viable immotile sperm, but requires experienced embryologist.




A




B

Fig. 10. STFT positive (A) and negative (B) sperms

### 2.6.5. Microfluidic devices

Optoelectronic tweezers (OET) based sperm assay can be used to select completely immotile but, viable sperm. OET uses a light induced dielectrophoretic force to distinguish live and dead cells as they respond differently to the electric field.

**Conclusion**

The various methods for selection of spermatozoa described here have their own pros and cons. The conventional selection methods such as swim up and density gradient centrifugation are most commonly used, but there is a great controversy exists in literature regarding their negative effects on sperm cells. However, microscopy-based techniques and other advanced methods like LAISS, STFT etc. are more expensive or technically complex to be implemented in the normal ART settings.

Actually, an ideal sperm sorting method in ART should efficiently select healthy, motile and morphologically normal sperms capable of fertilising oocytes. The procedure should be non-invasive as the same sperm has to be used for fertilisation. Hence, it is better to couple more than one technique to improve the quality and quantity of selected spermatozoa.

Microchips are more advantageous that they can offer the sperm sorting in an optimized automated procedure and reduce sperm loss due to complex protocols and multiple transfers. Thus, it is possible to avoid multiple manual steps and minimize the human errors.

Selection of best spermatozoa from the ejaculates of genetically valuable animals of rare breeds or endangered species may improve fertility in ART and thus facilitate the success of conservation programmes. Removal of pathogens during sperm selection procedure enhances biosecurity and also reduces the non-therapeutic usage of antibiotics in semen.

As future perspective, research should be focussed on the application of the advanced techniques in ART of animals also. And the complexity of the devices has to be reduced to be practically applied in a normal ART setting. Their cost effectiveness should also be considered. Selection of robust

spermatozoa from an ejaculate and therewith reduction of sperm concentration per dose are also to be studied for improving the artificial breeding programmes.

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# PROGNOSTIC MARKERS OF CANINE PYOMETRA

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***ABSTRACT-***

*Pyometra is a life-threatening systemic illness among female dogs, characterized by inflammation, bacterial infection and consequent pus accumulation in the uterus. Due to uterine rupture, endotoxemia, sepsis, multi-organ dysfunction syndrome and septic shock, a clinically stable pyometra can transform into an emergency in a few hours. Markers that can predict the complications, outcome and treatment response would be of use in clinical practice for optimizing therapy and increasing survival. Although various clinical and haemato-biochemical parameters act as prognostic/diagnostic markers, none of these is specific for pyometra and a putative marker is yet to be identified.*

***Key words****: Bitch, Pyometra, Prognosticmarkers*

## INTRODUCTION

Pyometra is described as a poly-systemic disease affecting sexually mature female dogs, mostly during diestrus, characterised by accumulation of pus in the uterus and sometimes affecting all layers of the uterus. It is usually diagnosed from four weeks to four months after oestrus and is

reported in almost 25 per cent of all intact bitches before they are 10 years old; in high risk breeds, incidence may reach up to 50 per cent.

Progesterone plays an important role in disease development, by stimulating proliferation and secretion of the uterine glands, decreasing contractions of the myometrium and diminishing the immune response. This will facilitate intrauterine bacterial attachment and optimal conditions for bacterial growth. Oestrogen further enhances the ill-effects of progesterone.

Gram-negative bacteria, mainly *Escherichia coli (E. coli)* are most often isolated from the uterus of the affected animals. Endotoxin is a lipopolysaccharide part of the outer cell wall of Gram-negative bacteria, which is released during bacterial growth or death. Endotoxin present in the blood stream is a potent inducer of systemic inflammation. Thus, dogs with pyometra may suffer from sepsis which is a serious condition, frequently leading to organ dysfunctions, septic shock and death. Although mortality rate in pyometra is relatively low (3-4%), in severe cases with shock, mortality rate may reach up to 35 per cent and can be as high as 50 per cent, if peritonitis is present.

Depending on the functional patency of the cervix, pyometra is classified as closed (without vaginal discharge) or open cervix pyometra (with vaginal discharge). Closed cervix pyometra is a medical emergency that requires rapid intervention to prevent overwhelming sepsis and the potential of patient death.

Although the most obvious clinical sign is vaginal discharge, which may vary from serosanguinious to mucopurulent, signs of pyometra are not limited to genital tract alone. Other frequently reported clinical signs include anorexia, vomiting, polydipsia, polyuria and lethargy. Clinical signs are often more severe in bitches when the cervical canal is occluded and these animals frequently have a distended abdomen and severe lethargy. Polyuria and polydipsia are common features of

pyometra. Traditionally, the treatment of choice for canine pyometra is ovario-hysterectomy (OHE).

Uncontrollable inflammation in pyometra could develop into severe sepsis and endotoxaemia, which further progress to disseminated intravascular coagulation, multi-organ failure, shock and death. The risk of endotoxaemia and uterine rupture could make a clinically stable pyometra, an emergency within a short period of time. Prompt prognosis would help to predict outcome and presence of complications, which is of value in clinical practice for optimizing therapy and increasing survival. A cage-side rapid and cost-effective prognostic test would be highly valuable in such situations.

Markers or variables useful for prognostication can be derived from case history or physical examination parameters, results from inflammatory response and various other laboratory analyses or even other biological factors (Hagman, 2014).Prognostic markers used in canine pyometra studies are listed below.

**1. Clinical markers**

Clinical signs and physical examination findings evaluated at the time of admission could be useful as predictive indicators. Cases presented with distended abdomen and without any vaginal discharge is an indication of closed-cervix pyometra and hence at higher risk. Vomiting and diarrhoea are strong indications of high endotoxin level and hence at high risk. Subnormal temperature is an indication of endotoxaemia and poor prognosis. Vomiting, polyuria and polydipsia are indications of renal affection and hence severity of disease. Pale mucus membrane indicates anaemia and is associated with three fold increased risk of prolonged hospitalisation.

Considerable variations in physiological parameters like temperature, pulse, respiratory rate and heart rate is an indication of systemic affection caused by endotoxaemia and hence high risk.

**Systemic Inflammatory Response Syndrome (SIRS)**

The presence of SIRS has been detected in over 50 per cent of affected bitches and has been linked with poorer prognosis. Assessment of SIRS is important in order to determine severity of the disease, optimise treatments and prevent fatal outcomes, especially in patients at a high risk of developing shock or multiple organ dysfunctions.

In humans, clinical criteria have been established in order to recognize SIRS and these criteria have shown a significant correlation with mortality and morbidity. The limits for clinical criteria used in human have been adapted for use in dogs. The clinical criteria for dog, as presented by Purvis & Kirby (1994), Hardie (1995) and Hauptman *et al*. (1997), is presented below

| Criteria | Purvis & Kirby (1994) | Hardie (1995) | Hauptman *et al.* (1997) |
|---|---|---|---|
| Temperature (°C) | <37.8; >39.7 | <38.0; >40.0 | <38.1; >39.2 |
| Heart rate beats/min) | >160 | >120 | >120 |
| Respiratory rate (breaths/min) | >20 | >20 | >20 |
| WBC ($10^3$/µl); band neutrophils | <4, >12; >10 % | <5, >18; >5 % | <6, >16; >3 % |

SIRS is identified clinically in dogs by the presence of any two of the four criteria.

**Ultrasonographic imaging for prognostic evaluation**

Trans-abdominal ultrasonography is a non-invasive, accurate procedure for the qualitative and quantitative assessment of canine pyometra. Ultrasound examination can evaluate endometrial integrity, variation of uterine wall thickness, uterine distension and cystic endometrial glands. The findings of ultrasound examination are valuable, because the treatment protocol will change in accordance with the severity of condition observed. An evaluation of the efficacy of the treatment is possible by serial assessment of uterine horn width and luminal contents by ultrasonography, together with clinical parameters, haematology and biochemistry after the start of treatment.

Local inflammatory reactions in the endometrium during pyometra result in increase in the intrauterine concentration of

PGE, leading to an increase of uterine perfusion. Doppler ultrasound is a useful tool for evaluation of treatment response by sonographic evaluation of the hemodynamic parameters. Pyometra can cause increase in uterine blood flow velocity, marked by low blood flow resistance and pulsatility. In comparison to CEH and normal diestrous bitches, animals with pyometra have an increased peak systolic velocity and end diastolic velocity and lower resistance index (Batista *et al*., 2016). The values will change in accordance with severity of uterine infection and help in prediction of prognosis and treatment result.

## 2. Haematological markers

In pyometra, the degree of systemic illness is reflected as alterations of haematological variables. Leucocytosis with neutrophilia and left shift are often usually observed in pyometra as an indication of active infection. Band cells are immature neutrophils and their high per cent indicates active inflammatory process. Presence of toxic neutrophils is a clear indication of sepsis and is associated with disease severity and case fatality. Toxic changes in the neutrophils are the collection of cytoplasmic alterations (including cytoplasmic basophilia, cytoplasmic vacuolation, Dohle bodies and toxic granulation), resulting from accelerated production in the bone marrow. When toxic changes are moderate to severe and many cells are affected, it will be accompanied by signs such as fever, vomiting, diarrhoea, depression, shock and sepsis.

**Different grades of toxic neutrophils**

| Severity of toxicity | Morphologic characteristics of toxic neutrophils |
|---|---|
| 1+ | Only few to moderate number of Dohle bodies are present in clear cytoplasm |
| 2+ or 3+ | Mainly variable intensity of cytoplasmic basophilia, cytoplasmic foaminess, but also giant neutrophils or toxic granulation |
| 4+ | Cytoplasm is too blue, vacuolated, and nuclei are too rounded to differentiate toxic neutrophils from reactive monocytes or reactive lymphocytes |

Leucopenia is the most important predictive variable, associated with increased risk for peritonitis.

A normocytic, normochromic anemia is thought to reflect the chronicity of the disease, decreased erythropoiesis caused by toxic suppression of bone marrow, non-availability of free iron due to sequestration in myeloid cells and loss of erythrocytes into uterus.

Thrombocytopenia may be a result of bone marrow toxic affection.

**3. Biochemical markers**

Pyometra being a systemic illness, vital organ functions can be altered, including kidney and liver, which will be indicated by elevated creatinine and blood urea nitrogen (BUN) concentrations, hypoalbuminemia, proteinuria etc. Impaired renal function might be due to tubule-interstitial inflammation or immune-complex associated glomerulo-nephritis, resulting in glomerular and tubular dysfunction. Increased serum concentrations of alkaline phosphatase, bilirubin and cholesterol may indicate intrahepatic cholestasis which also has been suggested as a possible consequence of endotoxaemia. Elevated BUN and creatinine levels are associated with higher risk of mortality.

**Serum endotoxin concentration**

Disseminated bacterial infection, endotoxaemia and coagulation disturbances may lead to dysfunction of several organs. The plasma endotoxin concentrations are related to outcome of pyometra. Okano *et al*. (1998) correlated the endotoxin concentrations with the prognosis of pyometra, as mentioned below.

| **Serum endotoxin concentration** | **Prognosis** |
|---|---|
| 3.4±2.8 pg/mL | Healthy dog |
| 9.5±11.3 pg/mL | Good prognosis |
| 74.2±18.3 pg/mL | Poor prognosis |

**Serum Prostaglandin $F_2\alpha$ metabolite (PGFM) concentration**

Uterine endometrium is known to synthesise and release prostaglandins; mainly prostaglandin $F_2\alpha$ ($PGF_2\alpha$). The systemic release of $PGF_2\alpha$ can be assessed by measurement of its more stable metabolite 15-keto-13, 14-dihydro-$PGF_2\alpha$ (PG-metabolite). In several species, including canines, increased plasma levels of PG-metabolite have been demonstrated in pathological inflammatory conditions of the uterus. Analysis of PG-metabolite could facilitate the differentiation of other conditions without inflammation (CEH/mucometra) from pyometra and possibly also predict severity of the condition (Hagman *et al.,* 2006).

**Serum Acute phase protein (APP) concentrations**

When the inflammatory cells (e.g. macrophages or granulocytes) are activated by different inflammatory stimuli, the acute phase response is triggered and different cytokines are released from the inflammatory cells, e.g. interleukin 6 (IL-6), IL-8, IL-1 and TNF-alpha. These cytokines (especially IL-6) then induces enhanced production of acute phase proteins (APPs) in the liver, such as C Reactive Protein (CRP), serum amyloid A (SAA), haptoglobin (Hp), fibrinogen or albumin. Blood concentrations of APPs have been widely used as diagnostic and prognostic tools in human medicine and are increasingly used in veterinary medicine also; in dogs, CRP and SAA being most often assessed in clinical studies. Since SAA and CRP concentrations have been shown to increase in trauma, infectious diseases and malignancy; their analyses are not specific for pyometra alone.

**Serum C-reactive protein concentration**

The CRP is produced in the liver by hepatocytes in response to inflammatory stimuli. Function of CRP is to bind many different molecules that are released or present during inflammation, such as different cell fragments, bacteria and chromatin. Cytokines (especially IL-6) induces an enhanced CRP-production in the liver within about 4-6 h; according to the intensity of inflammatory activity, the CRP concentration varies. Following treatment of dogs for infections, CRP concentrations drops within one day of treatment. This rapid response makes

CRP an almost real-time marker of inflammatory activity and response to treatment (Gebhardt *et al*., 2014).

**Serum Albumin concentration**

Hypoalbuminemia is a common finding in dogs with pyometra (negative acute phase protein). As a result of increased hepatic production of positive APPs and other inflammatory components, albumin production decreases during inflammation. Hypoalbuminemia may also reflect increased vascular permeability in response to inflammation or increased loss via kidneys. In humans with renal disease and who require hemodialysis, hypoalbuminemia is associated with increased risk of mortality. The value of albumin for prognostication in pyometra has not yet been explored.

**Serum lactate concentration**

Blood lactate analysis is clinically valuable in predicting prognosis and survival, evaluating tissue perfusion and treatment response in human and veterinary critical care settings. Lactate is end product of anaerobic metabolism of glucose; hyperlactataemia develops when the rate of elimination decreases than the rate of production. Increased level of blood lactate results from decreased tissue oxygenation due to hypo-perfusion, changes in the glycolytic enzyme system and hyper-metabolism during inflammatory process. In pyometra, lactic acidosis may be induced by hypo-perfusion, SIRS and septic shock. Early identification of tissue hypoxia is an advantage for rapid intervention. Not only hypovolemia or oxygen debt but also metabolic conditions such as hepatic malfunction with decreased lactate uptake or diabetic keto-acidosis may induce hyperlactatemia.

**Serum Cardiac troponin I concentration**

Myocardial damage can be caused by multiple conditions including ischemia, trauma, toxins or inflammation. Canine pyometra is known to lead to systemic inflammation, potentially affecting multiple organs in the body, including the heart. Cardiac-specific Troponin I (cTnI) is currently the most sensitive and specific marker of myocardial cell damage in the dog. It is a

protein that is expressed at high concentrations only in the myocardium. When cardiac myocytes are damaged, cTnI leaks into the bloodstream and can be detected in serum; half-life of cTnI is reported to be 120 min (Schober, 2005). In normal dogs serum concentrations of cTnI are low or, most often, undetectable. Although the most effective treatment for canine pyometra is OHE, anaesthesia and surgery may cause myocardial ischemia with subsequent myocardial cell damage, especially in individuals with systemic inflammation and impaired circulation (Schober, 2005). Hence, mild to moderate increases in cTnI appears to be common in dogs with pyometra before and after surgery (Pelander, 2008). Another possible reason for myocardial injury during anaesthesia could be direct toxic effects of the anaesthetic agents. Detection of damaged myocardium may be useful for the clinician to take actions to avoid adverse cardiac events, by monitoring the dog during the peri-operative period and intervene early when indicated.

**Serum Insulin- like growth factor-I concentration**

Insulin-like growth factor-I (IGF-I) is an anabolic peptide mediated by growth hormone which has many roles including control of cell proliferation, cell differentiation and anti-apoptosis. In endotoxaemia and chronic inflammation, IGF-I is down-regulated, leading to decreased circulating IGF-I concentrations. Thus IGF-I is act as a negative inflammatory marker in dogs. However, other factors such as age, gender, nutrition status and diseases such as *diabetes mellitus*, could influence concentrations of IGF-I in the circulation, which should be considered while using this parameter to assess inflammation or infection.

**Serum Iron concentration**

Plasma iron concentrations are regulated by the hormone hepcidin, originating from the liver. Production of hepcidin leads to decreased concentrations of iron in plasma. The production of hepcidin can be induced by cytokines (e.g. IL-6) and bacterial infection, leading to sub-normal serum iron concentrations.Thus iron is considered as negative marker in pyometra.

**Serum glucose level**

In surgically treated bitches, peritonitis is associated with hypoglycemia. The finding that hypoglycemia has predictive value is in agreement with the results of the study of septic human patients, showing that low blood glucose is associated with a high risk of mortality in sepsis caused by *E. coli.*

**Serum inflammatory mediators**

In pyometra, *E.coli* endotoxin, a lipopolysaccharide, binds to the receptor complexes such as cluster of differentiation 14 (CD14), myeloid differentiation protein-2 (MD-2) and toll like receptor 4 (TLR4), which will stimulate up-regulation of cascade that culminate in the secretion of pro-inflammatory cytokines including interleukins (IL-1, IL-6, IL-8) and tumour necrosis factor-r (TNFr) as well as secondary inflammatory mediators such as prostaglandins (PG), nitric oxide (NO) and reactive oxygen species (ROS). Elevated concentration of PGFM, endotoxin, IL-6 and IL-8 are noticed during the analysis of serum levels of inflammatory mediators in bitches with pyometra. High serum levels of IL-8 are observed in pyometra with SIRS, suggesting IL-8 as a useful biomarker of severity of uterine infection.

**4. Urinary markers**

Proteinuria is a common feature of pyometra-associated renal dysfunction and there is an association between proteinuria and progression of disease. Proteinuria can be quantified and monitored using urine protein and creatinine ratio (UPC). Dogs with pyometra and UPC more than one is likely to have clinically relevant renal histologic lesions and require monitoring even after OHE. Complementary to UPC, serum renal variables and other urinary biomarkers can be useful for evaluating functional renal abnormalities. Urinary C-reactive protein, albumin and immunoglobulin G provide information about alternation in glomerular permeability. Tubular dysfunction is reflected by increased concentrations of urinary retinol-binding protein and N-acetyl-b-D-glucosaminidase (NAG).

Urinary thromboxane B2 is useful to evaluate intra-renal hemodynamics (Maddens *et al.*, 2011).

## 5. Histological markers

Histopathological analysis of biopsy specimens of uterus and ovaries aids in grading of lesion, thus help for prognostic evaluation of the disease and establish the best therapeutic approach. The degenerative, haemorrhagic and other pathological changes in the uterus may be caused by the toxic factors present in the bacteria and lesions may vary accordingly. The increased thickness of the uterine wall in dogs commonly accompanies a CEH process; it is identified in early stage only by the histological exam in tissue sections. The CEH will predispose to pyometra even in young animals and also has increased chance of recurrence. Those cases with inflammatory cell infiltration, endometrial cyst formation, necrosis, oedema, haemorrhages and squamous metaplasia of endometrium are considered as hyperplastic pyometra. Atrophic pyometra is characterised by dense endometrial atrophy without myometrial or glands and stroma hyperplasia and endometrial cysts and indicates chronicity of the condition.

## 6. Bacteriological markers

Infection in pyometra could be caused by different types of bacteria (Gram +ve and Gram –ve); depending on the type of bacteria involved, severity of the disease also differs. Usually Gram –ve bacteria like *E. coli* causes severe endotoxaemia. Among the *E. coli,* those with genes for virulence factors *fim, sfa, afa1*, *cnf1*, *hlyA* and *pap* is responsible for more severe infection; α-hemolysin (*hlyA*) causes haemolytic *E. coli* infection. The *hlyA* is able to lyse erythrocytes and nucleated host cells at high concentrations. At sub-lytic concentrations, it can disrupt immune signalling and cyto-skeletal components. In both haemolytic and non-haemolytic *E. coli* affected pyometra cases, clinical signs, haematological and blood biochemical values are similar. But more extensive endometrial damage and metritis is noticed in the former infection. Therefore characterization of

virulence factor genes especially *hlyA* has importance in prognostication of the disease.

**Conclusion**

In pyometra, the degree of systemic illness is reflected as alterations of clinical, haematological and biochemical variables and hence these variables act as prognostic markers of the disease. Similarly, alterations in some of the urinary variables, histological changes and bacteriological studies also help for assessing severity of the condition and thus for predicting the chances of recovery/mortality.

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# TRANSITION OF DIGESTION IN DAIRY CALVES

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***ABSTRACT-***

*Calves when born have a physically and metabolically underdeveloped rumen and initially rely on milk to meet their nutrient demands for maintenance and growth. Beginning of solid feed consumption, acquisition of anaerobic microbes, establishment of rumen fermentation, increase in rumen volume, differentiation and growth of rumen papillae, ability of digestive system to absorb digested nutrients, absorbed nutrients entering different metabolic pathways, maturation of salivary apparatus and development of rumination behaviour are all needed as the calf weans from milk to solid feed.The provision of fermentable carbohydrates in starter feeds may affect rumen development and that forage supplementation is beneficial for promoting development of the gut and rumination behaviour in young calves. It is important to note that both the physical form of starter diets and their nutritional composition affect various aspects of development in calves. Various strategies to promote the physiological and microbiological development of rumen by modifying the diet, by adding feed additives and feeding management like intensive milk feeding, early weaning, socialisation or grouping with the peers has affected the growth and development of rumen.*

***Key words****: Calf, Milk feeding, Management, Rumen development*

## INTRODUCTION

Ruminants are considered as latecomers in evolution. They are very successful herbivores. They have a peculiar reticuloruminal sorting mechanism that retains food requiring further digestion but clears the forestomach of already digested material. When a calf is born, it has a pre-ruminant digestive system.They have the same four stomachs as an adult but the rumen is significantly smaller. In the calf, the largest part of digestive tract is the abomasum making up nearly 60-70 per cent of the stomach. By the time the calf is 3-4 months old, the abomasum makes up to 20 percent capacity and as the animal matures, that shrinks to only 8 percent of the stomach capacity. A primary objective of all calf rearing systems is to get the calf transformed to ruminant without setbacks to calf's performance. Rearing healthy calves is very important as it can have a significant impact on their growth and milk production performance in adult life. However, due to the poor immunity and the incomplete development of the digestive system in young calves, any interference from the external environment or changes to the nutrition can drastically affect the development of calves.

On many dairy farms, calves are separated from their dams at birth and reared artificially. Calves are born with a non-functional rumen and must initially rely exclusively on milk to meet the nutrient demands of growth and maintenance. The time for milk digestion in a calf is 12-18 hours. A smooth transition from liquid to solid feed allows calves to consume and digest sufficient solid feed to support growth during and after weaning; this transition requires the physical and metabolic development of the rumen and coincides with the development of the salivary apparatus, rumination behaviour, and several physiological adjustments at the gut, hepatic, and tissue levels (Khan *et al*., 2011). The nature of solid feed and the amount consumed can influence rumen development. Calf starter feeds, containing easily fermentable carbohydrates stimulate rumen development, including changes in the epithelium of the fore stomach. In contrast, calves reared by their dams in extensive housing

systems will not have access to starter feeds. Under forage-based livestock production systems, forage quality and availability determines the need for supplementary feeds to support growth of young ruminants. However, in nature, milk and pasture provide the majority of the stimulants and nutrients required for growth and development to young ruminants.

**Gastric digestion in young ruminants**

The development of gastric digestion may be considered as having four phases;

1) The newborn phase (0 to 24 hours)
2) The preruminant phase (1 day to 3 weeks)
3) The transitional phase (3 to 8 weeks)
4) The immediate pre-weaning and post-weaning phase (8 weeks to adulthood)

**The newborn phase (0 to 24 hours)**

At birth the fore-stomach (rumen) is small and non-functional. It represents only 39 percent of total stomach. It contains no microbes and the papillae are rudimentary. In the calf, the largest part of the digestive tract is the abomasum (fourth stomach). The abomasum secretes no acid or pepsinogen. So the colostral antibodies pass through the stomach undigested and are absorbed intact through intestinal mucosa by pinocytosis. The antitrypsin factor in colostrum also prevents their degradation in intestine. The facility to transport immunoglobulins without digestion and to absorb them intact is limited to first 24 to 48 hours of birth. Establishment of rumen microflora is necessary for the physiological development of the rumen and for the animal's ability to convert plant mass into products that can be utilized by the animal for maintenance and production. During the first hours of life, the forestomach is rapidly colonized by bacteria and the microbial density in the rumen quickly reaches concentrations as high as $10^9$cells/mL. Colostrum promotes the growth of microbes (mainly lactobacilli) and these gain access to the gut increasingly with each bouts of sucking. Faecal contamination facilitates various bacteria like

*E.coli, Streptococci* and *Clostridium* to gain entry in intestine within 8 to 16 hours after birth. Their access is also facilitated by the lack of abomasal acid.

**The pre ruminant phase (1 day to 3 weeks)**

During this period principal food is milk. In the first weeks of life, rennin is the predominant enzyme in the digestive system of the calf. Rennin allows the calf to efficiently utilise the proteins in milk. In time, as the level of the enzyme pepsin increases, the calf is able to utilise non-milk sources of protein. For this reason, milk replacers that contain non-milk protein should not be fed to the calf in the first three weeks of life. For the first three to four weeks of life, the enzyme lactase also predominates, and the calf can efficiently utilise lactose, the main carbohydrate in milk. Now the calf is unable to utilise starch.The saliva contains pre-gastric esterase that start hydrolysis of milk lipids. Sucking of milk causes closure of reticular groove and milk ends up in abomasum. The main determinant of reflux closure is the hunger drive of the calf and is not consistently affected by other factors such as the position of head and whether sucking is from a teat or from a bucket.

**The transitional phase (3 to 8 weeks)**

Bacterial fermentation begins in the rumen when a calf consumes water and starter concentrates. This generates large amounts of Volatile Fatty Acids (VFAs) in the form of acetate, butyrate and propionate. This production of VFAs leads to rapid rumen development. The time it takes for the calf to change from using just the abomasum to efficiently using all four stomachs depends on the type of food it is fed. If milk is freely available for a long time, the calf will have only a small appetite for dry feeds and rumen development is slow. If the feed management encourages the calf to eat calf starter, rumen development is enhanced and the calf reduces its dependence on liquid milk as a source of essential nutrients. If the calf is on a restricted liquid diet and has access to solid feed, this transition from pre-ruminant to ruminant digestion can be completed at about six weeks of age.

**The immediate pre weaning and post weaning phase**

During ruminant digestion micro-organisms transform carbohydrate, protein and all other fermentable substances into volatile fatty acids, ammonia, methane, carbon dioxide and microbial protein. The ruminant phase begins at about six to eight weeks of age. Unless the animal continues to receive milk reflex closure of reticular groove becomes erratic and is usually absent in old animals. At this point, solid feed is the sole source of feed, and the rumen accounts for approximately 70% of all stomach compartments. A calf will usually have full rumen development at 12 weeks of age. The ability of calf to eat and digest dry food will then be more or less similar to that of an adult animal. Pepsinogen replaces rennin and the digestive system can utilise non-milk proteins.

**Rumen Development**

Significant changes in rumen have to occur first before the calves can digest dry feed for their own growth needs. The specific changes include the development of the rumen organ and rumen epithelium, and the establishment of rumen microbiota.

Rumen development in new-born calves is one of the most important focus areas of calf nutrition. The esophageal groove, namely the rumino-reticular groove, is one of the unique features inside the gastrointestinal (GI) tract of calves. The majority of the liquid feed, such as colostrum, whole milk and milk replacer (MR), can bypass the rumen, reticulum andomasum, and flow directly into the abomasum as a result of the reflex closure of the esophageal groove. The abomasum of new-born calves is the only fully developed and functional stomach, and is also the most important digestive organ for calves at birth. The digestion of fat, carbohydrates, and protein is predominantly dependent on the digestive enzymes secreted by the abomasum and small intestine, which is similar to the digestive system in mono-gastric animals. Over time, with the increase in dry feed intake, the rumen begins to develop and starts to play more important digestive roles.

**Rumen Epithelium**

The epithelium of rumen plays the key role in rumen development, including absorption, transportation, short-chain fatty acid metabolism, and protection. The proliferation and growth of the rumen squamous epithelium promotes the growth of papillae in length and width, and increases the thickness of the rumen wall. However, papillae per square centimetre are not used as an indicator of rumen development. New-born calves have a smooth epithelium with no prominent papillae. Calves fed solely with liquid feed have been shown to have limited rumen development characterized by decrease in rumen weight, papillary growth, degree of keratinisation, pigmentation and musculature development. Increased intake of solid feed contributes to the rapid development of ruminal fermentation. As calves consume more starter feed, rumen digesta pH decreases, whereas volatile fatty acid (VFA) concentration gradually increases during the first two months. The molar proportion of acetate decreases during the first two months, and then starts to increase until nine months of age as forage intake increases. The presence and absorption of VFAs in the rumen provides chemical stimuli required for the proliferation of rumen epithelium. Intraruminal administration of acetate, propionate, and butyrate can stimulate the growth of rumen epithelium in young ruminants (Wang and Jiang, 2010). Among different VFAs, the effect of butyrate being the most prominent, followed by propionate. Rumen papilla proliferation is associated with increased blood flow through the rumen wall and a direct effect of butyrate and propionate on gene expression.

**Ruminal Microbiota**

At birth, the GI tract of ruminants is sterile. During the early hours of life, the fore-stomach becomes rapidly colonized with millions of bacterial population. The neonates acquire bacteria from the dam, partners, feed, housing and environment. By two days of age, the rumen microflora reaches $10^9$ cells/mL. The establishment of these rumen bacteria occurs long before young ruminants have access to concentrated feed or forage.

Several fungal units observed in weaned calves are also present in adults. As fungi mainly colonize fibrous solids, this may suggest an introduction of forage allows previously low-abundant or transient fungi to persist and multiply.

**Strategies to Promote Rumen Development**

Strategies to promote morphological structure and metabolic function of rumen in pre-ruminants are an ongoing issue. Numerous studies and approaches attempt to modulate rumen fermentation and the microbial community in young ruminants to accelerate rumen development. These approaches include alteration of diet composition and physical forms, addition of new types of feed additives, and introduction of variables in the feeding management.

**I. Diet**

**A. Liquid Feed**

Liquid feed may affect plasma concentration of hormones and growth factors, such as insulinand IGF-1, which play important roles in stimulating proliferation of rumen epithelial cells.Colostrum contains many biologically active substances, mainly polypeptide growth factors and steroid hormones, insulin, insulin like growth factor (IGF-1), and transforming growth factor (TGF). Intake of colostrum has been associated with the development, digestion, and absorption ability of the GI tract in the new-born calves. Moreover, a whole milk calf diet was shown to have a positive effect on milkyield during the first lactation of the adults compared to calves fed a milk replacer (MR) diet. Soybean protein can be used as an alternative to milk protein in formulating milk replacer. The abomasal pH declines more slowly and pH is higher in calves fed MR containing soy flour compared to calves given whole milk. Decreasing the pH of MR emulsion by addition of an acidifier reduces the pH of digesta pH in the rumen, reticulum, and omasum. Specifically, pH reduction of MR emulsion was found to be beneficial for the development of ruminal epithelium.

**B. Calf starter**

Feeding readily fermentable carbohydrates to calves increases VFA production in the rumen, which is necessary to stimulate the development of rumen epithelium. Calves fed milk-only diet during the first three weeks present with a different microbial community in their GI tract and faeces compared to calves given milk and solid feed. Diets differing in carbohydrate composition lead to differences in rumen fermentation patterns and VFA profiles which may have a variable effect on rumen development. For example, high concentrations of ruminal ammonia, acetate, propionate and butyrate were detected in calves fed corn- and wheat-based diets compared to calves fed barley and oat-based diets. Moreover, the fore-stomach weight and papillae growth were greater in calves fed corn- and wheat-based diets. The mucosal thickness was greater in veal calves fed starch and pectin-based diets compared to calves on neutral detergent fiber (NDF)-based diets. It was reported that the stimulatory effects of VFAs are different, with butyrate being most stimulatory followed by propionate and then acetate. Butyrate provides energy required for rumen wall thickening, formation of papillae and stimulating capillary development. Butyrate can also increase the blood flow during nutrient absorption and metabolism and can directly affect gene expression in the ruminal epithelium.

**C.Forage**

Forage is less energy-intensive than starter feed. The low digestibility of forage in the rumen increases gut fill and decreases voluntary intake of concentrated feed by calves, which results in insufficient levels of VFAs required to stimulate rumen growth. However, forage consumption is associated with positive effects of fiber on rumination and salivation in the GI tract. The inclusion of forage in the diet increases rumen pH calves. Importantly, intake of forage was negatively correlated with the severity of sub-acuteruminal acidosis (SARA), suggesting that a small quantity of consumed forage (0.080 kg/day) can alleviate rumen acidosis in calves. The empty rumen weight was greater in

calves supplemented with hay compared to calves fed a hay-free diet. During weaning transition, feeding dietary forage in calves mitigates ruminal acidosis and induces changes in ruminal bacterial diversity and abundance. Thus, two completely opposite opinions exist as to whether to feed forage to calves before weaning. To address this issue, several studies have been conducted to compare the effect of different initial time of forage provision on growth and rumen development in calves (Lin *et al*., 2018). Calves with hay supplementation initiated at two weeks of age showed the best productivity. Inclusion of forage in the starter feed was positively linked with muscular development of the rumen and morphological appearances of rumen epithelial cells, and caused decreased plaque formation. Different forage sources have different effects on stimulating chewing activity and saliva production. Supplementation of alfalfa hay in the starter diet was shown to be more effective than beet pulp in increasing rumen pH and stimulating chewing activity.

**II. Physical Form**

The physical form and particle size distribution of the diet exert significant influence on the anatomical and microbial development of the rumen. For example, calves fed ground diet had shorter papillae with a smaller surface area compared to calves fed the unground diet. Moreover, a decrease in cellulolytic bacteria and an increase in amylolytic bacteria were detected in calves fed finely ground diet. Consumption of ground diets can reduce ruminal pH and lead to rumen parakeratosis. Calves fed texturized starter feed containing whole corn had higher ruminal pH compared to calves fed diet with dry-rolling corn, roasted-rolling corn, or steam-flaked corn. Increasing particle score of alfalfa hay from 1 mm to 3 mm can affect non-nutritive oral behaviours in calves fed finely ground starter feed. Chopping of hay grass decreased chewing time of calves, meanwhile, the richness and diversity of rectal microflora was reduced.

## III. Feed Additives

### A. Probiotics

Probiotics are viable and beneficial microorganisms that help to maintain GI microbial balance and promote rumen development. Feeding probiotics to calves around weaning age may facilitate the development of rumen bacterial communities and help easy transition from liquid feed to dry feed and forage. Fermentation products of *Saccharomyces cerevisiae* have been shown to positively influence ruminal microbiota and improve ruminal morphology. An oral dose of *Megasphaera elsdenii* NCIMB 41125 given to calves at 14 days of age increased butyrate concentration in rumen, reticulo-rumen weight and papillae growth, suggesting an improvement in epithelial metabolism. However, feeding probiotics to calves has not always been shown to exert positive effects on the development of cellulolytic bacteria. Supplementation of *Candida tropicalis* in MR had no effect on the morphology of the fore-stomach and enzymatic activities of ruminal digesta. Overall, the effects of probiotics on rumen development in calves are inconclusive, and frequently driven by differences in viable probiotic bacterial numbers, probiotics species, administration methods, and health status of animals.

### B. VFAs

VFAs are the primary products of rumen fermentation and contribute to rumen epithelium development in calves. Infusion of sodium propionate or sodium butyrate greatly promotes the development of the rumen papillae in calves. Supplementation of MR with sodium butyrate was associated with increased reticulo-rumen weight and increased length and width of papillae. Branched-chain VFAs (BCVFA), such as isobutyrate, isovalerate and 2-methyl butyrate are naturally derived from the catabolism of branched-chain amino acids. Adequate levels of BCVFA are essential for the growth of some cellulolytic bacteria and digestion of structural carbohydrates in the rumen. Supplementation of isobutyrate and isovalerate in milk and concentrate feed can accelerate the growth of calves by

improving ruminal fermentation, rumen enzyme activities and growth of cellulolytic bacteria.

**C. Plant Extracts**

Plant extracts are considered as alternatives to feed antibiotics and growth promoters in ruminant nutrition. Plant extracts have been shown to favourably affect rumen microbiota and modulate ruminal fermentation. However, studies evaluating how plant extracts affect rumen development in young ruminants are limited. Research has revealed that adding *Aloe barbadensis* to milk was beneficial in increasing total VFA concentration and bacterial count in cross-bred calves (Kumar *et al*., 2018). Supplementation of mulberry leaf flavonoids in MR increased amylase activity in ruminal digesta and protease activity in abomasal digesta in calves. Supplementation of caraway and garlic in concentrated feed can improve rumen fermentation parameters by increasing total VFAs, increasing rumen pH and decreasing rumen ammonia in growing buffalo calves. Thyme and cinnamon essential oils were shown to decrease the molar proportion of acetate and lower the ratio of acetate to propionate, as well as increase the level of propionate in Holstein calves consuming a high-concentrate diet. Finally, cinnamon essential oil was shown to increase rumen molar concentration of butyrate. Plant extracts are among the most promising alternatives to antibiotics due to their extensive biological effects, and can be used in calf feed to prevent diarrhoea. However, the efficacy of plant extracts is subject to a series of factors, including the composition of active components, addition levels, and physiological status of animals. The use of different types of plant extracts at various inclusion rates in the diet deserves further research. Moreover, effects of plant extract on the colonization of microbial populations remains to be determined in calves.

**IV. Feeding Management**

Weaning age can influence the development of rumen in pre-ruminants. Calves weaned at six weeks of age had longer and wider papillae compared to calves weaned at nine weeks of age.

In early-weaned calves, the ruminal pH, molar proportion of acetate and the ratio of acetate to propionate were lower, but the molar proportion of propionate and butyrate were greater. Due to the differences in feeding and management during the pre-weaning period, rumen development of calves may vary in different experiments. Calves with a well-developed rumen are able to utilize grains and forage efficiently. The effect of weaning age may only be detected incalves with undeveloped rumen. Additionally, pair-housed calves were shown to consume more solid feed at an earlier age compared to calves housed individually. Intensive feeding of milk or MR may decrease starter feed intake, thereby delaying rumen development. Hence, the amount of milk supplied to calves is normally restricted to promote starter feed intake and rumen development in conventional feeding practices (Scha *et al*., 2018). However, calves fed limited amounts of milk had lower growth rates and abnormal behaviour due to reduced nutrient intakes. Enhanced MR feeding increased the concentration of plasma IGF-1 and insulin which may be beneficial for gastrointestinal growth in pre-weaning calves. Furthermore, increasing nutrient intake from milk or MR resulted in enhanced milk yield in the first lactation. Thus, intensive feeding practices have been widely adopted by producers.

**Conclusion**

Rumen development in calves takes place in four stages. Feeding readily fermentable carbohydrates to calves to increase VFA production can stimulate rumen development. A pellet or texturized starter feed is superior to a finely ground meal. Providing calves with high-graded forage, such as alfalfa hay, can reduce the occurrence of rumen acidosis and papillae keratinization. Moreover, additives like probiotics, volatile fatty acids and plant extracts like essential oils can be used in calf feed due to their potential advantages in rumen development. However, the types and the optimal inclusion rate deserve further study. More importantly, there is no fixed pattern of calf feed. The diet compositions and nutrient specifications should be matched with the feeding program and management to better

promote rumen development.The rumen is a unique part of the GI tract in ruminants. As the rumen develops and becomes colonized by microorganisms, a calf physiologically transitions from a pseudo-monogastric to a functioning ruminant. The development of rumen in calves can directly affect feed intake, nutrient digestibility and eventual growth of calves. Any changes in the early feeding regime and nutrition can influence rumen development, and thus, lead to long-lasting effects on subsequent growth, health, and milk production performance. Additionally, an early feeding regime and nutrition can influence rumen development and rumen microbial composition, ultimately exerting an effect on the lifetime milk yield in cattle (Soberon *etal*., 2012). The postnatal period is frequently referred to as the most sensitive window for rumen manipulation, although studies evaluating ruminal imprinting are still limited.

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# GENOMICS AND PROTEOMICS FOR IDENTIFICATION AND CHARACTERISATION OF BACTERIA

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***ABSTRACT-***

*Identification and characterisation of microorganism involved in a disease process occupy large fraction of time and work in a microbiology laboratory. The traditional approaches based on the pure culture of the microorganisms are laborious and require at least 36-48 hrs. The advent of new molecular technologies has resulted in shifting traditional techniques for identification and characterisation of bacteria towards a genomics and proteomics based approach as these new methods are rapid, offer high throughput and produce unprecedented levels of discrimination among strains of bacteria. Many of these techniques may find a place in the routine laboratory investigation in near future, if the lacuna like establishing an integrated database to handle large amount of data and developing appropriate standards for its use as routine test is solved.*

***Keywords:*** *Bacteria, Genomics, Proteomics*

## INTRODUCTION

Identifying the microorganism involved in disease process is one of the major challenges for a microbiologist. This is the crucial step of diagnosis which is mandatory in order to implement appropriate therapeutic measures (Urwyler and Glaubitz, 2015). It also allows knowing the etiopathogenic implication and the clinical evolution of the disease (Emerson *et al*., 2008). Different identification methods have been devised by the scientists since the first recognition of microbes in order to identify the species systematically in an evolutionary or phylogenetic context (Clarke, 1985). Compared to other organisms, these methods are more challenging in case of bacteria as they are small in size which attributes to their limited range of morphological attributes and asexual nature which makes it difficult to apply the classical definition of species. They also exhibit enormous biochemical diversity in both their metabolism and cell structure which has proved to be a useful clue for the taxonomy of some groups. As in any other area of biology, molecular revolution has had a great impact in identification, characterisation, taxonomy and systematics of bacteria also.

Bacterial identification involves different methods ranging from typing based on morphological and metabolic attributes to use of genomics and proteomics. Genotypic techniques have many advantages over phenotypic methods (Vandamme *et al*., 1996; Rossello-Mora and Amann, 2001; Tindall *et al*., 2010). The most important one is it is unaffected by the physiological state or growth phase of the organism as well as the composition of the medium. However, phenotypic techniques are a direct reflection of the metabolic activities aiding the survival, growth and development of organisms.

With the advent of new molecular technologies in genomics and proteomics, the field of bacteriology has witnessed a shift from traditional techniques for classification, identification, and characterisation to methods based on the elucidation of specific gene sequences and molecular

components of a cell (Emerson *et al*., 2008). The crucial framework for bacterial classification schemes was provided by molecular systematics. Despite the lack of a coherent species definition, the timely classification, characterisation, and identification of bacteria continue to be critical in many areas like disease diagnosis, food safety monitoring, public healthand identification of biological threat agents (Gevers, 2005; Achtman and Wagner, 2008).

The methods based on genomics and proteomics are rapid, offer high throughput, and produce unprecedented levels of discrimination among strains of bacteria thus forming an attractive alternative to conventional microbiological procedures for characterisation and identification of microorganisms. The main challenges for these techniques include developing appropriate standards and methods and routine application and establishing integrated databases that can handle the large amounts of data that they generate.

## 1. Genomics for bacterial Identification

Genomics is defined as an interdisciplinary field of science within the field of molecular biology which deals with the genes and their functions, and related techniques. It aims at the collective characterisation and quantification of genes, which direct the production of proteins with the assistance of enzymes and messenger molecules. Genomics also involves the sequencing and analysis of genomes.

Polymerase chain reaction remained the most widely used molecular method for bacterial identification since its development in 1983. The real time PCR took its place later in 1990s. The merit of PCR is that it enabled the identification of microorganisms regardless of their culturability. Multiplex PCR enables simultaneous detection of microbes which reduces the time and effort for a prompt diagnosis (Fournier *et al*., 2014).

Genotypic methods for bacterial identification can be classified to two broad categories namely pattern or fingerprint-based techniques and sequence-based techniques. Current protocols for the identification of bacteria may utilise a variety of

different fingerprinting or sequence-based methods, either alone or, more often, in combination. Continuous evolution is taking place in the technologies of these methodologies, to develop new techniques with a greater accuracy, easier to do and higher sample throughput (Emerson *et al*., 2008).

**Ia. Pattern based techniques/fingerprinting-based techniques**

At present, fingerprinting techniques are the most commonly used genotypic methods for bacterial identification. In pattern-based techniques a series of fragments are produced from chromosomal DNA of an organism, which is separated by size to generate a fingerprint that is unique to an organism and its very close relatives. Using this information, a library or database of fingerprints from known organisms can be created which helps in identification of unknown organism by comparing with these. Matching fingerprints occurs when the organisms are closely related, usually at strain or species level (Goyal *et al.,* 2017). Some of the commonly used fingerprinting techniques are as follows

1) ***Repetitive element polymerase chain reaction (rep-PCR)***

   In this technique, the polymerase chain reaction (PCR) primers target specific repetitive elements randomly distributed in the chromosomes of bacteria and helps in identification at the species and strain levels. Either a user created database or commercial individual databases can be used for comparison.

2) ***Amplified fragment length polymorphism (AFLP)***

   Restriction digestion of chromosomal DNA followed by PCR using adapters coupled to the restriction sites is used in AFLP. It is used for identification at species and strain levels of bacteria and archaea. The database for this is user created.

3) ***Riboprinting***

   Similar to AFLP, restriction digestion of chromosomal DNA is the basic principle of riboprinting. However, here PCR is replaced by a sensitive probing method to detect differences

in gene patterns between strains and species. User created and commercial universal database is available for this (Bruce, 1996).

***4) Random amplification of polymorphic DNA***

Short stretches of chromosomal DNA are randomly amplified using arbitrary short primers and is used for comparison of strains of known species. The data base is user created.

***5) Pulsed-field gel electrophoresis***

Pulse field gel electrophoresis involves cutting of the chromosomal DNA into large fragments with rare cutting restriction enzymes. The fragment thus determined is used for typing of pathogenic bacteria. Public universal database administered by the Centres for Disease, Control and Prevention (www.cdc.gov/pulsenet) is available for data comparison**.**

***6) Multiplex PCR***

Multiplex PCR is used for identification of more than one organism using unique PCR primer sets for diagnostic genes. These sets can be separated on the basis of amplicon size as a way of rapidly identifying more than one microbe at a time in a mixed sample (Settanni and Corsetti, 2007).

Polymerase Chain Reaction is utilised in all the above techniques in which multiple copies of short DNA fragments using defined sets of primers are created (Versalovic *et al*., 1994; Cocconcelli *et al*., 1995; Vos *et al*., 1995; Lin *et al*. 1996). DNA polymorphisms in related organisms created as an impact of various evolutionary mechanisms are utilised in all these techniques to identify bacteria in different ways and the applications include source tracking, authentication of isolates for archival purposes, taxonomy and systematics and determination of microbial population structures and community studies (Meays *et al*., 2004; Cleland *et al*., 2008; Vandamme *et al*., 1996; Gevers, 2005; Savenlkoul *et al*., 1999).

**Ib. Sequence based techniques**

In sequence-based techniques the sequence of a specific stretch of DNA associated with a specific gene is determined. Here, as in genotyping, a database of specific DNA sequences is generated, and then a test sequence is compared with it. The relatedness of organisms determines the degree of similarity, or match, between the two sequences. A number of computer algorithms have been created that can compare multiple sequences to one another and build a phylogenetic tree based on the results (Ludwig and Klenk, 2001).

***1. Multilocus sequencing***

The Multilocus sequencing is one of the newest and one of the most powerful methods developed to identify microbial species. This technique is similar to 16S rRNA gene sequence comparisons in principle. However, MLS involves sequencing of fragments of multiple "housekeeping" genes which are concatenated, into a long sequence that can be compared with other sequences. Housekeeping gene loci are present in most cells and tend to be conserved among different organisms. As a result, general-purpose primers can be designed that will work using PCR to amplify the same genes across multiple genera. However, in most cases, primers need to be designed for specific families or orders of bacteria as truly universal primer sets are not possible (Zeigler, 2003).

Currently, two multilocus sequencing strategies are used: multilocus sequence typing (MLST) and multilocus sequence analysis (MLSA). Multilocus sequence typing (MLST) uses a set of 6 to 10 genetic loci, with appropriate primers for each locus to allow PCR amplification and sequencing of the products (usually 400 - 600 bp)(Maiden *et al*., 1998). The resulting concatenated sequences are compared with a curated database of sequences for the same organism. The result provides a high-resolution identification of an individual strain that may reveal close evolutionary relationships among individual strains. This technique has proved useful in epidemiological studies, making it possible to track the outbreak of virulent bacterial pathogens.

Thus far, MLST, and the robust databases that have been created for it, has been applied only to a relatively small number of common pathogens, using highly prescribed conditions for each organism, both for PCR primers and for database analysis (Cooper and Feil, 2004).

Multilocus sequence analysis also involves sequencing of multiple fragments of conserved protein encoding genes. However, a more *ad hoc* approach is used for choosing the genes for comparative analysis. It uses a smaller subset (≤ 6) of genes or loci (Gevers, 2005). The technique is used mainly to identify organism to analyse the species relationships within genera of families. The major limitations of MLSA are the lack of standardisation, and absence of a central database.

**2. *Small-subunit ribosomal gene sequencing (SSU rDNA)***

The SSU rDNA gene is amplified and sequenced using conserved primers and the sequences are further compared with a database. It is the current gold standard in bacterial identification and determination of evolutionary relationships. However, strains or species within a genus cannot be distinguished using this technique. Public universal databases like Ribosomal Database Project (http://rdp.cme.msu.edu) and Greengenes (http://greengenes.lbl.gov) are available for comparison of sequence.

***3. Next generation sequencing (NGS)***

An evolutionary change has been made in genomics by the next generation sequencing by considerably reducing the sequencing time and cost. Designing of diagnostic and genotyping tools, development of culture media, detection of virulence and antibiotic resistence markers and outbreak investigation are some of the applications of NGS (Fournier *et al*., 2014). Several techniques and platforms can be used for the same like Ligation based sequencing, semiconductor sequencing, SMRT technology, Ionic current sequencing etc.

A foolproof technique is a daydream in molecular biology. Every technique has its own pros and cons. It applies to both fingerprinting techniques and sequence-based methods. When

the sequence-based methods helped in establishing broader phylogenetic relationships among bacteria at the genus, family, order and phylum levels, the fingerprinting-based methods proved to be good at distinguishing strain or species-level relationships (Vandamme *et al*., 1996). Hence, combined approaches by coupling these methods with other phenotypic tests are needed for describing new bacterial species (Gillis *et al*., 2001).

***Ic. Other techniques***

1. ***Microarray*** is another technology which simultaneously identify specific microbes as well as provide ecological context for the population structure and functional structure of a given microbial community. It works on the general principle of spotting probes for hundreds or thousands of genes onto a substrate and then hybridising sample DNA or RNA to it. The sample DNA or RNA is labelled with a fluorescent reporter molecule so that samples that hybridise with probes on the microarray can be detected rapidly.
2. ***Phylochip*** is a technique which utilises the small-subunit ribosomal gene as a target for both specific and very broad groups of bacteria (Liu *et al*., 2001, Wilson *et al*., 2002).
3. ***Geochip*** has been developed to identify microbes involved in essential biogeochemical processes such as metal transformations, contaminant degradation, and primary carbon cycling (He *et al*., 2007).

**II. Proteomics technologies in bacterial identification and characterisation**

Proteomics is the study of the expression of genes, as well as the structure and function of the resulting proteins. The proteome comprises the entire set of proteins expressed by an individual cell, an organism or a biological system of different organisms. Similar to gene sequence variations, there exist variations in amino acid sequences and the corresponding proteins. Hence, analysis of the protein from a microbe is considered as an indirect analysis of the coding parts of its genome (Karlsson *et al*., 2015). The traditional phenotypic

methods which determine the activity of specific enzymes, or metabolic functions have long been a spine of bacterial identification. The advent of new proteomics techniques allow rapid interrogation of biomolecules produced by an organism. Hence it can be considered as an excellent complement to classical microbiological and genomics-based techniques**.** In short, genomics assess the potential expression of a microorganism whereas; proteomics directly assess the actual expression.

The predominant proteomic technologies that have been utilised in the field of bacteriology includes protein detection and separation methods like one dimensional electrophoresis on polyacrylic gel or starch gel and two dimensional protocols like Polyacrylamide gel electrophoresis (PAGE) (O'Farrel,1975) and isoelectric focusing (IEF). Recent developments in proteomics have paralleled the development of mass spectrometry (MS). The latest techniques like matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), surface-enhanced laser desorption/ionization (SELDI) mass spectrometry, electrospray ionization mass spectrometry (ESI-MS), or the combination of mass spectrometry, gel electrophoresis, and bioinformatics can be used for proteomics studies of bacteria (Emerson *et al*., 2008). Fourier-transform infrared spectroscopy (FT-IR) is another proteomics technique which has been used to classify and identify bacterial samples (Al-Qadiri *et al*., 2006).

### *i) Mass spectrometry–based bacterial characterisation and identification*

Mass spectrometry (MS) is an important analytical technique with several applications like identification of unknown compounds, elucidating the structure and chemical properties of molecules, quantifying known compounds as well as physical measurement, chemical characterisation, and biological identification. The advent of soft ionization method like MALDI-TOF-MS and ESI-MS is one of the major

breakthroughs in MS for the analysis of biological molecules (Kallow*et al*., 2010, Welker *et al*., 2011).

***ii) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry***

MALDI-TOF-MS is the most commonly used mass spectral method for bacterial analysis with several advantages like i) it can be used to analyse whole bacterial cells directly, ii)produce relatively simple, reproducible spectra patterns over a broad mass range under well-controlled experimental conditions, iii) the spectra patterns contain characteristic information which can be used to identify and characterise bacterial species by comparing the spectra fingerprints of the unknown species with known library fingerprints, and iv) a number of known, taxonomically important protein markers can be used directly for identifying bacterial species (Emerson *et al*., 2008; Edwards-Jones *et al*. 2000;Pignone *et al*. 2006; Puchalski *et al.,* 2016).

***iii) Electrospray ionization mass spectrometry***

The analysis of cellular components can be achieved using this technique. The technique is used to analyse the proteins expressed by the bacteria which is extracted from the lysed cells. Both intracellular and extracellular proteins, carbohydrates, and lipids can be analysed using this technique. A major advantage of ESI-MS is its ability to perform tandem mass spectrometry. Here, the protein of interest can be fragmented for a second mass analysis which provides protein fragment sequence information, or a peptide fragmentation fingerprint, which is used to identify that specific protein. This has significantly increased the accuracy of protein identification and reduced the time of estimation. Also the ability of the method to identify target bacteria in mixed samples is of great value. Newer techniques like atmospheric pressure ionization (API) can also be used forbacterial identification (Krishnamurthy and Ross, 1996).

***Iii. Surface-enhanced laser desorption/ionization***

In SELDI, mass spectrometric analysis of protein mixtures retained on chemically or biologically modified chromatographic chip surfaces are utilised. These varied surfaces allow differential

capture of proteins based on the intrinsic properties of the proteins. The SELDI mass spectrometer produces spectra of complex protein mixtures based on the mass-to-charge ratio of the proteins in the mixture and their binding affinity to the chip surface. Differentially expressed proteins are determined from these protein profiles by comparing peak intensity (Emerson *et al*., 2008). This technology offers an alternative approach to the other techniques for exploring bacterial proteomes.

Although the use of mass spectrometry has great potential for identifying bacteria by their spectral profile, the reproducibility of bacterial spectra is still under question as many factors affect the reproducibility including sample preparation, matrix selection, and differences in instrument quality and performance. The physiological state of the cell is also said to have an influence on the results of mass spectral analysis (Yip and Lomas, 2002; Seo *et al*., 2004; Wunschel *et al*. 2005).

***2. Gel-based bacterial characterisation and identification***

Several gel based techniques are used for the bacterial differentiation. These techniques are based on the cellular protein content of the organism. The principle of lysing the cells and separating the protein complement using SDS PAGE, results in a migration pattern of the protein bands that is characteristic for a given bacterial strain. By comparing the migration pattern with the reference gel pattern in established databases helps in identification of bacteria (Vandamme *et al*., 1996). However, the demerits of SDS-PAGE analysis are i) slow and labor- intensive, ii) the application necessitates precise culture conditions that yield fairly large amounts of sample material. Hence, it is not particularly useful for rapid identification of bacteria like field conditions.

Two-dimensional gel electrophoresis (2DE) which is a combination of isoelectric focusing (IEF) and SDS-PAGE affords a high-resolution separation (up to several thousand spots in a single gel analysis (O'Farrell., 1975). Here, proteins are separated by IEF electrophoresis in a pH gradient in the first dimension,

followed by the second-dimension SDS-PAGE separation. Further the gel is stained with standard sensitive staining solutions so as to visualise and analyse the protein spots. Protein gel patterns or 2DE maps from known bacteria can be further scanned, analysed, and stored in a reference database (Emerson *et al*., 2008).

Though the proteomics is an upcoming field with several advantages, the building of a collective proteomics database with complete 2DE maps and mass spectra of known bacteria forms the major hurdle in this area.

**Conclusion**

Advanced genomics and proteomics technologies will continue to play a critical role in bacterial identification and characterisation in the present era. The characterisation of bacteria has a number of practical applications ranging from fundamental to bacterial systematics, taxonomy, and evolution. Rapid identification and discrimination of pathogenic microbes has a major impact in terms of correct diagnosis and timely disease treatment. Different genomic and proteomic techniques enable the identification of any microbe with great accuracy. In addition, in near future NGS methods might become a routine method to identify bacteria due to its cost effectiveness and speed. However, the major constrains in the field of genomics and proteomics includes the building up of databases, genome amplification biases and a need for an improved *de novo* assembly of DNA sequences of previously non sequenced microbes. These disadvantages can be overcome by continuous research and development in the field of bioinformatics and molecular biology which will hopefully occur in few years thereby solving many dilemmas in the field of bacterial identification, classification and evolution.

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# ADVANCEMENTS IN DIAGNOSTIC IMAGING OF ELEPHANT FEET

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***ABSTRACT-***

*Elephants, the largest terrestrial mammals, are the representatives of the only surviving family of order Proboscidea, which once had more than 180 members. The current three extant species are declared as endangered by International Union for Conservation of Nature and Natural Resources. Although there is improved husbandry initiatives and advances in knowledge of veterinary care for these species, there remain several areas that continue to be obstacles to optimum welfare and hence conservation. The limbs of elephants reveal many peculiarities both in structure and in kinematic patterns. Foot problems are a major cause of morbidity and mortality in elephants, but are underreported due to difficulties in diagnosis, particularly of conditions affecting the bones and internal structures. Diagnostic imaging creates the visual representations of internal structures of body. In elephants, these imaging which see the unseen helps in detecting the disease conditions or defects in feet and associated structures even at a very early stage, when there is no clinical*

*manifestation or the disease is undetected by the conventional methods of investigations. The era of diagnostic imaging started with the invention of X-ray and moved ahead thorough advances like Computed Radiography (CR), Direct Digital Radiography (DDR), Computed Tomography (CT), Magnetic Resonance Imaging (MRI), and Infrared Thermography (IRT). Some of them are of use in clinical practice for disease diagnosis in foot problems in elephants, while others help in research to understand normal anatomy and its deviations, pathological conditions, evaluation of husbandry practices etc. The future of diagnostic imaging made a quantum leap with the invention of 3D x-ray in humans, but still more researches need for its suitability animals. This doctoral seminar covers the current advances in diagnostic imaging with special emphasis to elephant feet.*

***Keywords**: Elephant feet, Diagnostic imaging*

## INTRODUCTION

Elephants are the largest living mammals of the land at present. They belong to family Elephantidae, the only surviving family of order Proboscidea, which once had more than 180 members (Kingdon, 2013). Elephants amazed scientists and amused general public by a number of characteristics and characters. Apart from that, due to the need of large area for conservation, elephant is considered to be the umbrella species, whose conservation will also protect a number of species occupying the same area. They are also considered as the flagship species and keystone species because of their importance in ecological role and impact on the environment (Choudhury *et al.*, 2008). Although the welfare of captive elephants has been improving through husbandry initiatives and advances in knowledge of veterinary care for these species, there remain several areas that continue to be obstacles to optimum welfare. Foot problems are one of the most important conditions that affect elephants. A 2012 report from "The Seattle Times" analyzed 390 captive elephant fatalities in US that happened over a period of 50 years, and found that chronic foot problems associated with captivity are the major cause of death. Indians

cared their captive elephants and tried all possible ways to ensure their wellness. We have "Hastyayurveda", written 2000 years ago, the one of the oldest book on treatment of elephant disease. But, the biggest handicap in treatment of these animals is the lack of proper and timely diagnosis. Early diagnosis can help to resolve this problem and can even help to prevent the disease condition and its progression. Early detection of foot disorders and timely interventions would have beneficial effects in animal's welfare. The management and treatment of such conditions will vary from stage to stage and thus early detection using advance methods can be an effective intervention to develop treatment regime and management protocol. Effective and timely usage of advancements in the fields of diagnosis and treatment is the need of the hour to ensure the conservation of these large mammals in captivity and their welfare. To carry out clinical diagnosis of foot related problems in elephant, diagnostic imaging is one of the most important tools. Diagnostic imaging still majorly depend upon Conventional Analogue Radiography, but advances like Computed Radiography (CR), Direct Digital Radiography (DDR), Computed Tomography (CT), Magnetic Resonance Imaging (MRI), and Infrared Thermography (IRT) are also of use. The imaging techniques are getting advanced day by day, thanks to the researches going on across the globe, the latest being the 3D colour X-ray. Digital imaging also finds new places in veterinary practice as wound or surface measurement apps or digital planimetry apps are getting into the practice scenario.

## 2. About elephants

Elephants, the largest extant terrestrial mammals, are the members of Taxonomic order Proboscidea, under the Afrotherian trunked mammals. The only living family in this order is Elephantidae whose members are large herbivorous mammals collectively called as elephants and mammoths. Their most striking anatomical features are the snout modified into trunk and teeth modified into tusks. The scientific classification of elephant is as follows.

| | |
|---|---|
| Kingdom: | Animalia |
| Phylum: | Chordata |
| Class: | Mammalia |
| Super order: | Afrotheria |
| *Clade:* | Paenungulata |
| Order: | Proboscidea |
| Sub order: | Elephantiformes |
| Super family: | Elephantoidea |
| Family: | Elephantidae |
| Sub family: | Elephantinae |

In this family only three species are currently recognized as surviving and they are the African bush elephant or African savannah elephant (*Loxodonta africana*) and African forest elephant (*Loxodonta cyclotis*) of sub-Saharan Africa, and the Asian elephant (*Elephas maximus*) of South and Southeast Asia.

African elephants have larger ears, a concave back, more wrinkled skin, a sloping abdomen, and two finger-like extensions at the tip of the trunk. Asian elephants have smaller ears, a convex or level back, smoother skin, a horizontal abdomen that occasionally sags in the middle and one extension at the tip of the trunk. African bush elephants are the largest species, while African forest elephants are the smallest species. The most important and versatile appendage of elephant is its trunk or proboscis which is highly muscular. Proboscis is a combination of the nose and upper lip (Shoshani, 1998). In early fetal life, the upper lip and trunk are separated. The tusks of an elephant are modified second incisors in the upper jaw. To support the animal's weight, an elephant's limbs are positioned more vertically under the body like concrete columns, than in most other mammals, limb bones are placed on top of each other and under the body, and joints are in straight line which enables elephants to stand still for longer periods. Long bones don't have marrow cavities and are filled with cancellous bone. Clavicle is absent in elephants and the acetabulum faces downwards.

## 2.1. Elephant feet anatomy and its diagnostic importance

Elephant locomotion is in some ways atypical of more familiar quadruped gaits. The duty factors of elephants are greater for the forelimbs than for the hind limbs. The main difference from most other animals is that elephants never change their footfall pattern to a gait that uses a whole body aerial phase. So, in a classical sense, they do not run (Hutchinson *et al.*, 2006). The skeletal posture of elephant is best termed sub-unguligrade because only the tips of the phalanges (via their nails) are in approximate contact with the substrate. Yet the functional posture is more plantigrade, especially in the more horizontally oriented pes, because the massive foot pad and associated structures (e.g. prepollex and prehallux or 'predigits') connect the proximal carpal/tarsal bones with the substrate (Panagiotopoulou *et al.*, 2012).

The limbs of elephants reveal many peculiarities both in structure and in kinematic patterns. All structures of the locomotor apparatus are integrated within a column-shaped, extended limb. The forelimbs support 60% of the body mass. The carpals/tarsals and metapodials are arranged and form an arch, similar to the human foot, and the toes are enclosed within a flexible sheath of skin (Fig.1). None of the phalanges touches the ground directly-the distal phalanges are separated from the sole, firmly attached to the corium of the respective nail/hoof. Cushions occupy the spaces between carpal/tarsal, metapodial and digital bones or tendons, muscles and ligaments, which cover the bones palmarly/plantarly, and the sole skin lies under all of these structures. These cushions are roughly comparable with the foot/heelpads in humans the *Pulvini digitales* (digital cushions) in hoofed mammals and the *Tela subcutanea* of the *Tori metacarpei*, *metatarsei* and *digitales* in domestic Carnivores (Fig 3). The cushions consist of sheets or strands of fibrous connective tissue forming larger metacarpal/metatarsal and digital compartments and smaller chambers which were filled with adipose tissue. In both the forelimb and the hindlimb a 6th ray, the prepollex or prehallux, is present. These cartilaginous

rods support the metacarpal or metatarsal compartment of the cushions (Weissengruber *et al.*, 2006a).

The cartilaginous prepollex, which resembles a slightly curved, elongated, blunt-ended cone, is attached to the basis of the *Os metacarpale primum* (first metacarpal) (Fig. 2a.). It extends laterodistally towards the central part of the metacarpal cushion. The prehallux is a mediolaterally flattened cartilaginous rod and its distal end is widened (Fig. 2b). It is attached to the *Ossa tarsale primum* and *metatarsale primum* (first distal tarsal and first metatarsal) and extends on the medial and plantar sides of the foot towards the sole. Owing to the positions of the prepollex or the prehallux, respectively, and to the attachment of the cushion capsules to these flexible cartilages, it seems likely that they mainly serve to improve the stiffness and the joint (tarsus, carpus) stabilizing effect of the foot cushion. Nevertheless, the cushion also presumably helps to distribute the animal's weight over the entire sole. When loaded, the cushion is compressed and expands medially, laterally and palmarly/plantarly.

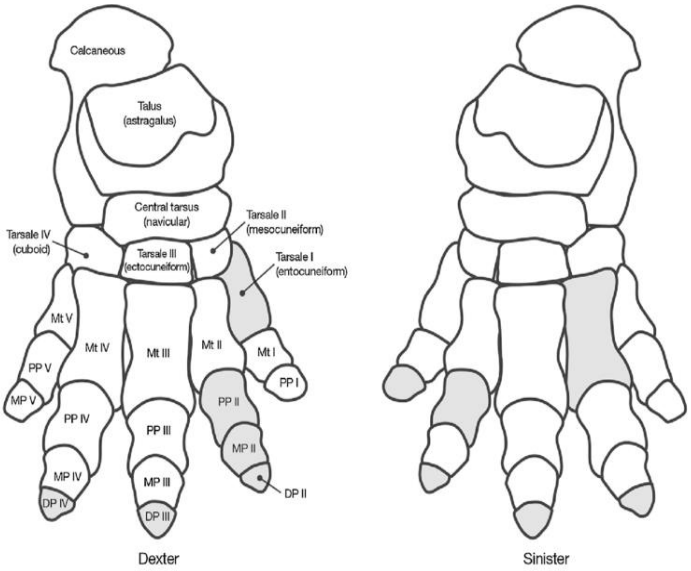


**Fig. 1** Schematic representation of the left and rightmanus of an elephant in dorsal view (Larramendi, 2014).

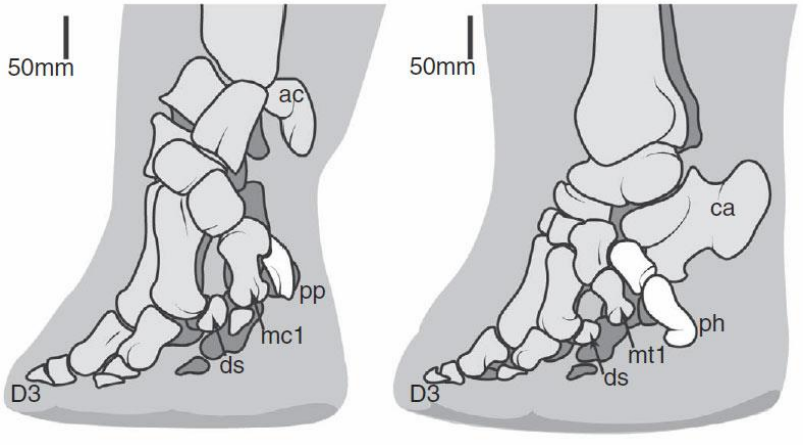


**Fig. 2a &b**. Elephant toe-forefoot and hind foot (Yong, 2011)

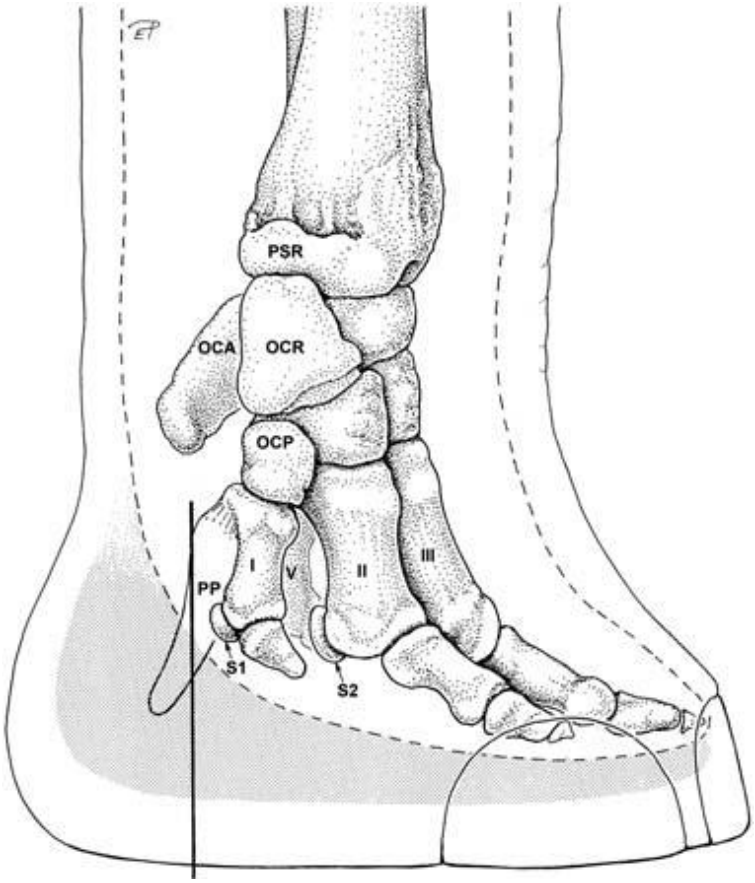


**Fig. 3** Medial aspect of the distal part of the left forelimb in an African elephant. Black line: position of transverse section,

broken line: outline of soft tissues of the locomotor apparatus (ligaments, muscles, tendons), stippled: position of the foot cushion. PSR, processusstyloideus radii; OCA, os carpi accessorium; OCR, os carpi radiale; OCP, oscarpaleprimum; PP, prepollex; I, first metacarpal bone; II, second metacarpal bone; III, third metacarpal bone; V, fifth metacarpal bone; S1, promimalsesamoid bone of the 1st digit; S2, medial proximal sesamoid bone of the 2nd digit. (Weissengruber *et al.*, 2006a)

Foot problems are a major cause of morbidity and mortality in elephants, but are underreported due to difficulties in diagnosis, particularly of conditions affecting the bones and internal structures (Regnault *et al.,* 2017). Fowler (2001) while evaluating foot conditions of Asian and African elephants states that 50 % of captive elephants will suffer from foot problems at some point in their life and that untreatable foot infections and arthritis are the major reasons for euthanization. Chandrasekharan *et al.* (2009), in an extensive study spanned over 30 years, recorded 939 cases among captive elephants out of which 404 were non-specific conditions. Among non-specific conditions, 55 cases of arthritis noted. They found that the working elephants were found to be more susceptible to different types of inflammatory conditions on the joints and all the four legs found to be affected. Digits 3–5 seem to be the areas of the most common occurrence of pathologies (Panagiotopoulou *et al.*, 2012).

**3. Diagnostic imaging**

Imaging is the visual representation of an object or its part or its form. Images can be the visual documents of event of that particular moment. The term medical imaging means creating images of human or animal body or its parts in order to aid in diagnosis or treatment. The biggest advantage of imaging is that it is non-invasive and less time consuming. It creates the visual representations of internal structures of body which may be hidden thus helping in clinical evaluation, analysis, diagnosis and treatment. The medical imaging can be of visible light or invisible light, the former being used in wound management or

dermatology while latter refers to radiology mainly. Visible light medical imaging involves pictures that can be seen and interpreted without special equipment while invisible light medical imaging need a technical person for acquiring, processing and interpreting the image. Diagnostic imaging refers mainly the invisible light medical imaging where radiography being the first and foremost one. These imaging which see the unseen helps in detecting the disease conditions or defects in an organ or any other part of body even at a very early stage, when there is no clinical manifestation or the disease is undetected by the conventional methods of investigations. This early diagnosis is of greater clinical importance as the medical or surgical intervention will be more and more difficult and prognosis will be poor as time and disease process advances. For example, Siegal-Willott *et al*. (2008) conducted radiographic evaluation of distal limb of juvenile Asian elephants to determine the relative times of growth plate closure and phalangeal ossification in the bones of the distal forelimb. They recommended consistent use of the proposed foot radiograph technique to facilitate foot disease recognition and as part of the annual examination of captive Asian elephants. Mumby *et al.* (2013) conducted a study to develop a radiographic protocol for elephant feet using digital radiography, and to describe the normal radiographic anatomy of the Asian elephant front and hind foot. Their protocol can be used for larger-scale diagnostic investigations of captive elephant foot disorders, while the normal radiographic anatomy described can improve the diagnostic reliability of elephant feet radiography. Udomtanakunchai *et al.* (2018) investigated for an appropriate procedure for radiography of elephant's limbs and concluded that equations suitable for designing the exposure technique are kVp equal to two times of sample thickness in centimetre plus source image distance in inches and the tissue correcting factor 5, and mAs equal to two-fifths of the sample thickness in centimetres. Thus diagnostic imaging holds a key and pivotal role in modern medical science and the innovations and research in the diagnostic imaging modalities are always

applauded by the some of the greatest recognitions of our time, like Nobel Prize.

### 3.1. X-ray

On November 8, 1895, German physicist Wilhelm Conrad Röntgen discovered X-rays and on December 22, he took the first x-ray picture, showing the skeletal composition of his wife's left hand. In January 1896 itself, doctors started using X-rays in clinical practice. Roentgen received the first Nobel Prize for Physics for this path breaking discovery in 1901. X-rays are high energy electromagnetic radiation which can penetrate to inside of objects. They are electromagnetic energy waves that act similarly to light rays, but at wavelengths approximately 1,000 times shorter than those of light. The use of X-rays in imaging internal structures or organs is known as Radiography. In conventional radiography, to create such an image, an X-ray generator produces a beam of X-rays and is projected toward the object. The X-rays or other radiation is either absorbed by the object, dependent on the object's density and structural composition, scattered to various directions or pass through it. The X-rays which pass through the object are captured behind the object by a detector (either photographic film or a digital detector). Such generation of flat two dimensional images is called projectional radiography. The image, thus obtained as a result of an X-ray beam transmitted through the patient's body, with modulation of intensity, and processing of data collected by the detector is the carrier of the diagnostic information. The X-rays which are captured using photographic film is known as Analogue Radiography also. Another diagnostic radiographic modality is fluoroscopy or x-ray movie which produces real-time images of internal structures of the body in a similar fashion to radiography, but employs a constant input of x-rays, at a lower dose rate. Fluoroscopy is used in image-guided procedures when constant feedback during a procedure is required. An image receptor is required to convert the radiation into an image after it has passed through the area of interest. In its simplest form, a fluoroscope consists of an X-ray source and a fluorescent screen, between which a patient is placed. However, since the 1950s

most fluoroscopes have included X-ray image intensifiers and cameras as well, to improve the image's visibility and make it available on a remote display screen.

Analog image detector of an X-ray unit consists of three main components: X-ray film, intensifying screen and light-proof housing or cassette. An ancillary part of this is the developing device along with reagents like developer, fixing agent, water for photochemical processing and the dryer. X-ray film is made of a thin, transparent, flexible polyester film and a thin layer of a photographic emulsion. The emulsion is coated with protective polymer layers and with a dulling agent. The photosensitive elements are silver halide micro crystals. Quanta of X-rays or visible light radiation energy emitted by the intensifying screens transfer their energy, as soon as they reach the silver halide crystals. If the energy is sufficient, crystal electron can be transferred from the valence band to the conduction band, where it can move freely throughout the crystal. The electron moves until it reaches the place of crystal structure distortion, where it is 'trapped'. The presence of the 'trapped' electron with a negative electric charge causes the attraction of positively charged silver ions, which further leads to origin of a metallic silver atom in that particular place. This place becomes a part of a latent image. As a result of this process, latent image sites may occur on the crystal, large enough (several to over a dozen of atoms of metallic silver) for the developer to initiate the process of reduction of the whole crystal to metallic silver. The amount of the developed metallic silver determines the level of blackening (optical density), creating a real image on the film (Oborska-Kumaszyńska and Wiśniewska-Kubka, 2010).

The major advantages of Radiography for diagnosis are as follows.

1. It is simple, fast and less costly compared to other modalities.
2. It is non-invasive, thus helps to avoid some in invasive exploratory procedures.

3. Aids in diagnosis and location of lesions, especially reliable diagnosis of a bone fracture and check for correct reduction of fracture dislocations.
4. Not only aids in initial diagnosis, but also helps in monitor the response to treatment, progress of disease or healing process.
5. Radiation risk is very low compared to CT scan.

The disadvantages are,

1. It doesn't provide 3-Dimensional information and hence more views required in orthogonal manner to locate the lesion correctly.
2. Limited range of densities can be demonstrated.
3. X-rays are carcinogens and continuous or cumulative exposure causes developmental problems, especially in fetus.

**3.2 Digital radiography**

Even though the basic things like techniques of X-ray generation remains more or less same since the discovery of X-rays, the dramatic changes in radiology happened during the last decade is in the image capturing techniques. From, hard copy analogue films, which stood us in good stead for more than a hundred years, it changed over to digital imaging (Kirberger and McEvoy, 2016).

A variety of digital imaging solutions based on various detector and readout technologies available nowadays. The common technical features of such systems include four major steps like,

1. Signal acquisition
2. Signal processing
3. Image distribution and archiving
4. Image presentation

At present, two types of digital image detectors exist namely, CR (Computed Radiography) and DDR (Direct Digital Radiography).

### 3.2.1. Computed Radiography (CR)

Computed Radiography uses a storage image plate covered by a cassette. A detector layer of photo-stimulable crystals of this plate absorbs X-ray energy and temporarily stores during exposure and thus a latent image is formed. The cassette is put into an image reading device where the image plate is removed to be scanned by a laser beam. This process sets the stored energy free as visible light which will be capture by photodiodes to convert it into a digital signal. The residual latent image which remains in the plate will be erased or removed using exposure to intense white light inside the reader for the reuse of image plate. This readout will take 20-40 seconds only (Ludewig and McEvoy, 2016)

### 3.2.2. Direct Digital Radiography (DDR)

Direct Digital Radiography (DDR) refers to direct digital registration of the image at the detector with no intermediate processing step required to obtain the digital signals. Here, flat panel detectors (FPD) or Charge-Coupled Device (CCD) used to convert X-rays into electrical charge by means of a direct readout process. The FPDs can be either direct conversion detectors or indirect conversion detectors. Direct conversion detectors are having photoconductor layer (amorphous selenium) which directly transforms incoming X-ray energy to electric charge. The layer underneath this layer is of electrodes for transmission of the released electrons to a Thin Film Transistor (TFT) array which forms the third layer. The TFTs sample and store the energy of electrons for readout process. In the case of indirect conversion detectors, in the scintollator layer, X-rays are converted into visible light. In the next step, a photo diode array produces electric charge from light. The next layer is an array of TFTs. The signals from flat panel detectors (direct or indirect) can be transferred to host computer by wired or wireless connection. CCD is an electronic chip with photosensitive layer and embedded electronics. The charge-coupled device requires optical coupling to focus the large field of view onto the small chip. Each dexel releases and stores electrons when struck by

light and the electric charge is determined by how much light it received during the exposure. There are charge amplifiers at the end of each column of the matrix that converts the charge to a voltage. The information is then digitized to give a grayscale value for each corresponding pixel.

The processing in digital radiography uses region-related specific mathematical algorithms. An instruction that telling computer how to display the processed image is called Look-Up Table (LUT). The images are viewed with the help of DICOM (Digital Imaging and Communication in Medicine) software. The computer system that manages the acquisition, transmission, storage, distribution, display and interpretation of medical images is called Picture Archives and Communication System (PACS). It includes imaging modalities, network and archiving components, workstations, software and interfaces.

### 3.2.3. Advantages and disadvantages of Digital Radiography over Analogue Radiography

Advantages of Digital Radiography over Analogue Radiography are,

1. It can be easily integrated into complex information systems. This allows digital storage and exchange of information which cane available at any time and everywhere.
2. For Teleradiology services or expert consultation for better interpretation, image data can be sent to experts worldwide within no time.
3. There is opportunity to monitor the performance during the imaging chain including post exposure stages, so that overall improvement in system performances can be achieved.
4. More diagnostic information from the image
5. Wider exposure range can be used to reduce dose.
6. Since film processing is avoided, image distribution can be faster, fewer workforces needed for film handling and no need of chemicals or dark rooms for processing.

The disadvantages are,

1. High cost for purchase and maintenance
2. Over exposures can be overlooked.

### 3.3. Computed tomography (CT)

It refers to a computerized X-ray imaging procedure in which a narrow beam of X-rays is aimed at a patient or animal and quickly rotated around the body producing signals that are processed by the computer to generate cross sectional images or "slices" of the body. These slices called tomographic images contain more information than conventional X-rays. CT scanner uses a motorized X-ray source that rotates around the circular opening of machine called gantry and digital X-ray detectors are placed opposite to the source. During the process, animal will be restrained and placed on a bed which slowly moves through the gantry. For every one full rotation of the source, computer generates 2D image slice. These slices can be viewed individually or used for Multiplanar Reconstructions (MPR) or Three-dimensional reconstructions (3D) by computer. This reconstruction will help in identification of location of affected area. CT permits un-obscured visualization of anatomical structures and pathology in transverse plane. It is particularly valuable in the evaluation of intra-articular abnormalities, subtle or complex fractures, bones or soft tissue tumours and in the detection of small bony fragments. While a loss of 30% bone density is required for a lesion to be visible on conventional radiographs, CT is reliably able to detect density changes of only 0.5-2%. This allows early diagnosis than radiographs, especially degenerative joint diseases and neoplastic diseases. Better soft tissue differentiation and absence of super imposition are the major advantages. More X-ray exposure is the major disadvantage.

Computed Tomogaphy (CT) is applied in elephant foot post mortem studies. Regnault *et al.* (2017) evaluated post-mortem computer tomographic (CT) scans of 52 feet from 21 elephants (seven African *Loxodonta africana* and 14 Asian *Elephas maximus*), describing both pathology and variant anatomy (including the appearance of phalangeal and sesamoid bones) that could be mistaken for disease. They found all the elephants in their study to have pathology of some type in at least one foot. The most common pathological changes observed

were bone remodelling, enthesopathy, osseous cyst-like lesions, and osteoarthritis, with soft tissue mineralisation, osteitis, infectious osteoarthritis, subluxation, fracture and enostoses observed less frequently. Most feet had multiple categories of pathological change (81% with two or more diagnoses, versus 10% with a single diagnosis, and 9% without significant pathology). Much of the pathological change was focused over the middle/lateral digits, which bear most weight and experience high peak pressures during walking.

### 3.4. Magnetic resonance imaging (MRI)

Raymond Vahan Damadian, an American physician, invented and patented Magnetic Resonance Imaging scanning machine in 1974 and performed first human body scanning in 1977. Magnetic Resonance Imaging (MRI) uses strong magnetic fields, magnetic field gradients, radio waves and computer to detailed cross-sectional images of tissues and organs within the body. The major difference from X-ray and CT is that MRI doesn't use ionizing radiation for imaging. The powerful magnets in the MRI use to polarize and excite the hydrogen nuclei or single proton of water molecule in tissues, producing a detectable signal, processing of which produces an image. Hydrogen atoms have an inherent magnetic moment (directional magnetic field) as a result of their nuclear spin. The characteristic movement of charged particle is known as precession. When placed in a strong magnetic field, these magnetic moments tend to align with the magnetic field. Proper stimulation by a resonant Radio-Frequency pulse (RF) which is applied perpendicular to the magnetic field, can force the magnetic moments of the nuclei partially or completely. When the applied RF-excitation is removed, the magnetic moments of the nuclei precess and realign. This return to equilibrium is known as relaxation. During relaxation, the nuclei lose energy by emitting their own RF signal. This signal is referred to as the free-induction decay (FID) response signal. The FID response signal is measured by a conductive field coil placed around the object being imaged. This measurement is processed or reconstructed to obtain 3D grey-scale MR images (Mackiewich, 1995). Two types of

relaxation occur, like realignment of protons with the magnetic field or dephasing of spinning protons (loss of resonance). Two signals can be detected. T1 signals relates to the speed of realignment and T2 signals relates to the speed of dephasing. Different protons in the body realign and diphase with varying rapidity, depending upon in which tissue the proton can be found out. For example, protons in fat realign quickly with high energy that produces high T1 signals, water in body diphase slowly that produce high T2 signals.

The advantages of MRI are,

1. No ionizing radiation
2. Variable thickness in any plane
3. Better contrast resolution
4. Many details even without i/v contrast

The major disadvantages are,

1. Time consuming process compared to other modalities
2. Very expensive
3. Movements during scanning cause blurring

The usage of MRI in live elephants is not yet reported, but, post-mortem MRI evaluations were performed to find our normal anatomy. Weissengruber *et al.* (2006b) have done experiments on African and Asian elephants including radiology and Magnetic Resonance Imaging (MRI) to evaluate and described the distinct morphological features of the elephant knee joint together with biomechanical analyses and pathological–anatomical discussion.

### 3.5. Infrared thermography (IRT)

Infrared Thermography (IRT) or Infrared Thermal Imaging is an emerging diagnostic imaging method. This is a true non-invasive imaging method as all other modalities discussed above uses radiations that pass through the body before capturing the image. It is non-contact in nature and can be used for imaging from a distance. It works on the principle that, all objects with a temperature above absolute zero emits radiation, especially infrared radiation, recording of that makes it possible to view one's environment with or without illumination. Visual

displays of the amount of infrared energy emitted, transmitted, and reflected by an object is called Thermal images, or Thermograms which is accomplished using special soft ware. These radiations are imaged using special imaging cameras called thermal imaging cameras. These cameras use sensing device consisting of an array (typically rectangular) of light-sensing pixels (that respond to mid and long wavelength infrared) at the focal plane of a lens called Focal Plane Array (FPA).

The story of infrared radiation began in 1800, when Sir William Herschel, the astronomer, detected heating rays beyond the visible red of the spectrum. After his death in 1840, his son John Herschel made the first thermal image from sunlight using the evaporograph technique. He used the term thermogram to describe the image, which is still in common use today (Ring, 2004). In animals and humans, body heat is generated by metabolism and by muscular activity and keeps the core temperature at a defined, slightly oscillating level (about 37 °C). The organism's heat loss depends on ambient factors and results of conduction, convection, IR radiation, and of evaporation (sweating) of the surface, the skin (despite of breathing and other mechanisms). Inside the organism heat is transported by convection (blood flow) and by conduction (Berz, 2007). The association between disease and body temperature is as old as medicine itself. Hippocrates defined fever in different forms, such as malignant, benign and acute. It was claimed that if wet mud was applied to the skin and one area dried rapidly while the remainder was still moist, an underlying tumour may be suspected. The same principle applies in the use of diagnostic Infrared Thermography. For example, the classical inflammatory process involves raise in temperature in the affected area. This heat can be detected at an early stage which aids in early diagnosis of some pathological conditions. Medical infrared imaging entails the use of high-resolution infrared cameras and sophisticated computer processing to produce a topographic heat map display which bears a resemblance to the visible image of the body. Modern computerized thermography produces an

accurate and reproducible high-resolution image that can be analyzed both qualitatively and quantitatively for minute changes in skin surface heat emissions. Infrared imaging does not replace any other form of imaging, but is designed to be used in addition to other tests to provide physiological information that cannot be obtained from any other examination procedure.

When viewed through a thermal imaging camera, warm objects stand out well against cooler backgrounds; humans and other warm-blooded animals become easily visible against the environment, day or night. As a result, thermography is particularly useful to the military and other users of surveillance cameras. Being a distant recoding and non-invasive method, Infrared Thermography gained more popularity among wildlife. Hilsberg (2002) reported diagnosis of inflammation, lameness evaluation and foot problems in elephants using Infrared Thermography. A study by Avni-Magen *et al.* (2017) in zoo elephants in Isarel concluded that thermography can be an effective diagnostic tool for early diagnosis of inflammatory processes and useful for regular and continuous monitoring of zoo elephants in general. Hilsberg-Merz (2008) opined that, tracking inflammatory processes in both wild animals and zoo animals is a major advantage of the IRT method. In their observations, it is best used in animals without long hair such as elephants, giraffes and other large herbivores.

The major advantages of Infrared Thermography are,

1. Non-invasive, non-contact and distant imaging.
2. No immobilization or restraint required for most of the recordings.
3. Completely safe for the technician and subject as it is not using radiations.

The disadvantages are,

1. All subjects must undergo acclimation with the recording environment.
2. The images are hard to interpret even with experience.

Infrared imaging can lead to misreading information taken by the camera when temperature having very close range and due to this objects can become indistinguishable (Battalwar *et al.*, 2015).

### 3.6. Colour 3D X-ray

After 120 plus years, x-ray imaging got a remarkable update with 3D, full-colour images that reveal far more than just the bones. In 2018, Father-son duo researchers namely, Anthony Butler and Phil Butler of Otago University in New Zealand developed a new type of medical imaging scanner that works on Medipix3 technology developed for the Large Hadron Collider at CERN (European Agency for Nuclear Research) to produce far more detailed results. The original concept of Medipix is that it works like a camera, detecting and counting each individual particle hitting the pixels when its electronic shutter is open. This enables high-resolution, high-contrast, very reliable images, making it unique for imaging applications in particular in the medical field. The spectroscopic information generated by the Medipix3 enabled detector couples with powerful algorithms to generate 3D images. The colours represent different energy levels of the X-ray photons as recorded by the detector and hence identifying different components of body parts such as fat, water, calcium, and disease markers.

### 3.7. Digital planimetry

Wound healing is a multi-factorial process consisting of angiogenesis, deposition of extracellular matrix, contraction and epithelialisation. The success of wound healing is commonly evaluated using the parameter of 'time to complete healing'. Management strategies, especially those which are expected to provide small, nonlinear incremental benefits, require an objective and easily reproducible method of quantification (Williams *et al.*, 2017). Cutaneous wound measurements are important to track the healing of a wound and direct appropriate therapy. The most commonly used method to calculate wound area is estimation by multiplying the longest length by the widest width (Rogers *et al.*, 2010). The method of wound area

measurement using a planimetric software (or a graphical software with appropriate functions) and digital photographs is popular as being easy to perform and inexpensive. The wound is photographed with a ruler or a marker of known dimensions placed at the skin near the wound edge and the image is transferred to a computer and opens in planimetric software. The ruler or marker is used for calibration of linear dimensions at the image. Once the wound border is manually traced with a computer mouse the area of wound is calculated and displayed (Foltynski *et al.*, 2015). Khoo and Jansen (2016) observed by reviewing the literature between 2000 and 2014 that, the precision and reliability of digital planimetry over the more conventional methods of ruler measurements and acetate tracings are consistently demonstrated.

The advantages are,

1. Non-invasive and thus animal friendly
2. Requires very less time compared to other techniques
3. More reliable
4. Even smartphone apps can be used for measurements
5. Any number of images can be taken

The disadvantages are,

1. The rulers used should be parallel to camera lens
2. The camera lens axis should be perpendicular to the wound plane

**Conclusion**

Elephants are one of the most fascinating animals on earth and the extant species of a big order. At present, they are classified as endangered in terms of conservation. The anatomical peculiarities which are suitable for a life in the wild cannot suit its captive life. One of the important problems that face by elephants in captivity and aged elephants in wild is foot disorders that too affecting the bony and cartilaginous structures. Misdiagnosed, undiagnosed or lately diagnosed conditions in foot disorders lead to irreparable injuries and often death or euthanasia. Only few diagnostic modalities like X-ray hold good in the case of elephant feet considering the wild

nature, difficulty in restraint, size and thickness of tissues etc. Hence advanced diagnostic imaging modalities like CT and MRI are yet to be introduced in clinical practice. But, still they find their own space in research which aids in diagnosis. At the same time, advances like wireless digital radiography and non-invasive infrared thermal imaging are emerging as promising diagnostic tools. A huge scope for the future research in this area awaits researchers.

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# ROLE OF BIOACTIVE FACTORS IN MILK

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***ABSTRACT-***

*Milk and colostrum contain nutritional and non-nutritional bioactive factors. The non-nutritional factors are hormones, immune factors and growth factors. The growth factors include EGF, BTC, IGF-I, IGF-II, TGF-β1, TGF-β2, FGF1 and 2, and PDGF. These growth factors are resistant to gastric digestion and pasteurisation. They can exert local and systemic effects on the gastrointestinal tract. A large amount of growth stimulating substances in the colostrum promotes intestinal cell proliferation. Colostrum intake promotes the absorptive capacity of small intestine. This leads to improved serum glucose level in calves fed colostrum immediately after birth. It results in elevated insulin status. The higher insulin level triggers IGF-I and the postnatal somatotropic axis. It stimulates the anabolic processes resulting in increased growth rate. Development of intestinal epithelium and the intestinal barrier of the mucosal immune system occur during the first week of life in calves. Lactoferrin, cytokines and immunoglobulins are the factors present in lacteal secretions which can activate the immune system of young ones.Therefore inorder to explore the effects of bioactive factors in milk, an intensive milk feeding programme is required to realise the potential for growth and development of pre-weaning calves. (EGF-Epidermal growth factor, BTC- Betacellulin,IGF-Insulin-like growth*

*factor, TGF- Transforming Growth Factor(TGF-β1 and TGF-β2), FGF-Fibroblast Growth Factor (FGF1 and FGF2), and PDGF-Platelet Derived Growth Factor)*

***Key words**: Bioactive factors, Milk, Colostrum*

**INTRODUCTION**

Bioactive factors are substances having an effect on a living organism. Antibiotics, enzymes, and vitamins are all bioactive substances. Milk forms the primary source of nutrition for young mammals. Calves rely on colostrum intake immediately after birth for nutrient supply and for establishing passive immunity. In addition to its high content of nutrients colostrum contains non-nutritive bioactive factors designed to help the body battle and face a lifetime of attack by micro-organisms and environmental toxins. The nutritive factors include lactose, proteins, lipids, vitamins and minerals. Non-nutritive factors are hormones, immune factors and growth factors.

Colostrum and milk contain growth factors, immune factors and hormones that have beneficial effects on growth and development of the young one. All of these molecules are important for the growth, multiplication, maturation or repair of different cell types in the neonate and adult. Moreover, all of these growth-promoting factors are signalling molecules released by cells to communicate with each other. Hormones can be described as substances that are released into the extracellular medium by the cells of one tissue, to be carried to a new site of action (endocrine function), where they induce a specific response. Generally, they are not included in the growth factor families. Cytokines are proteins or glycoproteins, which are produced by many cell types and have profound bioactive effects, in a very minute range of concentration (10 to 1000 pg·mL–1), on other cells within a short distance, or often even on the cells from which they originate. Hence, cytokine effects tend to be local, where they are involved in autocrine or paracrine functions. It also includes molecules which are principally responsible for the coordination of the immune response

(enhance or suppress immunity), and generally include the interleukins (IL) series, tumour necrosis factors (TNF) and interferons (IFN).

Growth factors are proteins or polypeptides that bind to receptors on the cell surface, with the primary result of activating cellular proliferation and/or differentiation. Many growth factors are capable of stimulating cellular division in numerous different cell types, while others are specific to a particular cell type. Certain growth factors, such as TGF-β, can also inhibit the growth of specific cells (macrophage and lymphocyte). As cytokines, growth factors are involved in autocrine or paracrine functions.

**Bioactive factors and stage of Lactation**

The concentration of growth factors in milk and colostrum varies widely according to the period of lactation. It is known that the growth factor concentration in colostrum is the highest during the first hours of calving and generally declines substantially in a time-dependent manner following peak concentration. In addition, the total protein concentration rapidly declines during the first four days of lactation.

**Bioactive Factors in milk and colostrum**

Bioactive factors in milk are broadly classified into nutritional and non-nutritional factors. The nutritional factors include water, lactose, milk proteins, lipids, vitamins, minerals. Non-nutritional factors include hormones, immune factors, and growthfactors.

Hormones present in milk include insulin, prolactin, estrogen and androgens. Recently, there has been concern that consuming milk containing elevated amounts of estrogen could affect blood levels of the hormone in humans, leading to an increased risk of some cancers.

Immunoglobulins are the most well-known immune factors in milk. They include IgG, IgE, IgD, IgM and IgA. Lactoferrin is an immune protein with strong anti-microbial function. Cytokines have the potential to alter oral tolerance via their impact on the development of the newborn's immune system and gastrointestinal tract. Some cytokines were also

quantified in bovine colostrum or milk. These cytokines are G-CSF, IL-1, IL-1β, IL-6 and INF-γ, TNF-α and IL-18. Their main action is to activate the immune system of young ones.

Many growth factors were identified in colostrum and milk and partly associated with the growth-promoting or growth inhibitory activity. At the present time, the growth factors that have been identified in bovine colostrum and milk are the following: Epidermal Growth Factor (EGF), Betacellulin (BTC), Insulin-like Growth Factors (IGF-I and IGF-II), Transforming Growth Factor (TGF-β1 and TGF-β2), Fibroblast Growth Factor (FGF1 and FGF2), and Platelet Derived Growth Factor (PDGF) .

**Growth factors in milk and colostrum**

**1. Epidermal growth factor (EGF) and betacellulin (BTC)**

EGF and BTC are members of the EGF family.They were detected in sufficient amount in milk to induce physiological effects. EGF family members stimulate the proliferation of epidermal, epithelial and embryonic cells. They decrease crypt fission in the small intestine or colon during repair but promote crypt hyperplasia. They also act as differentiation factors for some cell types. These growth factors inhibit the secretion of gastric acids and modulate the synthesis of a number of hormones. They are involved in wound healing processes.

**2. Insulin-like growth factor (IGF)**

The insulin-like growth factors are single-chain polypeptides structurally similar to insulin. IGF-I and IGF-II have been identified in most species. IGFs are present in the circulation and throughout the extracellular space almost entirely bound to members of a family of high affinity IGFBPs (IGF binding proteins). Generally, the in vitro effects of the IGFs are either acute anabolic effects on protein and carbohydrate metabolism, or longer term effects on cell replication and differentiation of numerous cells. IGFs also present the capacity to inhibit cell death in some cells; for example, in hematopoietic cells. Administration of IGFs to humans causes hypoglycemia, improvement in nitrogen balance, lowering of cholesterol and

potassium, and improvement in renal functions. In animals, IGF-I was also shown to have a positive effect on wound healing.

**3. Transforming growth factor-beta (TGF-β)**

The transforming growth factors-β (TGF-βs) family comprises multifunctional growth and differentiation factors that act on most cell types. Their activities are dependent upon the cell type, stage of proliferation and environment. The physiological function of TGF-β2 in milk is unknown but it could be a mediator of mucosal immunity or gut epithelial differentiation in the neonate. TGF-βs are recognized to stimulate proliferation of some cells, especially in connective tissue, whereas they act as a growth inhibitor of some other cells, such as lymphocytes and epithelial cells. TGF-βs play an important role in embryogenesis, tissue repair, formation of bone and cartilage, and in the control of the immune system.

**4. Platelet-derived growth factor (PDGF)**

PDGF plays an important role during embryogenesis, in particular for the development of the kidneys, blood vessels, lungs, and central nervous system. It has a growth-promoting activity on mesanglial cells, pericytes, alveolar fibroblast, and glial cells. PDGF has been shown to have angiogenic effect and to promote wound healing processes.

**5. Fibroblast growth factor 2 (FGF2 or basic FGF)**

Twenty-two members of the FGF family have been identified in humans and other vertebrates. FGF2 plays an important role in proliferation, differentiation and survival of cells of almost all organ systems. Also, it stimulates the growth and development of the new blood vessels (angiogenesis), normal wound healing, tissue development and hematopoiesis.

**Stability of Milk Growth Factors**

Growth-promoting activity in human and bovine milk has been found to resist pasteurization and even heat treatments more severe than pasteurization. The growth-promoting activity of milk is also retained following removal of milk fat and decaseination.

**Bioactive Factors in colostrum and milk and their effect on calves**

**B. Effect on growth and development of gastrointestinal tract**

Bovine colostrum has an overall importance for the postnatal development of the gut. The high concentrations of hormones, growth factors and cell-modulating factors in colostrum stimulate growth of villi in the small intestinal mucosa in calves. Colostrum feeding promotes mucosal cell growth and protein synthesis in the enterocytes of neonatal mammals. The amount of overall ingested colostrum corresponds to the villus size in the intestinal mucosa, leading to a greater villus size in repeatedly colostrum-fed calves. When feeding colostrum extract, that is, a fraction originating from first colostrum including most of the growth-promoting peptides, together with a milk-based formula, the villus size is stimulated when compared to a milk-based formula feeding with similar protein and energy as in colostrum but no growth-stimulating peptides. This finding supports the general assumption that bioactive peptides, such as IGF-I, or hormones, such as insulin, are involved in the growth-stimulating effect on the intestinal mucosa of neonatal calves.

Biofactors in milk and colostrum stimulate crypt cell proliferation in the intestinal mucosa of calves. When comparing colostrum feeding and milk-based formula feeding (same nutrient content but no growth-promoting bioactive factors as colostrum during the first 3 days after birth), the greater stimulation of cell proliferation corresponded to the greater villus growth in colostrum than formula-fed calves on day 8 of life. The cell turnover of the intestinal mucosa depends on cell proliferation and programmed cell death (apoptosis; mainly seen at the villus tips). Milk intake reduces apoptosis of epithelial cells and therefore prolongs the lifespan of the epithelial cells.

A more distinct stimulation of mucosal cell proliferation is observed when feeding a colostrum extract instead of a single growth-promoting peptide. This finding indicates that not a single factor but the interaction of the large amount of growth-

stimulating substances in the colostrum promotes intestinal cell proliferation and growth. Receptors for IGF-I, IGF-II and insulin (IGF1R, IGF2R and InsR, respectively) are present in the intestinal mucosa throughout the total gut in neonatal calves.

**C. Effect of bioactive factors in milk on glucose metabolism**

Due to its growth-stimulating effect of bioactive factors in the small intestine, milk intake promotes the absorptive capacity of the small intestine. Measurements of glucose absorption in calves clearly indicate a greater absorption after feeding with colostrum instead of formula or MR. Therefore, the intake of first colostrum during the first hours after birth is of great importance for glucose absorption and the postnatal glucose status in neonatal calves. In contrast, digestive enzymes and mucosal transporters with respect to carbohydrate digestion, such as lactase and SGLT1 and GLUT2, seem to be less affected by colostrum feeding.

First-pass glucose uptake in the splanchnic tissue on days 2 and 7 of life is greater in formula-fed than colostrum-fed calves, indicating a greater glucose utilisation in the splanchnic tissue (gastrointestinal tract and liver) of calves not fed with colostrum. Nutrient absorption is generally impaired in formula-fed calves, leading to increased glucose utilisation in the splanchnic tissue, whereas colostrum-fed calves are able to use greater amounts of digested fat and protein as energy fuel in the splanchnic tissue. Thus, growth-promoting substances of ingested colostrum do not affect endogenous glucose production in neonatal calves. Nevertheless, the increased plasma glucose concentration and the greater hepatic glycogen content in colostrum-fed calves indicate an improved glucose status by colostrum feeding. The improved glucose status in calves fed colostrum immediately after birth and for 3 days is a result of enhanced glucose absorption and probably of less glucose utilisation in the splanchnic tissue. The better glucose status is not a result of increased endogenous glucose production.

**D. Bioactive factors and their effect on the maturation of somatotropic axis**

Accelerated maturation of the somatotropic axis occurs due to elevated glucose availability and the improved insulin status in colostrum-fed calves (Blum, 2006). The stimulation of gastrointestinal hormones due to colostrum feeding may contribute to the elevated insulin secretion in the calves (Inabu *et al.*, 2019). The elevated insulin status due to colostrum feeding in neonatal calves is probably the trigger for stimulating endogenous IGF-I and the postnatal somatotropic axis because glucose and insulin stimulate the hepatic gene expression of the growth hormone receptor and IGF-I as well as IGF-I secretion. On the other hand, studies in neonatal calves and piglets indicate no intestinal absorption of colostral IGF-I or insulin. Thus, the endogenously produced IGF-I determines the IGF-I status of the calf. Therefore, the nutrient supply is responsible for the maturation of the neonatal somatotropic axis, and the IGF-I in milk promotes intestinal development of the calf but does not contribute to systemic IGF-I availability (Ontsouka *et al.*, 2016).

**E. Effect of bioactive factors on growth and endocrine growth regulation**

The improved growth and development along with protein accretion in calves fed intensively with milk or MR are confirmed by the stimulation of the somatotropic axis. Important elements of the somatotropic axis are growth hormone (GH), IGF-I and several IGF-binding proteins (IGFBPs). The postnatal interaction of GH, IGF-I and IGFBP affects body growth and organ development in mammals, including the development of the mammary gland and immune function (Akers, ). The stimulation of the postnatal somatotropic axis depends on the nutrient supply and reflects the glucose and insulin status of the animal. Plasma IGF-I and IGFBP-3 concentrations are elevated, and the IGFBP-2 concentration is decreased during growth in well-nourished animals as compared to animals of same age with restricted feed intake. A key factor in maturation of the somatotropic axis is the increased expression of the GH receptor,

particularly in the liver, with age. The GH receptor mediates GH action on IGF-I synthesis and secretion and is stimulated by insulin. The glucose, insulin, IGF-I and IGFBP-3 plasma concentrations are much greater, and hepatic gene expression of the GH receptor and IGF-I is higher in intensively milk-fed calves than in calves with restricted milk intake. No signs of impaired insulin response are seen during enhanced milk feeding in calves (MacPherson *et al.*, 2019). During the first weeks of life, the elevated concentrate intake in restrictively milk-fed calves cannot compensate for impaired nutrient intake due to reduced milk feeding. Hence, the somatotropic axis is not stimulated during early postnatal life when concentrate and forage feeding are favoured instead of milk feeding. In particular, the elevated IGFBP-2 plasma concentration in milk-restricted-fed calves indicates an impaired nutrient intake (Schäff *et al.*, 2016). These changes in the somatotropic axis are reflected by a depressed growth rate during the weaning process. To prevent a growth depression during weaning in calves with an intensified milk-feeding programme, a delayed weaning age or individual weaning based on solid feed intake is recommended (Welboren *et al.*, 2019). Parameters of the somatotropic axis may provide useful information on the metabolic status and may help to avoid detrimental weaning programmes in calves.

**Development of the preweaning calf due to intensive milk feeding**

After the colostrum period, the calf depends on the intake of liquid feed in the form of milk or high-quality MR for nutrient supply. Although it is a common feeding strategy to increase solid feed intake as soon as possible in the preweaning period by reducing milk feeding, solid feed intake during the first 3 weeks of age is low, and the digestion of solid feed is impaired due to the immature forestomach in the postnatal period. Thus, sufficient milk or MR supply during the first weeks of life is a prerequisite for calf growth and development. The World Organisation for Animal Health (OIE) defines animal welfare in the Terrestrial Animal Health Code as a state where the animal is healthy, comfortable, well nourished, safe, able to express natural

behaviour and not suffering from pain, fear and distress (OIE, 2017). Feeding calves limited amounts of liquid feed during the first weeks of life results in a lack of expression in natural suckling behaviour followed by hunger and stress for the calves. Calves should be allowed to drink unlimited amounts of milk or MR for several weeks during the preweaning period more than double the liquid feed intake compared with restricted amounts of 4 to 6 kg/day of MR or milk. Therefore, an intensive milk-feeding programme contributes to the overall well-being of preweaning calves (OIE, 2017).

**Extraction of Bioactive factors and recent applications**

A number of methodologies for the extraction of milk growth factors from milk, colostrum or whey have been developed. Cation-exchange chromatography has been widely used because of the basic nature of the growth factors. Also, microfiltration has been used for the concentration of some growth factors from colostrum, while ultrafiltration was successful only in separating IGF-I from IGF-II in whey. Growth factor extracts from milk, colostrum or whey have been used as therapeutic preparations for wound healing and in the treatment of inflammatory gut disorders. More recent applications are related to bone tissue regeneration and treatment of inflammatory skin diseases such as psoriasis.

**Conclusion**

Colostrum and milk contain bioactive factors which can exert immunological and growth stimulating activity in newborn calves. While feeding calves with other milk formula or milk replacers, extracts of bioactive factors has to be included in the liquid feed to promote growth of healthy calves. Intensive colostrum and milk feeding influence not only the postnatal and pre-weaning development and growth of calves but also the performance and health in later life.

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# SEMEN PROTEOMICS OF RUMINANTS IN RELATION TO FERTILITY

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***ABSTRACT-***

*Semen proteomics is getting importance in the prediction of fertility status of animals especially ruminants. Most of the spermatozoan proteins and seminal plasma proteins are contemporary with each other. Seminal plasma proteins are associated with sperm membrane protection, prevention of oxidative stress, anti-microbial activity, capacitation, acrosome reaction, oocyte binding and early embryonic development. Thus, they can be positively or negatively correlated with fertility. Binder of sperm proteins, lipocalin-type prostaglandin D synthase, phospholipase A2, osteopontin, acidic seminal fluid protein, P25b, a-L-fucosidase, cathepsin D, the inhibitor of metalloproteinase-2, C-type natriuretic peptide, albumin, sulfhydryl oxidase etc. are the proteins of seminal plasma which are positively correlated with fertility. The seminal plasma proteins which are negatively correlated with fertility include spermadhesin Z13, clusterin, ubiquitin, tissue factor pathway inhibitor 2, 5′ -nucleotidase, galectin-3-binding protein etc. Some of the sperm specific proteins identified in ruminant spermatozoa are pyruvate kinase, cyclooxygenase, mitochondrial ATP synthase F1 Beta subunit, phospholipase C, adenylate kinase-1, A-kinase anchor protein-4, phosphatidyl ethanolamine-binding protein 1, HSP90, SP22 etc.*

*Cryopreservation is reported to result the change in semen proteome of some ruminants. Deleterious effects associated with such changes in semen proteome can be resolved in many ways. The proteomic difference between X and Y spermatozoa is important in sex sorting and immunosterilisation of spermatozoa. For the improvement of ruminant fertility, more proteins should be identified along with their functions using precise technologies.*

***Keywords**: Ruminants, Semen proteomics, Fertility*

## INTRODUCTION

Reproductive efficiency of the male is one of the most important factors influencing sustainability of livestock. Artificial insemination (AI) using cryopreserved semen is very widely and conveniently used especially in ruminants. Hence, it is very important to ensure the quality of semen used for AI. Even though different parameters have been used for the assessment of semen quality, they are not enough to predict the fertility status of a semen sample much accurately. Here comes the relevance of semen proteomics. The proteins present in sperm and seminal plasma regulate the events associated with sperm maturation, motility, oviductal sperm reservoir, capacitation, acrosome reaction, fertilisation, early embryonic development etc. With the advanced technologies like mass spectrometry, large number of proteins could be identified in the spermatozoa and seminal plasma of different species of animals especially in ruminants such as bull, buffalo, ram, buck etc. And, it is clearly understood that the protein profile of spermatozoa and seminal plasma can be used as markers of fertility as they are different in fertile and infertile animals.

The seminal plasma proteins and spermatozoan proteins can be separately included in two categories even if most of them are contemporary with each other.

### Seminal plasma proteins

Seminal plasma is a complex mixture of secretions originated from testis, epididymis and male accessory sex glands (seminal vesicles, ampulla, prostate and bulbourethral glands). During transportation, spermatozoa acquire and interact with

seminal plasma proteins which help them to achieve motility, oocyte binding as well as penetrating capacity and fertilizing ability. Such proteins are also potentially involved in sperm membrane protection, prevention of oxidative stress, anti-microbial activity, capacitation, acrosome reaction and early embryonic development. The seminal plasma proteins may modulate the fertilising ability of spermatozoa positively or negatively.

**Binder of sperm proteins (BSPs)**

They are secreted by the seminal vesicles and belong to the family of heparin-binding proteins and represent approximately 70% of the total protein content of bovine seminal plasma. They were previously called as bovine seminal plasma proteins (BSPs) The most common BSPs are three acidic proteins, designated as BSP1, BSP3 and BSP5 (BSP-A1/-A2, BSP-A3 and BSP-30 respectively). Each BSP is composed of a unique N- terminal domain followed by two fibronectin type II (FN2) domains in tandem that are separated by a short linker polypeptide chain.

| **BSP protein homologues isolated from seminal plasma of ruminant species** | |
|---|---|
| Bull | BSP-A1, BSP-A2, BSP-A3, BSP-30kDa |
| Goat | GSP-14kDa, GSP-15kDa, GSP-20kDa, GSP-22kDa |
| Ram | RSP-15kDa, RSP-16kDa, RSP-22kDa, RSP-24kDa |
| Bison | BiSV-16kDa, BiSV-17kDa, BiSV-18kDa, BiSV-28kDa |

BSP1: It is also known as PDC-109 (Protein with N-terminus aspartic acid D and carboxy terminus Cystine, having 109 amino acids) is a mixture of BSP-A1 and BSP-A2. BSP-A1 and BSP-A2 have identical amino acid sequence but differ only in glycosylation. They are thus considered as a single protein. BSP-A1 contains neutral sugars, galactosamine and sialic acid whereas BSP-A2 contains galactosamine and sialic acid in less quantity and it does not contain neutral sugars.

PDC-109 protein could be identified in seminal plasma of Murrah buffalo bulls and cattle. Each FN2 domain of PDC-109

contains a heparin binding site and choline phospholipid binding site.

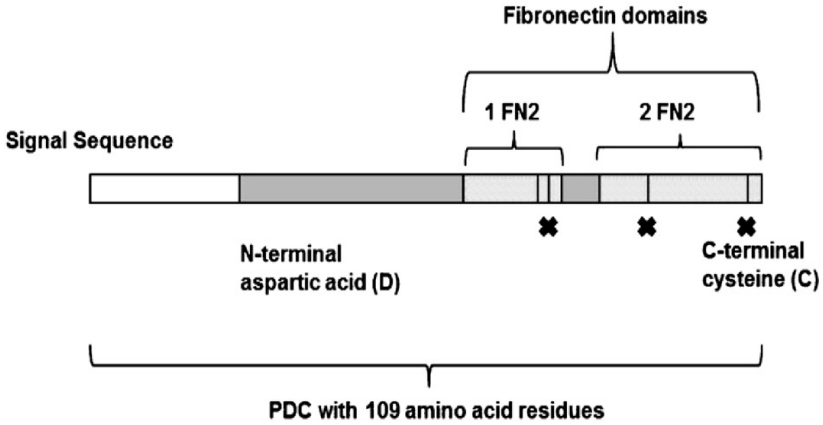


**Fig. 1.** Schematic diagram of PDC-109 protein structure (x: heparin and choline phospholipid binding sites)

BSP-A3 is not a glycoprotein whereas BSP-30 is the most glycosylated member of this family. At ejaculation, sperms are mixed with seminal plasma for a short period and then BSPs bind to sperm (Souza *et al.,* 2008) and remove some (5-8%) cholesterol (1st cholesterol efflux) from the sperm membrane. These proteins do not interact directly with cholesterol, but initial binding takes place by the interaction of proteins with phospholipids containing choline like phosphatidyl choline (PC) and sphingomyelin which will, in turn, interact with cholesterol. This first cholesterol efflux induced by BSP proteins may slightly destabilize sperm membrane (priming).

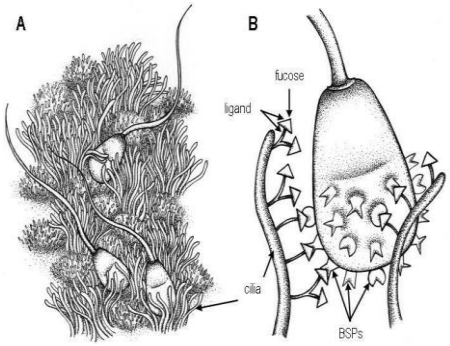


**Fig. 2.** Schematic diagram of oviductal sperm reservoir (Suarez *et al*., 2016)

BSP proteins also coat the spermatozoa and mediate sperm interaction with the oviduct epithelium and formation of the oviductal sperm reservoir by enabling spermatozoa to bind with the fucose-containing glycoconjugates on oviductal epithelium (Suarez *et al*., 2016). This binding maintains the sperm motility until the oocyte reaches the site of fertilization. After the arrival of oocytes, the capacitation factors such as high-density lipoprotein (HDL) and heparin in the follicular and oviductal fluid interacts with the sperm-bound BSPs and stimulates the second cholesterol efflux, resulting in further decrease in the cholesterol: phospholipids ratio, provoking reorganization of membrane. During capacitation, BSPs are lost from the sperm and thereby the spermatozoa are released from the oviductal sperm reservoir.

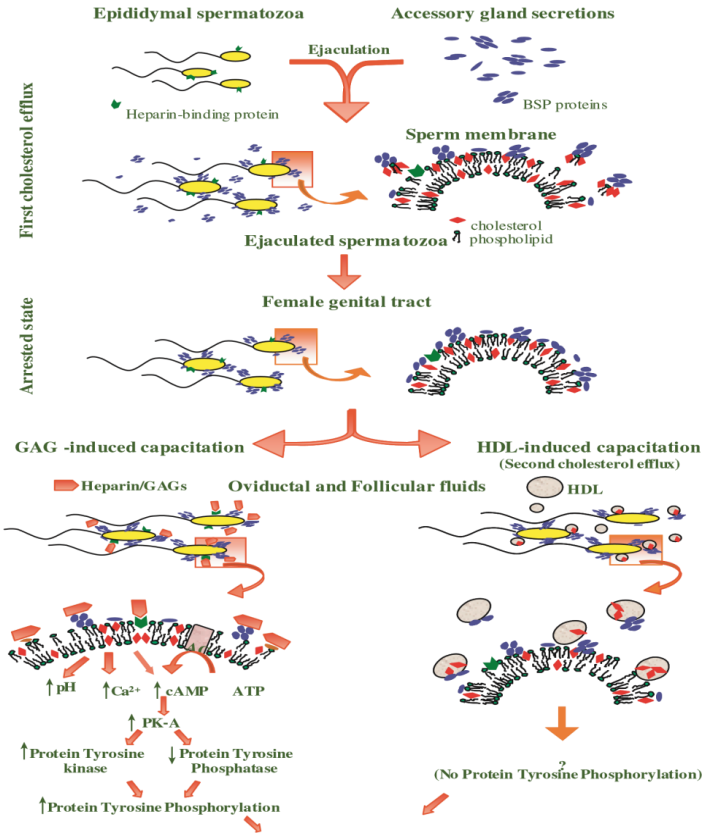


**Fig. 3.** Mechanism of BSP-mediated sperm capacitation (Manjunath *et al.*, 2007)

The altered permeability of sperm membrane allows calcium to enter for the activation of phospholipase A2. This converts phospholipids to lysophospholipids that are known to destabilize membranes. The influx of calcium increases plasma membrane Ca2+-ATPases activity. Thus, PDC-109 has a strong stimulatory effect on the activity of sperm plasma membrane-bound Ca2+-ATPases which regulates sperm motility through intracellular calcium concentration. This will help the sperm transit through the female reproductive tract, starting from the release of sperms from oviductal sperm reservoirs till penetration of zona pellucida.

At the same time, destabilization of sperm plasma membrane by lysophospholipids triggers membrane fusion which leads to regulation of the surface expression of sperm ZP receptors. Adhesion to zona pellucida then triggers the acrosome reaction.

BSP1 affects both fertilization and early development of bovine embryos *in vitro* (Rodriguez-Villamil, 2016). BSP 5 has receptors located in sub cortical cytoplasm of early embryos and plays roles in cell division and is thus associated with fertilization.

BSPs may also have some deleterious effects during the sperm storage. The cholesterol efflux induced by BSP proteins is dependent on exposure time and concentration. *In vitro* experiments confirm that BSPs are needed for proper sperm function but, when cells are exposed to high amounts of BSPs and for long periods of time, they excessively lose membrane cholesterol and phospholipids and become less viable.

**Lipocalin-Type Prostaglandin D Synthase (L-PGDS)**

(L-PGDS) is a fertility-associated seminal plasma protein. LPGDS have been identified on elongating spermatids, sertoli cells, *rete testis*, efferent duct epithelial cells along with epididymal epithelial cells and the apical ridge of the acrosome on ejaculated bovine sperm. It is involved in both development and maturation of spermatozoa. Higher expression of L-PGDS in seminal plasma is associated with high fertility in bulls. This may

be due to its ability to function as a trans-membrane lipophilic carrier protein to maintain the blood-testis and blood-epididymal barriers (Viana *et al.,* 2018).

**Phospholipase A2**

Phospholipase A2 (PLA2) is found in the plasma membrane and acrosome of ejaculated bull sperm. It plays important roles in the late maturational events of spermatozoa, the acrosomal reaction and sperm egg fusion (Sato *et al.,* 2010). PLA2 attached to sperm membranes synthesizes arachidonic acid, which is converted to prostaglandin E2, leading to events related to acrosome reaction.

The membrane-bound PLA2 may be stimulated or inhibited depending on the local concentration of BSPs. This is because BSPs may sequester choline phospholipids on the sperm surface and thereby block PLA2 from acting on these phospholipids and prevent sperm from undergoing a premature acrosome reaction (Manjunath *et al.,* 2007). There is also evidence that PLA2 stimulates immune cells and has antimicrobial activity in the seminal plasma. The expressions of PLA2 are more in semsatoinal plasma of high fertility bulls than in low fertility bulls.

**Osteopontin**

Osteopontin (OPN) is a highly acidic and fertility-associated glycoprotein present in bull seminal plasma. This protein is expressed in the ampullae, seminal vesicles and epididymis of different species.

Different isoforms of OPN have been detected in different portion of bull reproductive tract with different functions (Erikson *et al.,* 2007). In the seminiferous tubules it is synthesized by sertoli and germ cells and involved in binding these germ cells to the basement membrane of the seminiferous tubule and to adjacent sertoli cells.

OPN promotes capacitation of sperm and increase sperm viability possibly by blocking apoptotic pathways (Erikson *et al.,* 2007). OPN interacts with the membrane of ejaculated bull sperm through integrin and/or CD44 receptors and also affects

sperm-oocyte binding and early embryonic development. OPN remain bound to sperm until it reaches the site of fertilization.

Link of sperm-bound OPN with integrin and/or CD44 receptors on oocyte membrane inevitably trigger intracellular signalling for post-fertilization events (Souza *et al.,* 2008). Addition of antibodies against OPN to the IVF medium causes significant reductions in the percentage of fertilization of oocytes. OPN is associated with an increased capacity of sperm to penetrate the oocyte and prevention of polyspermy in porcine and bovine (Erikson *et al.,* 2007).

OPN is also associated with male fertility indirectly by protecting epithelial surfaces of the accessory sex glands from bacterial infections by binding to the integrin receptors on the epithelial surface.

**Acidic Seminal Fluid Protein**

The aSFP is present in the secretions of ampulla and seminal vesicles of bulls. Bovine aSFP belongs to the spermadhesin protein family. The aSFP binds loosely to the sperm surface at ejaculation and restrict motility of spermatozoa as an energy preserving mechanism. But, in the female reproductive tract, loosely attached aSFP is diluted out, allowing for rapid restoration of sperm motility.

The aSFP also protect spermatozoa from oxidative damage by diminishing lipid peroxidation. Higher concentration of aSFP is found in semen from high freezability bulls as compared to semen from low freezability bulls.

**P25b**

P25b, secreted by the epididymal epithelium is the member of xylulose reductases family. It is attached to the testicular sperm surface during the epididymal transit and is associated with the plasma membrane covering the acrosomal cap of spermatozoa. It may be involved in the binding the spermatozoa to the egg and acquisition of sperm fertilizing ability. This protein is considered as a marker of epididymal maturation of spermatozoa.Low levels of P25b are found in spermatozoa from bulls of lower fertility.

**a-L-fucosidase**

This protein is involved in the modification of carbohydrate moieties of sperm membrane proteins during epididymal transit and is found in lower amounts in the seminal plasma of bulls with more percentage of abnormal spermatozoa.

**Cathepsin D**

Cathepsin D, found in cauda epididymal fluid, participate in the proteolytic remodelling of membrane components of sperm during epididymal transit. Intensity of cathepsin D is greater in high-fertility bulls than in low-fertility bulls.

**The inhibitor of metalloproteinase-2 (TIMP-2)**

Thisis a heparin binding protein found in bovine seminal plasma (Moura *et al.,* 2007) influences fertility of bulls through inhibition of metalloproteinase activity in semen. However, precise role and mechanism of action of TIMP-2 in relation to bull fertility are currently under investigation.

**C-type natriuretic peptide (NPPC)**

NPPC belongs to a family of small peptides involved in vasodilatation (Potter *et al.,* 2009). NPPC from seminal plasma binds to the receptors in the acrosome and tail of spermatozoa. This stimulates guanylate cyclase and thus intracellular cGMP and sperm motility. NPPC also interacts with natriuretic peptide A, which promotes trophoblasts implantation and artery remodelling in uterus (Viana *et al.,* 2018).

**Albumin**

This protein facilitates cholesterol efflux from sperm membrane and promotes capacitation. Albumin also binds to free radicals, protecting sperm against lipid peroxidation. In low fertility bulls, it is possible that lower albumin concentrations cause increased production of reactive oxygen species and limited antioxidant defences in seminal plasma, contributing to poor semen quality and reduced fertility. Scrotal insulation causes reduction of albumin and low semen quality (Hunter, 2009).

**Sulfhydryl oxidase (QSOX1)**

QSOX1 is positively affecting fertility through its interaction with several types of cell membrane glycoproteins and with albumin, the most abundant protein of the cauda epididymal fluid (Moura *et al.,* 2010). Albumin protects sperm cells against harmful effects of lipid peroxides. QSOX1 also interacts with vascular endothelial growth factor A and insulin growth factor 1 and improves blastocyst formation in the bovine species.

**Seminal plasma proteins negatively correlated with fertility**

**Spermadhesin Z13**

Spermadhesin is abundant in seminal plasma of bulls of low fertility and is considered as an anti-fertility factor. Spermadhesin Z13 has 50 % homology with the aSFP. This binds to the surface of spermatozoa. Lower fertility of bulls with high levels of spermadhesin Z13 is due to the adverse effect of this protein on sperm motility.

**Clusterin (CLU)**

Clusterin is a disulfide-linked heterodimeric protein and is also known as sulfated glycoprotein-2, testosterone-repressed prostate message-2, apolipoprotein J and complement lysis inhibitor. It was first identified in ram *rete testis* fluid where it showed signs of clustering with rat sertoli cells and erythrocytes, hence its name.

In the male reproductive tract, it is produced by sertoli cells and epididymal epithelial cells. It binds and agglutinates abnormal spermatozoa in bulls and prevents oxidative damage to the sperms. The presence of clusterin on ejaculated sperm may indicate improper spermatogenesis or irregular epididymal maturation. Clusterin contributes to removal of defective spermatozoa and is an indicator of poor semen quality in bulls (Shojaei Saadi, 2013) and rams.

CLU also interacts with galectin-3 binding protein, another protein found at high levels in the seminal plasma of low fertility bulls.

**Ubiquitin**

In the male reproductive tract, ubiquitin is secreted by epididymis. In the bull epididymal fluid, prostasome-like secretory particles, epididymosomes transfer epididymis-secreted proteins to the bull sperm plasma membrane. Aryl sulfatase A is one of the sperm surface proteins ubiquitinated in the defective spermatozoa.

Increased ubiquitin levels in bull sperm are indication of both poor semen quality and fertility in bulls. The ubiquitin is a suitable marker of sperm abnormalities because it covalently links to the surface of defective mammalian spermatozoa.

**Tissue factor pathway inhibitor 2 (TFPI2)**

TFPI-2 in seminal plasma interacts with kallikreins, a group of proteins that convert kininogen into kinin, promoting increase in sperm hyper motility. However, further studies are still needed to confirm if TFPI-2 has any correlation with low fertility.

**5′ -nucleotidase (NT5E)**

NT5E is a glycosylated enzyme present in seminal plasma of bulls of low fertility (Viana *et al.,* 2018).

**Galectin-3-binding protein**

Galectin-3-binding protein is a member of beta-galactoside binding lectins (Kovak*et al.,* 2014) which interacts with clusterin and is also over expressed in low fertility dairy bulls.

**Spermatozoan proteins**

Large number of proteins could be identified in the spermatozoa of different species of ruminants. The protein profile of spermatozoa can also be used as markers of fertility as they are different in fertile and infertile animals. Spermatozoa are transcriptionally inactive so the only comprehensive method to understand the molecular functions in spermatozoa is via proteomics.

Most of the spermatozoan proteins get absorbed from the seminal plasma either during their epididymal transit or

after ejaculation. These proteins are mainly associated with membrane stabilization and sperm-zona binding.

***Location of sperm proteins***

Various proteins are localized in the surface of sperm head, mid piece and tail. The proteins in sperm head are mainly associated with pre-fertilisation events like capacitation and acrosome reaction and oocyte binding. The proteins identified from mid piece and tail of spermatozoa are found to be related to sperm metabolism, energy production, tail structure, motility etc.

Some of the sperm specific proteins identified in ruminant spermatozoa as follow:

***Pyruvate kinase (PKM2)***

Pyruvate kinase (PKM2) is higher in spermatozoa of bulls with high fertility. It catalyses the production of pyruvate and ATP from phosphoenol pyruvate which act as the energy source for the spermatozoa.

***Cyclooxygenase (COX3)***

The mitochondrial protein, COX3 is also higher in spermatozoa of high fertility bulls. This protein is the part of the electron transport chain and it is essential for the energy production of spermatozoa.

***Mitochondrial ATP synthase F1 Beta subunit (ATP5B)***

It is also a mitochondrial protein associated with ATP production in the mitochondrial membrane which is used for sperm motility and capacitation. This protein is also positively correlated with fertility.

***Phospholipase C (PLC)***

Phospholipase C is the spermatozoan protein important for acrosome reaction, fertilisation and embryo development. PLC catalyses the production of inositol triphosphate (IP3) which binds to the IP3 receptor gated calcium channel located on the acrosome membrane and activates the extra cellular calcium influx required for the acrosome reaction.

**Adenylate kinase-1 (AK1)**

This is more abundant on sperm from high-fertility bulls. It is mainly located in sperm flagella and promotes motility of spermatozoa (Kasimanickam *et al.,* 2012). Higher abundance of AK1 ensures the availability of additional energy for hyperactivation which is critical to fertilization, because it enhances the ability of sperm to detach from the oviductal epithelium and penetrate the zona pellucida.

***A-kinase anchor protein-4 (AKAP4)***

A-kinase anchor protein-4 is significantly higher in high fertility bull spermatozoa. This is a major fibrous sheath protein of the principal piece of the sperm flagellum. This recruits protein kinase A to the fibrous sheath and helps local phosphorylation to regulate flagellar function and thereby motility of spermatozoa.

***Phosphatidyl ethanolamine-binding protein 1 (PEBP1)***

It is present in higher abundance in sperm from high fertility bulls, which is released during capacitation or as a membrane-bound, glycol phosphatidyl inositol (GPI) anchored receptor for a decapacitation factor (Gibbons *et al*, 2006).

***HSP90***

HSP90 is positively correlated with freezability and fertility of bull spermatozoa. It is a major cytoskeletal protein, present in the tail region of spermatozoa which is associated with sperm motility. Its poor expression leads to low motility and sperm defects, reproductive abnormalities and infertility (Li *et al*., 2016).

***SP22***

SP22 is a testis specific protein of bull which is localized in the equatorial segment of sperm head, the important region for recognizing and fusing of oocytes.

**Semen proteome and cryopreservation**

As the seminal plasma is diluted 10-20 times during the processing for semen preservation, the spermatozoan proteins are critical for fertilisation. Cryopreservation induced damages

may be resulted from the loss of sperm surface proteins which are necessary for fertilization. Inactivation of membrane-bound enzymes and different distribution of proteins within the membrane have been shown to occur at different steps of ram semen cryopreservation (Marti *et al.,* 2008).

A threefold decrease of P25b, a spermatozoan protein associated with fertility, is observed in cryopreserved bull spermatozoa compared to fresh spermatozoa. Frozen thawed buffalo semen had a 50 % reduction in the quantity of Osteopontin (Pero *et al.,* 2007). Pini *et al.* (2018) found out that 24 proteins decreased in frozen thawed ram spermatozoa, compared to fresh spermatozoa.

Cryopreservation may also result in generation of reactive oxygen species which may result in degradation of spermatozoan proteins (Meyers*,* 2012).

***Tools for semen proteomics***

Methods based on gel electrophoresis and mass spectrometry have been used for the identification of proteins in the spermatozoa and seminal plasma. More recently, the more efficient proteomic approaches based on LC-MS/MS have been used for the study of protein mixtures.

**Applications of semen proteome in relation to fertility improvement**

***a. Sequestration of the seminal plasma proteins to minimize cryoinjury to spermatozoa***

Exposure of spermatozoa to high amounts of BSPs and for long periods of time may result in loss of viability of spermatozoa due to excessive loss of membrane cholesterol and phospholipids (Menezes *et al.,* 2017). Sequestration of those proteins can minimize such deleterious effects.

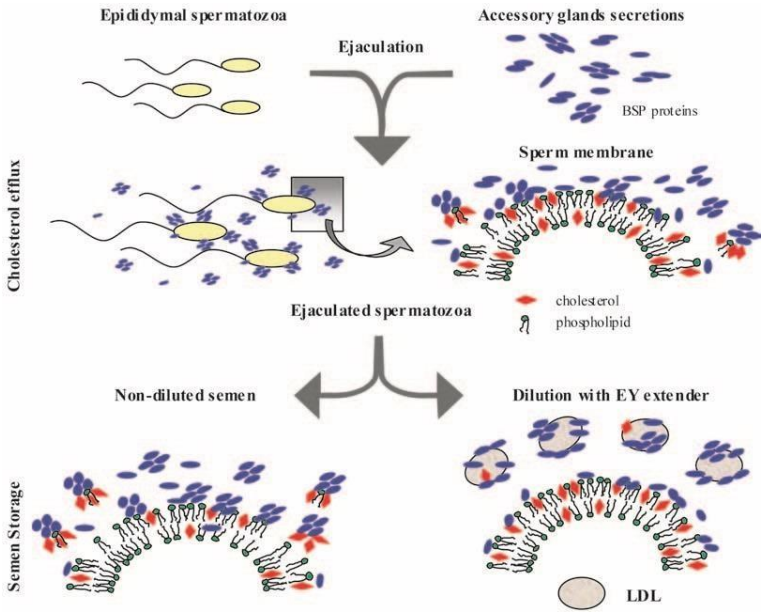


**Fig. 4.** Mechanism of sperm protection by egg yolk (Manjunath *et al.,* 2007)

BSPs bind to the low-density fraction (LDF) of the lipoprotein component of the egg yolk (EY) in a rapid, specific, saturable and stable manner even after freezing and thawing of semen. This may prevent their detrimental effect on sperm membrane, and it is crucial for sperm storage (Manjunath *et al.,* 2009). Thus, scavenging of the BSPs by LDF on dilution of semen with extender containing EY protects sperm from deleterious effects of BSP proteins present in seminal plasma.

Milk caseins also prevent the detrimental effects of BSP proteins on the sperm membrane during preservation.

Fn-2 type protein chelating agents like choline chloride can be used along with the extender for improving the freezability of semen from goat (Shiny, 2011). Choline chloride has the same receptor moiety as that of sperm surface receptors to Fn2 type proteins.

Sequestration of free floating PDC-109 protein in seminal plasma is possible by collection of bovine ejaculates in tubes coated with anti-PDC-109 antibodies (Srivastava *et al.,* 2013).

***b. Supplementation of cholesterol to compensate the cholesterol efflux caused by BSPs***

The spermatozoan membrane destabilization due to cholesterol efflux induced by BSPs can be compensated to some extent with the supplementation of cholesterol. As a hydrophobic molecule, cholesterol is not soluble in aqueous extenders. Hence, cyclodextrin, a cyclic oligosaccharide obtained by enzymatic degradation of starch, is used for incorporation of cholesterol (Moce *et al.,* 2010). Cyclodextrins contain a hydrophobic centre capable of incorporation and removal of cholesterol from sperm membrane. Cyclodextrins, if pre-loaded with cholesterol, can insert cholesterol into cell membranes. Methyl-β-cyclodextrin has highest affinity for cholesterol than other cyclodextrin molecules. Sperm should be incubated with cholesterol loaded cyclodextrin in a lipid free medium, as lipids interfere with the uptake of cholesterol by spermatozoa. Increased cryosurvival rates have been reported with cholesterol supplementation of spermatozoa in ruminants like ram, bull and buck (Farshad *et al.,* 2010).

However, higher amount of cholesterol incorporated sperm membrane may account for reduction in the motion parameters due to increase in the membrane rigidity and as a result of toxicity due to oxidation process of cholesterol (Farshad *et al*., 2010).

***c. Post-thaw supplementation of whole seminal plasma***

Supplementation of seminal plasma to the frozen thawed semen is reported to increase the semen quality in different species including ruminants. Seminal plasma of heterologous species can also be used. Supplementation of 40 per cent boar seminal plasma in cryopreserved ram sperms increased pregnancy rate in ewes following intracervical AI (Fang *et al*., 2018).

***d. Post-thaw supplementation of proteins lost during cryopreservation***

Supplementation of specific fertility related protein, which is found to be decreased during cryopreservation, to the thawed

semen is a good approach to improve the quality of semen to be inseminated. Addition of such proteins to cryoprotective media can also be a choice to protect sperm damage during cryopreservation (Mogielnicka Brzozowska *et al.,* 2015). Incorporation of the seminal plasma protein, osteopontin (OPN) into the frozen thawed buffalo sperms improved their quality (Boccia *et al*., 2013).

***e. Supplementation of IGF-1 to the extender***

IGF-1 has a positive effect on spermatozoa by reducing the oxidative stress along with improving the motility. IGF-1 already present in seminal plasma gets diluted on extending the semen. Hence, supplementation of IGF-1 to the extender reduces the lipid peroxidation and thereby ROS induced protein degradation (Selvaraju *et al.,* 2016).

***f. Sexing of spermatozoa based on the spermatozoan proteins***

X and Y sperms express different proteins which provide a new way to separate sperm by using immunological methods (Sang *et al.,* 2011).

***g. Immunosterilisation based on the spermatozoan proteins***

Immunisation against sperm surface antigens can be used as a mode of Immunosterilisation in animals. Immunisation against PH-20 and LDH -C4 reduced fertility in rabbits (Yitbarek *et al.,* 2014). This area should be more explored in ruminants.

**Conclusion**

Semen proteome is associated with fertility related events. Difference in their presence between fertile and infertile animals direct to their usage as biomarkers of fertility. Deleterious effects associated with the cryopreservation induced changes in semen proteome can be resolved in many ways. The proteomic difference between X and Y sperms have a very important application of sex sorting and immunosterilisation. As future perspective, more proteins should be identified and their biological roles in sperm function should be explored with more precise technologies.

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# THE ROLE OF REVERSE OSMOSIS IN DAIRY PROCESSING

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***ABSTRACT-***

*The membrane separation process has gained popularity in food industry, particularly in dairy industry. The different separation techniques like Ultrafiltration, Microfiltration, Nano-filtration, Reverse osmosis and electro dialysis are used for various purposes such as for the fractionation of milk, to extend the shelf life of milk and milk products, for instance control bacterial growth, pre-concentrate milk and whey proteins, to produce whey protein concentrate and valuable by-products, to fractionate whey and lactose intermediates, to improve cheese yields and product consistency. The separation processes are based on the ability of the semipermeable membrane which acts as a selective barrier to differentiate between molecules on the basis of chemical composition, size and shape. The liquid portion which pass through the membrane is referred as 'permeate' and other components which retained is known as 'concentrate' or 'retentate.' This process is mostly governed by the pressure driven across the membrane and concentration gradient of the liquids. The membrane separation technique has got several advantages that it act as non-thermal technology which minimizes the adverse effect such as, denaturation of proteins, changes in sensory attributes*

*and changes in phase. Thus, this is an environment friendly technology due to the low consumption of energy in filtration and improves the economics of dairy by reducing the cost of production. This review paper deals with the large field of application of Reverse Osmosis for the processing of milk and milk products.*

***Keywords**: Reverse Osmosis (RO), Total Solids (TS), Dairy Industry, Concentration.*

## INTRODUCTION

The Reverse Osmosis technique was first developed by Srinivasa Sourirajan in 1960. RO as a membrane filtration process in the initial stage found applications for treating brackish and seawater, desalination and biochemical sectors. But now its use in dairy sector has dramatically increased. RO is commercially used for concentrating the solutions or for dewatering. Among other membrane separation technologies, RO membrane has the smallest pore size ($10^{-4}$-$10^{-3}$ micrometer) and hence it demands highest operating pressure. In RO process, the solvent is passed through the suitable semipermeable, nonporous membrane and selectively separates the solutes of >150 Daltons molecular weight from the solvent by applying an external pressure greater than the osmotic pressure of the solvent. As the small solutes in the solvent exhibit higher osmotic pressure, inorder to overcome this osmotic pressure RO are designed for pressures between 35–100 bar. The desirable characteristics of membrane module such as high physicochemical stability, superior selectivity for solvent, less fouling and durability are the factors responsible for the high throughput and efficiency of RO process.

### Applications in Dairy Industry

**1. Processing of Liquid Milk**

**a. Bulk transportation of RO concentrated milk**

The transportation of liquid milk from chilling plant to far away city dairies was not feasible and was very expensive. RO is capable of removing 70% water present in milk and this concentration of milk reduces the cost of bulk milk transportation without any changes in the chemical and sensory characteristics. The Processing cost for the concentration of buffalo milk to 1.5

and 2-folds is 3.64 and 4.64 paise/kg of milk, respectively.The RO method is inexpensive, versatile, and energy-efficient, and it can also be used to enhance the evaporation process. According to the experiments, the most cost-effective RO concentrations for skim milk and whole milk were 22% and 30% TS, respectively.

**b. Use of RO Milk**

RO milk can be used for the manufacture of fluid products like flavoured milk, skim milk, high protein milk and plain milk. Whole milk was concentrated twofold using RO technology then stored at 50°C for 24 hour and diluted with water followed by pasteurization, homogenization and flavor addition for natural milk composition without any significant difference in organoleptic quality of diluted milk and products manufactured from it. The reconstituted whole milk was kept in the refrigerator for 9–12 days under refrigerated condition. RO concentrated milk can be used for preparing long shelf life UHT treated milk concentrates (fivefold) without any additives.

**c. Traditional Dairy Products**

RO concentrated milk is also utilized for the manufacturing of traditional dairy products such as khoa, basundi, chakka, shrikhand, rabri and kheer. Traditional methods, such as boiling milk in an open pan, were exceedingly expensive and necessitated a lot of fuel and energy. However, Khoa made from 1.5-2 fold concentrated cow or buffalo milk using RO proved energy efficient without any difference in the chemical and sensory attributes. The energy required to concentrate 1 kg milk to 65 percent TS using an open pan boiling method was 136 Kcal, whereas RO concentrated milk used just 20 Kcal. As a result, there was a reduction in energy use.

**d. Fermented Dairy Product**

The use of RO has expanded to include the production of yoghurt, dahi, and other fermented foods. Yogurt is made by standardising milk to 12-14 percent total solids by adding RO retentate and milk powders. RO retentate improves sensory properties; culture growth, acetaldehyde content, viscosity, and acid generation in addition to increasing TS. Due to its better

buffering capability, the pH drops in yoghurt made from RO retentate were slower.

**e. Frozen Dairy Product**

The RO technique was found to be energy efficient for the standardisation of ice cream mixes in numerous investigations. The use of RO retentate to replace 50–100% of the milk solids not fat (MSNF) content in ice cream mixes increased viscosity, specific gravity, protein, ash, freezing point, and melting resistance. As a result, the amount of stabiliser required fell dramatically since the protein in RO retentate had a larger water binding ability, but there were no negative effects on TS or fat. However, due to the high lactose content, total substitution of SNF with RO retentate causes sandiness and coarse texture faults in ice cream. To compensate for these flaws, lactose to protein ratio of 1.25–1.45 had been recommended in ice-cream mixes.

**f. Dried Milk**

In modest pilot scale equipment, Glover (1971) found that whole milk from cows was concentrated 2-fold by reverse osmosis. The concentrate contained all of the milk's major ingredients. Protein was found to be a primary cause of membrane blockage in the experiment. Ultrafiltration of milk to remove protein was tested in the lab with the goal of devising a two-stage method for milk concentration that included ultra filtration, reverse osmosis, and recombination of the concentrates from the two processes. The findings suggest that reverse osmosis alone may efficiently concentrate milk, but more research is needed to speed up the process. It's possible that some improvement could be obtained by first concentrating the protein by ultrafiltration (as demonstrated by the laboratory-scale experiment), and such a combination of the two procedures of ultrafiltration and reverse osmosis is now being investigated.

Since RO milk concentrates is done at ambient temperature, no nutrient degradation, protein denaturation and Maillard browning was measured. Calcium has a crucial role in determining the heat stability, gel strength, and zeta potential of milk powder, however calcium content increases during RO concentration, resulting in reduced heat stability in RO

concentrates. Report by Abbot *et al.* (1979) shows that no significant changes between the heat stability of RO and conventional concentrates. But according to Syrios *et al*. (2011), RO concentrated skim milk thus obtained were spray dried and powders were reconstituted to 25% TS solutions. Reconstitution of RO concentrate powder showed lower thermal stability at sterilization temperature (115°C for 15 min) therefore addition of 0.2% trisodium citrate and 0.1% disodium hydrogen phosphate improved the sterilization stability of both the retentates and could be successfully used for production of heat stable skim milk powder (SMP). Additionally SMP obtained from RO concentrate is whiter as RO concentration decreases the extent of Maillard reaction. And had 12 months shelf life without any adverse effect on any of the powder properties. Furthermore, Whole milk powder made from RO retentate had a greater free fat content (about 57 mg/g) and undesirable lipolytic taints due to fat globule rupture during RO retentate transit. Nevertheless, thermal inactivation of lipase prior to RO treatment was effective in averting high FFA. And such powder could be used in products where free fat is desired.

Balde and Aider (2017) conducted a study on skim milk, which was concentrated using cryoconcentration (CC), reverse osmosis (RO), and vacuum evaporation (VE), then spray-dried. The study demonstrates that vacuum evaporated milk powder contains some black particles; whereas powders made from cryoconcentrated milk and reverse osmosis concentrated milk do not. The CC-powder and RO-powder had the largest powder particle sizes, while the VE-powder had the smallest. The CC-powder was identical to the RO-powder in colour, whereas the VEpowder was slightly brownish. Milk reconstituted from CC-powder had the largest mean particle size of the protein micelles in reconstituted skim milk in water at a concentration of 25% (w/v), followed by milk reconstituted from RO-powder. The reconstituted skim milk had the greatest whiteness index of all the milks produced with CC-powder and RO-powder.

## 2. Processing of Whey

Whey is a byproduct of cheese, casein, and paneer, and it contains 5.5-6.7 percent Total Solids, which includes 12 percent protein, 70-75 percent lactose, 1% fat, 1 percent latic or citric acid, and 8-10 percent inorganic acids. The presence of a high amount of protein and lactose results in a significant pollution load, indicating the necessity for efficient whey processing. In a batch process, RO is used to concentrate whey to about 18 percent total solids, and in a multistage process, it is used to concentrate whey to around 26-28 percent total solids. Fouling of the membrane occurs during whey concentration as a result of a decrease in flow as a function of increasing lactose concentration and the precipitation of remaining protein components. Prior to RO concentration, whey protein is denaturated by heating to 50°C to avoid fouling. According to Rektor and Vatai (2004), whey should be defatted and sterilised using microfiltration before being concentrated by RO to produce the best results. The cost of steam for evaporating whey in the production of Gouda cheese whey concentrate was 55 percent of total costs, but this was lowered to 25 percent by employing the RO process.

## 3. Waste Water Treatment

RO techniques have also proven useful in the treatment of dairy effluent, allowing for the recovery of milk components and the production of reusable water. According to Koyuncu *et al*. (2000), RO tests on dairy waste water using a two-pass RO membrane can yield high-quality permeate. Although flux values decreased throughout each RO run, virtually 100% Chemical Oxygen Demand (COD) reductions were obtained. The dairy effluent was treated with RO until 90-95 percent of the water was recovered, resulting in a Total Organic Carbon (TOC) removal rate of over 99.8% and lactose removal rates of above 99.5 percent, 95 percent for multivalent ions, 87 percent for monovalent ions, and 96 percent for nitrogenous matter. Finally, purified water was discovered to have a comparable quality to vapour condensates from dairy processing, allowing it to be reused for cleaning, heating, and cooling.

## 4. Cleaning Process of RO Membrane plant

The cleaning process removes unwanted deposits/soils from the membrane and its supporting components. The foulants are deposited over/into the membrane surface during operation, forming a gel-like coating. Cleaning-in-place (CIP) methods and chemicals are used to clean membrane systems on a regular basis, maintaining adequate turbulence to dislodge foulants. Depending on the membrane material, module arrangement, and type of deposits, a variety of cleaning solutions are employed. To remove fat deposits, nonionic detergents or surfactants are utilised, whereas acid and alkaline detergents are used to clean inorganic salts and proteins, respectively. Cellulose acetate (CA) RO membranes are cleaned using a combination of enzymes and detergents when operating below 40°C and in the pH range of 3–8. UF membranes, on the other hand, can comfortably take 100–200 ppm of accessible chlorine, whereas CA membranes are susceptible to high chlorine concentrations (> 50 ppm). Because of the cleaning required by enzyme soaking, RO systems typically require 3–4 hours of CIP. Measuring the water flux rates during washing is a common way to determine the level of membrane cleaning. All membranes, regardless of procedure, make, or material, must be properly cleaned and sterilised prior to and after each run as per the membrane manufacturer's recommendations for optimal results, effective separation, and longer membrane life (Deshwal *et al*., 2021).

## Conclusion

As a result, RO has more prospective applications in the concentration of milk, whey, and the treatment of dairy waste water. This procedure is cost-effective since it minimizes the amount of energy required, lowering manufacturing costs, and reducing the volume, lowering transportation costs. The nutritional and sensory properties of RO concentrates are identical to those of the original, and they help to increase the yield of the products. Newer membrane modules with higher performance in terms of high flux rates, less fouling, and physically resistant and improved tolerance to cleaning solutions

(especially chlorine) should be produced through research and development. This could help industrial RO plants to save money on both construction and operating costs. Reverse osmosis may undoubtedly play a significant role in the Indian dairy sector in the future due to its simplicity, better competitiveness, process or product innovation, and environmental friendliness.

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# AN OVERVIEW OF SPERM AND EMBRYO SEXING TECHNOLOGY

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***ABSTRACT-***

*Recent developments in assisted reproductive technologies such as artificial insemination (AI), multiple ovulation and embryo transfer(MOET), and sex sorting of semen and embryos serve as effective tools in optimising genetic improvement efforts in livestock sector. Sperm sexing is a current breakthrough, wherein X- and Y- bearing spermatozoa are separated before insemination based on the difference in their DNA content to obtain off-springs of pre-determined sex. Sexing is done by techniques like flow cytometer, fluorescent dye based sorting, albumin gradient/ Percoll gradient/gradient swim down, identification of H-Y antigen, centrifugal counter current distribution, free flow electrophoresis, genetic approaches etc. Both invasive and non-invasive methods are employed for sexing of embryos. Karyotyping/cytogenetic analysis, embryo sexing using Y-specific DNA probes, PCR based methods, fluorescence in situ hybridization and Loop mediated isothermal amplification assay are the invasive*

*embryo sexing methods. Non-invasive techniques such as detection of X-linked enzymes, detection H-Y antigens, hormonal assay and sexing based on cleavage and development are also widely practised.*

***Key words**: Sperm sexing, embryo sexing, invasive methods, non-invasive methods*

## INTRODUCTION

Producing embryos of desired sex by sex identification at the time of conception has greater developmental potential in livestock industry. When production of males is desirable for meat industry, dairy sector prefers production of female calves for increasing milk production and herd replacement. Thus, outcome of an animal's pregnancy is significant as it could lead to animal wastage owing to wrong sex. Sex ratio is genetically controlled, though it is natural mating or artificial breeding, but the probability is almost fixed as 51: 49 in favour of male calves, even with the most efficient breeding programmes. The genetic sex of an individual is determined at the time of fertilization. Thus separating the X bearing sperms from Y bearing will help in predetermining the sex of embryo thus increasing the chance of producing a female animal. Embryo sexing helps to enhance the efficacy of embryo transfer technology by transfer of desired sex. Sex can be determined by different invasive and non-invasive techniques with different efficiency and merits.

### Sexing of sperm

Sexed semen technology can be effectively utilized at both farm and stud levels for the generation of productive (female) animals. High-value bulls and heifers born to selected sire and dam will help maximize the genetic gain and production.

### History of semen sexing

Sex sorting technology was developed by the United States Department of Agriculture researchers and was patented as "Beltsville Sperm sexing technology". Hoechst 33342 dye which could easily penetrate the cell membranes and bind to DNA, quantitatively distinguishing X and Y sperms was used for sexing. Advances in flow cytometry continued and soon high-

purity sorting was possible with input rates of greater than 40000× and Y-sperm cells per second which could then be sorted at a high purity of either sex at over 8000 cells/s. Dr. George Seidel and his co-workers from Colorado State University have been credited with the large scale field level application of this technology in cattle (Seidel, 2012) and other species (de Graaf *et al*., 2014). In the year 2001, sexed semen was first commercialised in United States, with a license granted to Sexing Technologies (ST), Texas. Even today, this technique of using flow cytometer and measuring DNA content of sperm through the fluorescence of the DNA bound Hoechst 33342 remains the only, commercially viable method to sex-sort mammalian sperm before insemination. Significant improvement in handling of semen, sorter technologies and automation has considerably enhanced its efficiency so that it could now be compared favourably with conventional semen (Vishwanath *et al*., 2014). Sexed semen is widely used in species like dairy and beef cattle, pigs, sheep, goats, deer and horses (de Graaf *et al*., 2014). The technology is also effectively utilized in exotic and conservation efforts of dolphins, rhinos, whales, brown bears.

**Advantages of semen sexing**

- Producing only female calves helps the farmers to save resources that would have been shared with unwanted males and also increase supply of replacement heifers.
- Opportunity to sell surplus heifers to other farmers/farms
- Sorted semen technology will speed up genetic improvement by increasing efficiency of progeny testing (PT) programme and increasing efficiency of embryo transfer and IVF programme
- An economic way to increase herd strength with no risk of introducing diseases by purchasing heifers from outside (improves bio-security).
- As dead, dying or damaged sperm cells are removed during the sorting process, only viable sperm are available which helps the sexed semen to be successful even at a low concentration (than conventional semen).

- By producing more female calves using sexed semen, the incidence of dystocia especially in maiden heifers can be reduced considerably.

Currently, sexed bovine sperm can be purchased from UK, Canada, USA, Mexico, Argentina, Brazil and China; the licensing and commercialization of this technology is under process in many other countries (Garner, 2013).

**Basic principle of semen sexing**

Semen with either X or Y bearing sperm which can be used to produce progenies of desired sex with 80-90 per cent reliability can be called as sexed semen. The considerable difference of 3.8 per cent DNA between X and Y sperm forms the basis of sex sorting. Thus, on average, the difference in size between X and Y sperm is about 4% in cattle with some subtle differences between breeds (Garner *et al*., 2013).

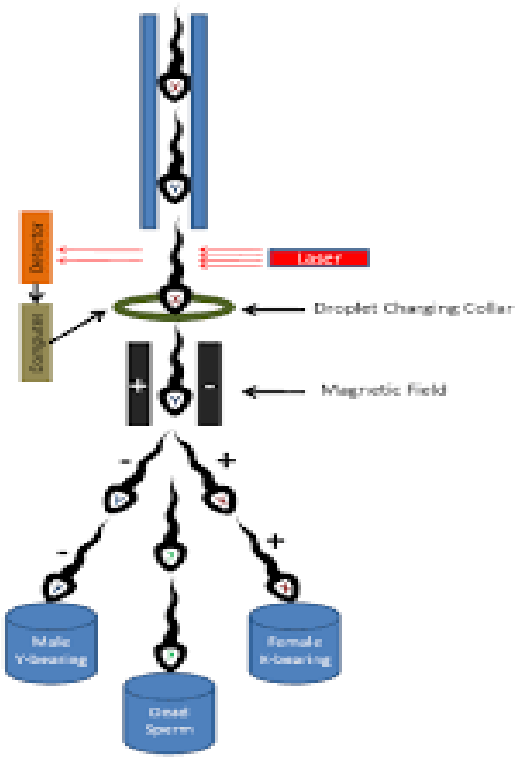
**Methods of sperm sexing**

***Flow cytometry***

Among the different methods, flow cytometry based sorting has emerged as the most efficient method of sperm sorting with 90 per cent accuracy. The technique based on fluorescent emission of Hoechst 33342 dye is well standardized, patented and commercialized in countries like USA, Europe *etc*. Hoechst 33342 will diffuse through intact cell membrane and selectively bind to the A/T base pairs within the minor groove. The absorption and fluorescence emission spectra of H33342 are about 350/460 nm and this shift makes it a convenient marker to differentiate X and Y sperms based DNA content (Garner, 2009). DNA quantification is done using two fluorescence detectors that measure the intensity of the signal from the H33342 bound to the DNA when excited by a laser.

The jet in air flow cytometer allows the sperm to flow through in a single file and creates a terminal droplet and a differentiating droplet charge to separate the two populations of sperm. The charged plates at the discharge point deflect the two separated populations to opposite streams which allow easier collection. The sorted populations are distinguished by

fluorescence histograms on the flow cytometer and the software also facilitates elimination of dead and moribund sperms.



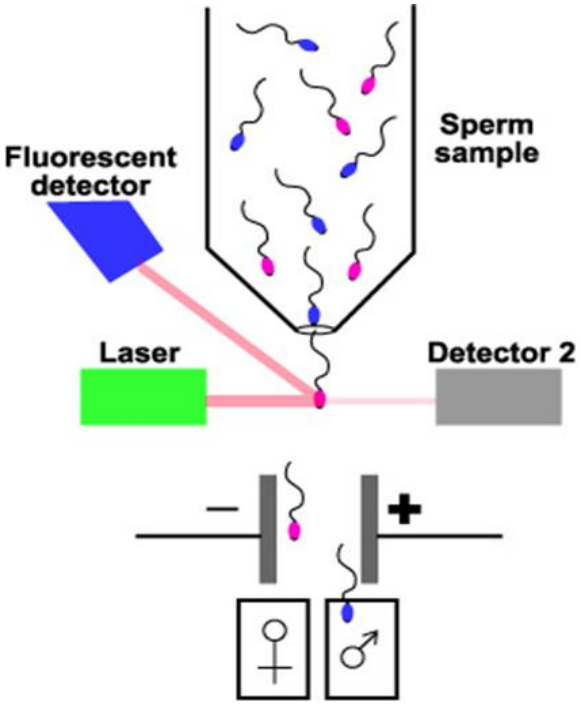

***Fluorescent dye based sorting**:*

The technique was developed by L. Johnson (ARS-USDA, Beltsville, MD) in 1989, wherein fluorescent dyes capable of binding to DNA are used to differentiate the sperms since X sperms have 2.8-7.5% more DNA than Y sperms.

**Procedure**

1. Stain the sperms with DNA dye (X sperms bind more dye compared to Y).
2. Release a single sperm in a drop.
3. The dye is excited using a laser beam so that the sperm gives off light proportional to its DNA content
4. The drop is charged depending on the light intensity (X sperm give off more light than Y sperm)

5. The drops are then passed through a pair of electrodes where the charged drops get sorted into two different tubes.




***Alternative methods for sperm sexing***

Besides the difference in size, DNA sequences also vary between X and Y chromosomes and are unique to each type. Fluorescent in situ hybridization (FISH) techniques can be used to specifically probe for X or Y sperm but requires the disintegration of the head of the sperm. Functionalized gold nanoparticles also were used to locate and non-invasively bind to Y-chromosome-specific sequences. Other popular methods of semen sexing; though not recommended for commercial production, includes Albumin Gradient/Percoll gradient/ Gradient swim down, Identification of H-Y antigen, Centrifugal counter current distribution, Free flow electrophoresis, Genetic approaches etc.

Some publications provide convincing evidence of manipulating sex ratios by nutritional (Herrmann *et al*., 1999), genetic (Roche *et al*., 2006), physical and immunological methods

(Rosenfeld, 2012). The most recent one is a report on sex sorting of buffalo semen using swim-up technique and validation by RT-PCR (Ul-Husna *et al*., 2017).

**SexedULTRA™ process**

Sexed semen is considered inferior to conventional semen owing to the physiological changes resulting from extended holding time before staining, exposure to laser beam (to induce fluorescence) and electrical field (for drafting as a relatively pure population into separate vessel). The SexedULTRA™ process has been designed to be less harsh on sperms during the different processing stages particularly, buffering pH changes and managing oxidative load during the sorting process (Lenz *et al*., 2016). Using the SexedULTRA™ media improved sperm motility and acrosome integrity has been reported (Gonzalez-Marin *et al*., 2016). SexedULTRA™ semen when used in in-vitro fertilization (IVF) trials resulted in greater number of freezable embryos compared with the XY method (13.2% and 9.2%, respectively) (Gonzalez-Marin *et al*., 2016).

**Difference in the processing of sexed semen**

Sorted sperm are collected into tubes containing buffers for the protection of cells. After sorting, tubes are slowly cooled to 5°C and extenders containing cryoprotectants are added before centrifugation to obtain concentrated sperm pellets. After equilibration, semen is loaded into straws and frozen in a programmable freezer. Post-thaw quality is evaluated based on sperm motility and acrosome integrity after 3 h of incubation at 35°C. Purity check is done using an analytical sorter where the histogram differentiates the relative populations of X and Y. Typically the purities are around 90% of the desired sex. The technique is challenging since the sperms are subjected to multiple processes during the sorting process before the final freezing and storage.

**Physiological changes in sexed sperms**

In vitro functional studies using chloro-tetracycline analysis and protein tyrosine phosphorylation revealed that sorting resulted in a more advanced membrane state, which

resembled in vitro capacitation (Bucci *et al*., 2012). These changes were more evident in bull spermatozoa than boars (Bucci *et al*., 2012). On subjecting to computer-assisted sperm analysis, sorted spermatozoa also showed altered motility characteristics, velocity and amplitude of lateral head displacement in bulls and sheep ( de Graaf *et al*., 2006), and ability to penetrate cervical mucus (de Graaf *et al*., 2006). Sorted ram spermatozoa also bind in fewer numbers to oviduct epithelial cell monolayers in vitro (de Graaf *et al*., 2006) and detach more rapidly than non-sorted spermatozoa. The change in the ability of sex-sorted sperm to bind to oviduct cells reflects partial capacitation and that sex-sorted sperm perhaps need less time to complete capacitation in the oviduct than non-sorted sperm (Winters *et al*., 2017). It was evident from field trials that conception rates similar to conventional semen could be achieved with non-cryopreserved sex-sorted semen by adjusting the insemination time closer to the time of ovulation. Even in in-vitro fertilization technique, the overall calving rate following the transfer of embryos obtained from non-sorted or sex-sorted sperm was similar (Ruiz Lopez *et al*., 2013).

**Semen sexing in other species**

Recently sexed or sorted semen has gained popularity in different species like sheep, goat, horses, pigs *etc*. with varying success rates.

In goats, even though sex-sorting technique is successful, fertility rate was comparatively lower than conventional non-sorted semen (Bathgate *et al*., 2013). Since inseminations are mostly trans-cervical in caprines, the challenge to address is the delivery of a fertile dose of sex-sorted semen. Ovines were the first species in which comparable fertility of sex-sorted semen was first demonstrated (de Graaf *et al*., 2007). Sex-sorted, frozen-thawed ram spermatozoa were superior in fertility to that of non-sorted, frozen-thawed controls when inseminated in super-ovulated ewes (de Graaf *et al*., 2007). In non-superovulated ewes a lower dose of one million motile sex sorted sperms showed equivalent fertility to conventional semen (de Graaf *et al*., 2007).

One of the reasons for better results in sheep could be due to laparoscopic inseminations (LAI) where the spermatozoa are placed at the tips of the uterine horns. Hence, sex-sorted semen could be used as a breeding option both at the elite stud level as well as the commercial farm level.

In equines, hysteroscopic and deep uterine insemination (DUI) of sex-sorted sperm have been used and both have reported indifferent results (Lindsey *et al*., 2005). A recent publication citing a pre-sort storage at ambient temperature, followed by sex-sorting and cryopreservation showed no difference in fertility to conventional stallion sperm by hysteroscopic inseminations (Gibb *et al*., 2017). Although fertilization rates are comparable with conventional semen, the primary issues related to cryopreservation and general early embryonic death after hysteroscopic or DUI remain, and these challenges need to be addressed before sex-sorted stallion sperm become a routine option.

In porcines, the speed of flow cytometric sperm sorting are the main limitations since 2.5 to 3.0 billion sperms in 75 to 100 ml of extender are used for insemination. Techniques to reduce the number of sperms and to deposit the spermatozoa closer to the site of fertilization as in deep uterine insemination are being tried out for better results.

**Sexed semen as a powerful tool for genetic improvement**

Sexed semen is a boon especially for commercial dairies. Selection of females was practically impossible even in large diaries due to the fact that at replacement rates of 40%, accounting for calf losses and a sex ratio of 50/50, dairy farmers need to keep every single born female simply to maintain herd size. Therefore, genetic progress through the dam-to-dam pathway has essentially been zero. Sexed semen offers a way of introducing selection in that pathway for the first time by skewing the sex ratio. Therefore, making use of the available technologies can, therefore, enhance genetic progress in the dam-to-dam pathway by a factor 3 to 5 (Heuer *et al*., 2017).

**Limitations of the technology**

Limitations in terms of both technology and implementation hinder the wide acceptance of sexed semen even though fertility has been assured. Reduced fertility, when using sorted sperm, has been attributed to the damage of spermatozoa caused by the sexing process. This includes staining and incubation of spermatozoa with Hoechst 33342, sperm dilution, exposure to high pressure and laser light, rapid projection into the collection tube, and centrifugation to concentrate sorted sperm. Extraneous factors like concentration of semen, site of deposition, oestrus detection and insemination skills, timing of AI, thawing procedure errors *etc*. are to be considered while assessing the fertility rate of sexed semen (Galma, 2021).

**Technological limitations –**

- High cost of machines and equipments required for sexing
- Lower sorting efficiency and speed
- Requirement of highly skilled technical staff
- Damage to the sperm due to shear force, electrostatic charge, droplet formation
- Reduced freezing potential of the sorted sperm

**Implementation limitations –**

- High cost of the sex sorted semen which include the cost of the intellectual property right (Rs. 1500-4500/- dose as compared to Rs. 15-20/- dose for conventional semen)
- The conception rate with sex sorted semen is almost 10-15% lower which is critical in Indian conditions where, artificial insemination coverage (20-25%) and conception rate (25-35%) by AI is also low. Numerous studies have shown conception rates can vary from approximately 60-90% of conventional semen (Loggan, 2019)
- There is no standard operating procedure to perform insemination with sexed semen. This is another area of concern as the sperm concentration of sexed semen ranges between 2 and 4 million/dose whereas it is 20 million/dose

in conventional semen. Managing lower sperm concentration will be a challenge in the field under Indian condition.

**EMBRYO SEXING**

The genetic sex of the zygote is determined at the time of fertilization. Those ova fertilized by spermatozoa bearing a Y chromosome become genetic males, while those fertilized by X-bearing spermatozoa become genetic females. Even though there exist a variation in the morphology of sex chromosomes, they are unidentifiable in each of the domestic species. This dimorphism forms the basis of sex determination by cytological methods. In mammals, sex is determined genetically by the presence of the SRY gene that encodes the testis-determining factor on the Y-chromosome. Complex interactions between genes such as AMH (Anti- Mullerian Hormone), WT1 (Wilm's tumor suppressor gene), SF1 (Steroidogenic factor 1) also plays inevitable role in sex determination.

**Methods of embryo sexing**

Embryo sexing starts with the collection of embryos produced either in vivo or in vitro on day 6.5 after first AI. Only those embryos graded excellent or good are selected. They are further washed with phosphate buffered saline thrice and are placed in a drop containing 200 mM sucrose under micro manipulator. Zona pellucida is cut open with a micro blade. Blastomeres are sucked out with fine aspiration needle and washed in Potassium chloride and transferred to an eppendorf tube.

The sexing methods can be grouped as invasive and non-invasive. Non-ivasive methods are considered superior since embryonic integrity is maintained, however, accuracy is lower when compared to invasive techniques. Also, advances in DNA probing have optimizedthe lower embryonic viability encountered earlier with invasive techniques.

**Invasive Methods**

***a. Karyotyping/cytogenetic analysis***

Cytogenetic sexing or karyotyping was the initial technique to produce sexed rabbits, calves and sheep. The technique involves culturing cells in a medium containing colcemid, which is a mitosis arresting agent. The cells are allowed to swell to view the dispersed chromosomes with specific banding patterns, later fixed and stained with a permanent DNA dye, such as Giemsa. The slides are then examined under a microscope. The identification of Y chromosome is easy due to its small size. Detection of Barr bodies in inactive X chromosomes in females are used to differentiate sex in cytogenetic analysis using Aceto-orcien staining. But there are chances that the female embryos may be mistaken for males due to the absence barr bodies before the complete inactivation of X chromosomes. In chromosome analysis, cell nuclei are expanded using salt solution and individual sex chromosomes and autosomes are examined microscopically.

Cytogenetic analysis is a reliable method as embryos produce *at least* one readable metaphase set of chromosomes if they were recovered after day 10. Even though it is a time consuming affair, it is inexpensive, easy to perform and would effectively identify chromosomal abnormalities before transferring embryos. Other disadvantages of the technique include accidental harm caused to the embryos, time consumption, requirement of well-trained cytogeneticist etc. Poor spreading and over scattering of metaphase chromosomes may hinder accurate sexing.

***b. Embryo sexing using Y-specific DNA probes***

The Y-specific probe technique involves the biopsy of embryonic cells and hybridization of ideal cellular DNA to a labelled sequence of DNA, specific to the Y chromosome and thus the male sex of the embryo. The generation and characterization of Y -specific DNA probes requires isolation of Y chromosomes, isolation of Y-specific sequences, and determination of sequence copy number and localization of the probe sequence on the Y

chromosome. DNA probing is undoubtedly the most accurate method of sexing embryos, but more widespread commercial application is limited by the fact that embryos have to be probed individually, necessitating skilful micromanipulation. Since it is an invasive technique, embryo viability may get compromised. There are some Y-linked gene transcripts like sex-determining region Y (SRY), found only in the early male embryos at 2- to 4-cell stage in human, murine and bovines that might represent useful markers of sex (Hamilton *et al*., 2012).

***c. PCR based embryo sexing***

Polymerase chain reaction (PCR) opened up new possibilities for embryo sexing. It facilitates the amplification of Y -chromosome- specific repetitive sequences and thus the sex of embryo can be determined in relatively short time with high (almost 100 %) accuracy. Now PCR is the most preferred method to determinefetal sex in early pregnancy using DNA extracted from maternal plasma (Cruz *et al*., 2012) since it is cost effective, simple and reliable (Malik *et al*., 2013). Sexing of embryo by this method include biopsy of embryo, amplification of DNA fragments (one species specific and one male specific) and analysis of amplified products and interpretation.

**Biopsy of embryo:** The embryos are collected on day 6.5 after first insemination. Only the embryos with excellent or good grade during compact morula to early blastocyst stage are biopsied, with the help of a micromanipulator. Two to eight cells from cultured embryos are collected and rinsed three times in DMPBS. Transfer cells in 2 µl DMPBS with 18 µl sterile triple distilled water and 2 µg Proteinase- K. DNA is released by heating for 1 hour at 55°C and proteinase-K is destroyed by heating at 99°C for 15 min.

**Amplification involves,**

1. Template denaturation at temperature 94-97°C for 90 sec.
2. Primer annealing (50-72°C) for 90 sec.
3. Extension of the annealed primer at 72°C for 180 sec, by Taq DNA polymerase.

These 3 steps are repeated for 40 cycles. After the last cycle, the samples are incubated at 72℃ for 7 minutes. The PCR mixture contains template DNA, 2 sets of primers, 4 deoxyribonucleotides Taq DNA polymerase and buffer.

***PCR-RFLP***

PCR-RFLP also involves PCR based genotyping. A pseudo-autosomal region, common to both X and Y chromosomes is amplified. These homologous regions are called ZFY (in Y chromosomes) and ZFX (in X chromosomes). The amplified product is then digested with restriction enzymes. This takes advantage of the restriction enzyme fragment length polymorphism between ZFX and ZFY. The digested DNA is separated by electrophoresis. ZFX and ZFY DNA are identified by their different digestion patterns.

***d. LAMP (Loop mediated isothermal amplification) assay***

Use of a loop-mediated isothermal amplification (LAMP) technique is also used for bovine embryo sexing (Khamlor *et al*., 2015). It involves specific DNA amplification under isothermal conditions. DNA polymerase, with its high strand displacement activity, enables auto-cycling strand displacement DNA synthesis within the range of 60–65℃. Loop-mediated isothermal amplification (LAMP) is a DNA amplification method that can amplify a specific DNA sequence within the range of 60 to 65℃ (Hirayama *et al*., 2013).

LAMP employs a set of four specific primers that recognize a total of six distinct sequences on the target DNA. In addition, loop primers are used to accelerate LAMP reaction. An inner primer initiates primary DNA synthesis, and the following strand displacement DNA synthesis by an outer primer releases a SS-DNA derived from the inner primer. The initial step produce a stem-looped DNA structure which is a characteristic DNA structure in LAMP and then an extremely large amount of DNA is amplified from a stem-loop DNA by the auto cycling reaction. Accordingly a white precipitate of magnesium pyrophosphate is produced. Therefore, amplification of a target sequence can be judged by measurement of the turbidity in the reaction solution.

### *e. Embryo sexing by fluorescence in situ hybridization (FISH)*

Fluorescence in situ hybridisation (FISH) facilitates sensitive detection of specific nucleic acid sequences, or more simply, identification of individual chromosomes in metaphase and interphase nuclei from many different cell types. FISH detect could detect mosaicism and aneuploidy at the same time determine the embryo's sex. The biopsies of the embryos were obtained by cutting the embryos at expanded blastocyst stage with a micro razor blade to partly cut the zona pellucida to obtain a few cells from the trophoblast for sex determination. Blastomeres are fixed individually and biopsies are poured into 0.075M potassium chloride with 0.5 bovine serum albumin (BSA), left for 10 minutes and then placed into clean slides coated with poly L-Iysine. Approximately 10 µl fixative (acetic acid: methanol, 1:3) is dropped onto the cells and specimens are air-dried by continuous blowing for 30 minutes. Genetic sex determination is based on the fact that numerous polymorphisms are present on two homologous copies of the amelogenin gene on the X and Y chromosomes (Luptakova *et al*., 2011).

The bovine Y-chromosome-specific DNA sequence used as a probe is termed BC1.2. Approximately 50 ng of digoxigenin - labelled probe prepared by PCR is added to 10 µl of a hybridization mixture. The mixture is composed of 50 formamide, 10 dextran sulphate and 2 mg/ml BSA in 2 X SSC (0.3M sodium chloride, 0.03 M sodium citrate, pH 7.0). Ten microlitres of the hybridization mixture is dropped onto the specimens which is then by pre-heated at 72℃ and denatured at 72℃ for 8 minutes on an aluminium block. Immediately after denaturation, the slides are transferred to an incubator maintained at 38.5℃ and hybridized for 5 minutes. The slides are then washed in 0.5 XSSPE (75 mM sodium chloride, 5 mM monobasic sodium phosphate, 0.5 mM EDTA, pH 7.4) at 72℃ for five minutes followed by a wash in PN buffer (0.1% sodium phosphate, pH 8.0, supplemented with 0.1% Nonidet-P40) for two minutes at room temperature. The digoxigenin is detected by incubation with 2µl/ml anti- digoxigenin-fluorescin in PN buffer

in 5 per cent non-fat dry milk at 38.5°C for five minutes. The free anti-digoxigenin -fluorescin is removed by three changes of PN buffer at room temperature. Finally, the preparations are counterstained with propidium iodide (0.3µl/ml). The slides are examined under an epifluorescence microscope with a 4-MWIB (excitation 460 to 490 nm) mirror unit. The nuclei that are male show Y -chromosome specific Signals, whereas the female nuclei do not show any signal.

In contrast to FISH, contaminations from the previous assay pose significant risk while using PCR for embryo sexing owing to its high sensitivity.

### 2.1.2. Non –Invasive methods

#### *a. Detection and quantification of X-linked enzymes*

Embryos theoretically can be distinguished as male or female by measurement of the gene dosage for X-linked enzymes (Bondioli, 2014). To maintain an equivalent number of genes between the sexes, one of the X-chromosomes in homogamatic female is inactivated in each cell during early embryonic life. X-inactivation happens during a brief period between activation of the embryonic genome and X-inactivation, in which genes from both X chromosomes in the female are transcribed. This is reflected in the cellular concentration and activity of certain X-linked enzymes which will be twice as high in female as in male embryos. X-linked enzyme activity is compared to autosomal-linked enzyme activity to account for individual variation in embryo metabolism.

Based on the hypothesis that the ratio of X-linked enzyme activity to autosomal enzyme activity will be higher in female than in male embryos, the activity of glucose-6-phosphate dehydrogenase (G6PD), hypoxanthine phosphoribosyl transferase (HPRT) and adenine phosphoribosyl transferase (APRT) activities were subjected to study in mice embryos. The study on G6PD showed that the sex of 72 per cent (62/86) female embryos and 57 per cent (54/95) of male embryos could be correctly identified. Tiffin *et al*. (1991) studied glucose and glutamine metabolism in bovines and inferred greater glucose

and glutamine metabolism in female embryos in comparison to male embryos. Even though X-linked enzyme activity measurement methods have shown promising results, the collection and examination of small amounts of enzymes remains as its limitation.

***b. Detection of H-Y (male) antigen***

The technique involves the immunological demonstration of a sex specific antigen; histocompatibility Y-antigen (or H-Y antigen) for sexing embryos. Though the function of this antigen is not known, the gene has been mapped to the long arm of the Y chromosome and is considered to be one of the many genes involved in organizing the indifferent gonads into testes. Methods for detecting H-Y antigen on embryos are cytotoxicity assay and immunofluorescent assay. In cytotoxicity assay, embryos are exposed to dilute H-Y antiserum and complement. Embryos expressing H-Y antigen show a degree of cell lysis and thus are recognized as males. The immunofluorescent assay system requires antibodies to cell-surface molecules specific to male tissues. Embryos are incubated for 30-60 minutes with antibodies, and then for an additional 30-60 minutes with an antibody to the first antibody containing the fluorescent dye-fluorescin isothiocyanate (FITC). Embryos are then briefly examined with a fluorescence microscope. Male embryos will emit fluoresce. The advantages of the technique are its speed and lack of need to biopsy embryos.

***c. Hormonal assay for sexing blastocyst***

Fetal endocrinology studies suggested that fluid collected from blastocoel could be subjected to hormonal assay for sex determination of embryos. The hormone selected must be measurable, must exist in blastocoelic fluid and must differ between sexes unambiguously. The hormonal assay could be used effectively for predicting fetal sex between 90 and 150 days of gestation in cattle. Pig embryos produce estrogens as early as day 12 (well before gonadal differentiation). Similarly the pre-attachment horse embryo produces estrogens and androgens as

early as day 14. But variations in steroid production with sex remain unknown. The method needs more explorations.

d. ***Sexing based on cleavage and development***

The cells of female embryos have proportionately more amount of DNA as compared to male embryo cells. If the amount of DNA is more, the time required for its duplication also will be more and hence a longer cell cycle.This difference is expected to affect the cleavage and development rate of male and female embryos. The male embryos may cleave early and develop fast to attain morula and blastocyst stage than female embryos. Recent reports on cleavage and development in bovine embryos produced both in vivo and in vitro also reported faster cleavage and development in male embryos than females. The limitations of this method are; Cleavage time of in-vivo produced embryos cannot be determined, besides the variation in developmental rate is minute and needs high skill in separation of fast and slow embryos. DNA probing is undoubtedly the most accurate method of sexing embryos, but commercial application is limited as the embryos have to be probed individually, which require skilful micro-manipulation. Since it is an invasive technique, it may result in decreased embryo viability.

**Conclusion**

Assisted reproductive technologies like sex sorting of semen and embryos serve as effective tools in optimising genetic improvement efforts in livestock sector. Sexing will promote production of off-springs with predetermined sex. Though a recent breakthrough, reduced fertility rates and high cost of technology limits its popularity. Flow cytometry, is the widely used method of sexing with almost 90 per cent accuracy especially in in-vitro fertilization; but the process is too slow to provide enough sperm for artificial insemination. Embryo sexing using cytological methods is very accurate though invasive in nature and will remain applicable at least for testing the results of other effective methods available or emerging in future.

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# REACTIVE OXYGEN SPECIES: IT'S ROLE IN BULL SPERM PHYSIOLOGY AND FERTILITY

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***ABSTRACT-***

*Among various causes of male infertility, oxidative stress has been attributed to affect sperm motility and capacity for fertilization. The main destructive aspects of oxidative stress are the production of reactive oxygen species (ROS), which include free radicals and peroxides. Due to their highly reactive nature, ROS can combine readily with other molecules, directly causing oxidation that can lead to structural and functional changes and result in cellular damage. Production of ROS is a normal physiological process and is a by-product of oxidative respiration. Within the physiological limits, it is beneficial for many of the cellular functions including sperm capacitation, acrosome reaction and sperm-oocyte fusion. Enzymatic and non-enzymatic anti-oxidant systems in the body will maintain ROS levels within normal limits. The body's complex antioxidant system is influenced by dietary intake of antioxidants, vitamins, and minerals such as vitamin C, vitamin E, zinc, taurine, hypotaurine, and glutathione. In bovine semen, ROS are generated primarily by dead spermatozoa leukocytes and immature spermatozoa. The susceptibility of ruminant spermatozoa to oxidative stress is a consequence of the abundance of polyunsaturated fatty acids in sperm plasma membrane, which makes them vulnerable to lipid peroxidation.*

*Cryopreservation of semen and thawing procedures as well as assisted reproductive techniques carry the risk of generating more ROS. An antioxidant that reduces oxidative stress could be useful in the management of male infertility. Interestingly, the agents that produce ROS may develop as next generation contraceptives.*

***Keywords:*** *Reactive oxygen species, Bovine, Infertility*

## INTRODUCTION

Defective sperm functions are the most prevalent causes of male infertility and a difficult condition to treat. Many environmental, physiological, and genetic factors have been implicated in the poor sperm functions and infertility. Thus, it is very important to identify the factors/conditions which affect normal sperm functions. Among various causes, oxidative stress (OS) has been attributed to affect the fertility status and physiology of spermatozoa. The term oxidative stress is generally applied when oxidants outnumber antioxidants. The imbalance between the production of reactive oxygen species (ROS) and a biological systems ability to readily detoxify the reactive intermediates or easily repair the resulting damage is known as oxidative stress. The main destructive aspects of oxidative stress are the production of ROS, which include free radicals and peroxides. The production of ROS by sperm is a normal physiological process, but an imbalance between ROS generation and scavenging activity is detrimental to the sperm and associated with male infertility. Physiological levels of ROS influence and mediate the gametes and crucial reproductive processes, such as sperm-oocyte interactions, implantation and early embryo development. Against ROS attack, sperm cells are well equipped with a powerful defense system of antioxidants (Agarwal and Saleh, 2002). Antioxidants are the main defense factors against oxidative stress induced by free radicals.

### Reactive Oxygen Species (ROS)

ROS are formed as necessary by-products during the normal enzymatic reactions of inter and intracellular signaling. Mammalian spermatozoa represent a growing list of cell types that exhibit a capacity to generate ROS when incubated under

aerobic conditions, such as, hydrogen peroxide ($H_2O_2$) the superoxide anion ($\bullet O_2-$), the hydroxyl radical (OH•), and hypochlorite radical (OHCl•). Due to their highly reactive nature, ROS can combine readily with other molecules, directly causing oxidation that can lead to structural and functional changes and result in cellular damage (Agarwal *et al*., 2005).

**Types of ROS**

ROS represent a broad category of molecules that indicate the collection of radicals (hydroxyl ion, superoxide, nitric oxide, peroxyl, etc.) and non-radicals (ozone, single oxygen, lipid peroxides, hydrogen peroxide) and oxygen derivatives. Reactive nitrogen species (nitrous oxide, peroxynitrite, nitroxyl ion, etc.) are free nitrogen radicals and considered a subclass of ROS. Nitric oxide (NO) has been shown to have detrimental effects on normal sperm functions inhibiting both motility and sperm competence for zona binding (Agarwal and Prabakaran, 2005).

**Free Radicals**

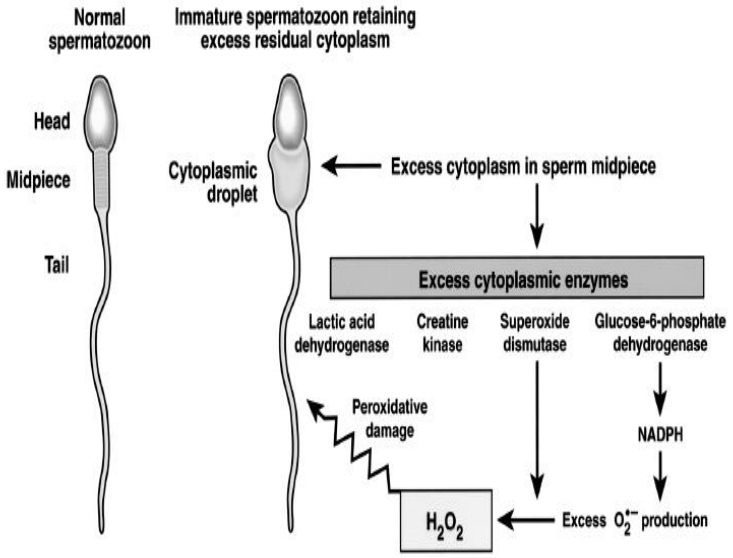
Free radicals are short lived reactive chemical intermediates, which contain one or more unpaired electrons. They induce cellular damages when they pass this unpaired electron onto nearby cellular structures, resulting in oxidation of cell membrane lipids, amino acids in proteins or within nucleic acids. Free radicals are also known as a necessary evil for intracellular signaling involved in the normal process of cell proliferation, differentiation, and migration. In the reproductive tract, free radicals also play a dual role and can modulate various reproductive functions. Excess of free radicals generation frequently involves an error in spermiogenesis resulting in the release of spermatozoa from the germinal epithelium exhibiting abnormally high levels of cytoplasmic retention (Rhee, 2006).

**Origin of ROS in Male Reproductive System/Sources of ROS**

In male, two ROS generating systems are possibly involved; a hypothetical NADPH oxidase at the level of sperm membrane and NADH-dependent oxido-reductase(diphorase) at the mitochondrial level. In bovine semen, ROS are generated

primarily by dead spermatozoa via an aromatic amino acid oxidase (AAAO) catalyzed reaction. Leukocytes and immature spermatozoa are the two main sources of ROS. Leukocytes, particularly neutrophils and macrophages, have been associated with excessive ROS production and they ultimately cause sperm dysfunction (Garrido *et al*., 2004).

**Immature spermatozoa and ROS**




**Positive and Negative Effects of ROS**

The production of ROS is a normal physiological process but an imbalance between ROS generation and scavenging activity is detrimental to the sperm and associated with male infertility. ROS generated by spermatozoa play an important role in normal physiological processes such as sperm capacitation, acrosome reaction, maintenance of fertilizing ability, and stabilization of the mitochondrial capsule in the mid-piece in bovine. Controlled generation of ROS may function as signaling molecules (second messengers) in many different cell types; they are important mediators of sperm functions. Evidences suggest that ROS, especially superoxide anion ($O_2$) is required for the late stage of embryo development such as, two germ cell layers and egg cylinder. Although a significant negative correlation between

ROS and IVF fertilization rate has been found, yet, controlled generation of ROS has shown to be essential for the development of capacitation and hyper-activation; the two processes of sperm that are necessary to ensure fertilization. In vivo physiological concentrations of ROS are involved in providing membrane fluidity, maintaining the fertilizing ability and acrosome reaction of sperm. The maintenance of a suitable ROS level is, therefore, essential for adequate sperm functionality. ROS cause adverse effects on the sperm plasma membrane, DNA, and physiological processes, thereby, affecting the quality of spermatozoa. The axosome and associated dense fibers of the mid-piece in sperm are covered by mitochondria that generate energy from intracellular stores of ATP depletion. Excessive ROS impairs motility and capacity of fertilization (Goncalves *et al*., 2010).

**Lipid Peroxidation**

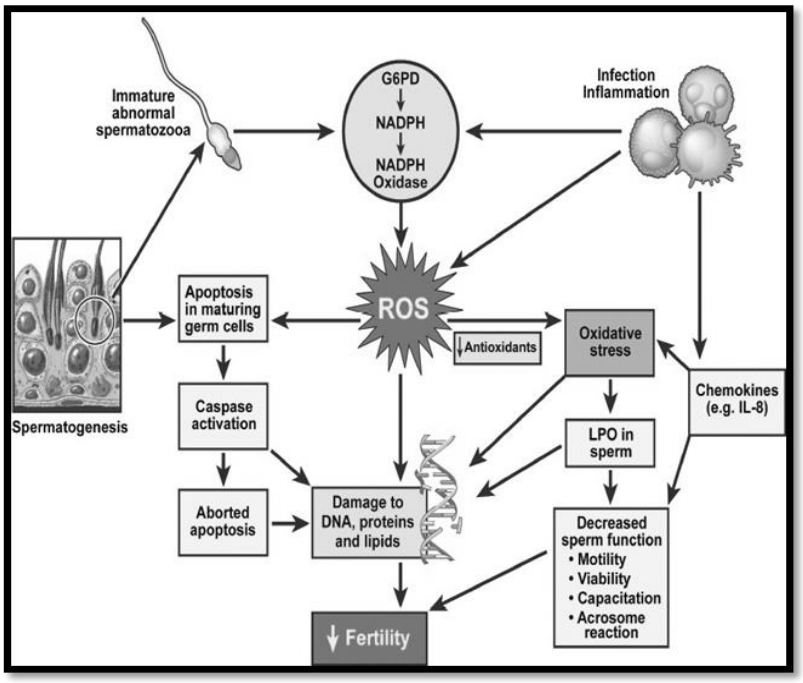
The mechanism of ROS-induced damage to spermatozoa includes an oxidative attack on the sperm membrane lipids leading to initiation of lipid peroxidation (LPO) cascade. Mammalian spermatozoa are known to be susceptible to loss of motility in the exogenous oxidant, as a consequence of LPO. The susceptibility of ruminant spermatozoa to oxidative stress is a consequence of the abundance of PUFAs in sperm plasma membrane, the presence of which gives the membranes fluidity and flexibility which help the sperm to engage in membrane fusion events associated with the fertilization. Unfortunately, the presence of double bonds in these molecules makes them vulnerable to free radicals attack and the initiation of LPO cascade. This results in a subsequent loss in membrane and morphological integrity, impaired cell functions, along with impaired sperm motility and induction of sperm apoptosis. Concerning the chemistry of LPO in spermatozoa, it implies that once this process has been initiated, its propagation is impeded, leading to accumulation of lipid peroxides in the sperm plasma membrane. Supplementation of transition metal ions such as Fe2+ to the sperm suspension results in a sudden acceleration of LPO and loss of sperm functions such as motility and viability. The key intermediates in spontaneous LPO are the lipid

hydroperoxides generated by a chain reaction initiated and their subsequent utilization. Peroxidation of PUFAs in sperm cell membrane is an autocatalytic, self-propagating reaction, which can give rise to cell dysfunction associated with the loss of membrane functions and integrity (Sharma and Agarwal, 1996).

**Lipid Peroxidation-Detrimental Effects on Sperm Functions**

Lipid peroxides are spontaneously generated in the sperm plasma membrane and are released by the action of phospholipase A2. They are capable of inducing DNA damage and decrease in fertility during storage of semen. The peroxides are generally associated with decreased sperm functions and viability, but, have also a significant enhancing effect on the ability of spermatozoa to bind with homologous and heterologous zona pellucida (Twigg *et al*., 1998).

**Spermatozoal damage from excess ROS**



**Strategies to Reduce Oxidative Stress**

**1. Antioxidants**

Spermatozoa are protected by various antioxidants and antioxidant enzymes in the seminal plasma or in spermatozoa itself to prevent oxidative damage. An antioxidant that reduces oxidative stress and improves sperm motility could be useful in the management of male infertility. Antioxidants are the agents, which break the oxidative chain reaction, thereby, reduce the oxidative stress. Vitamin E (antioxidant) may directly quench the free radicals such as peroxyl and alkoxyl (ROO) generated during ferrous ascorbate-induced LPO, thus it is suggested as major chain breaking antioxidant. Antioxidants, in general, are the compounds and reactions which dispose, scavenge, and suppress the formation of ROS, or oppose their actions. Mn2+ enhances sperm motility, viability, capacitation and acrosome reaction by decreasing the oxidative stress. Extracellular addition of Mn2+ ions also enhances the level of cAMP by stimulating Ca2+ or Mg2+ ATPase which leads to activation of calcium channel opening, thereby depositing more Ca2+. Thus, Mn2+ promotes the acrosome reaction. Thiol groups also play an important role in detoxification and anti-oxidation of ROS, besides maintaining the intracellular redox status. These groups serve as defense mechanisms of sperm cells to fight against oxidative stress. Recent studies demonstrate that supplementation of cryopreservation extenders with antioxidants has been shown to provide a cryo-protective effect on bull, ram, goat, boar, canine, and human sperm quality, thus improving semen parameters, for example, sperm motility, membrane integrity after thawing (Miller *et al*., 1993).

**2. Enzymatic Antioxidants**

Enzymatic antioxidants are also known as natural antioxidants; they neutralize excess ROS and prevent it from damaging the cellular structure. Enzymatic antioxidants are composed of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase (GR) which also causes reduction of hydrogen peroxides to water and alcohol.

SOD spontaneously dismutase superoxide anion to form oxygen and $H_2O_2$while catalase converts $H_2O_2$ to $O_2$ and $H_2O$. SOD protects spermatozoa against spontaneous $O_2$ toxicity and LPO. SOD and catalase also remove oxide radical generated by NADPH oxidase in neutrophils and play a major role in decreasing LPO and protecting spermatozoa against oxidative damage. Catalase presence in sperm has been demonstrated for ram and cattle and it has a potential role in ageing process and control of oxidative stress in cells, mainly resulting from $H_2O_2$.

**3. Non-enzymatic Antioxidants**

Non-enzymatic antioxidants are also known as synthetic antioxidants or dietary supplements. The body's complex antioxidant system is influenced by dietary intake of antioxidants, vitamins, and minerals such as vitamin C, vitamin E, zinc, taurine, hypotaurine, and glutathione (Agarwal*et al*., 2005).

**Cryopreservation/Freezing Thawing-Oxidative Stress**

Semen cryopreservation is an important procedure which allows specific advantages to livestock industry. The improvement of semen cryopreservation techniques requires in depth knowledge of the gamete physiology and the biochemical processes occurring during semen collection, processing, and freeze-thawing. Freezing/thawing of sperm sample is routinely performed in cattle breeding industries in order to perform artificial insemination. These procedures are known to produce ROS in sperm samples. During cryopreservation, semen is exposed to cold shock and atmospheric oxygen, which in turn increases the susceptibility to lipid peroxidation due to higher production of ROS. As the sperm plasma membrane is one of the key structures affected by cryopreservation, sperm cryopreservation and thawing is associated with increased ROS production and decreased antioxidant level. Both freezing and thawing cause tremendous alterations in cell water volume. Spermatozoa discard most of their cytoplasm during the terminal stages of differentiation and lack the significant cytoplasmic component containing antioxidants that counteract the damaging effect of ROS and LPO. Due to this, spermatozoa are susceptible

to LPO during cryopreservation and thawing, which confers considerable mechanical stress on the cell membrane. It has been noted in humans that ROS level has a positive correlation with the extent of apoptotic sperms. Despite recent morphological advances, cryopreservation exerts detrimental effects on spermatozoa that lead to a significant decrease in sperm viability and motility, and, ultimately in decreased cryopreserved sperm rates. The fertility potential of cryopreserved mammalian spermatozoa is lower than that of fresh sperm (Yousef *et al*., 2003; Said *et al*., 2005).

Cryopreservation induces extensive biophysical and biochemical changes in the membrane of spermatozoa that ultimately decrease the fertility potential of the cells. Procedure of cryopreservation increases premature capacitation of spermatozoa. These alterations may not affect motility but reduces life span, ability to interact with the female reproductive tract and sperm fertility. Freezing and thawing processes also lead to the generation of reactive oxygen species. Excessive production of ROS during cryopreservation has been associated with the reduced post thaw motility, viability, membrane integrity, antioxidant status, and fertility and sperm functions. The post thaw motility of the cryopreserved buffalo semen is poor and the success rate of IVF with buffalo sperm is only 10%–20% as compared to cattle which is 30%–35%(Reddy *et al*., 2010).

**ROS and Assisted reproduction techniques**

It is found that ART carries the risk of generating ROS in germ cells and since selection in female tract is bypassed, there is chance that genetically damaged sperms may fertilize an oocyte (du Plessis *et al*., 2008). ROS may originate from male/female gamete, embryo or from surroundings, which includes cumulus cells, leukocytes and culture media. Various anti-oxidants are added to media to improve the developmental ability of embryo. Sperm preparation by centrifugation is associated with ROS generation. ROS levels in semen is considered as an important predictor of IVF success (Agarwal *et al*., 2008).

## ROS and contraception

As mentioned earlier, lipid peroxidation, induced by $H_2O_2$ impairs all sperm functions, including sperm-oocyte fusion and 25-40 per cent of infertile men have high levels of ROS. There exists a possibility that $H_2O_2$ or reagents producing them on contact with sperms may develop as next generation contraceptives.

## Conclusion

Production of ROS is a normal physiological process and is a byproduct of oxidative respiration. Within the physiological limits, it is beneficial for many of the cellular functions including sperm capacitation, acrosome reaction and sperm-oocyte fusion. Enzymatic and non-enzymatic anti-oxidant systems in the body will maintain ROS levels within normal limits. Excess ROS production or lower anti-oxidant levels lead to oxidative stress and the resultant cellular damage, leading to pathological conditions including infertility. Oxidative stress is one of the main causes of infertility in man. Evaluation of oxidative stress and the use of antioxidants are not routine in veterinary clinical practice. The immediate need is to simplify and validate the evaluation of ROS and oxidative stress status so that it can be performed routinely without the use of sophisticated equipment. Also, it is important to establish reference values for ROS above which antioxidants could be used for male infertility treatment. The dose and duration of these antioxidants should also be determined and standardized. The possibility of development of an ROS based contraceptive has to be worked out.

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# 3D PRINTING OF HYDROXYAPATITE BASED COMPOSITES FOR BONE ENGINEERING

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***ABSTRACT-***

*3D Printing promises to produce complex scaffolds according to computer design using patient-specific anatomical data. Since its initial use as pre-surgical visualization models and tooling molds, 3D Printing has slowly evolved to create one-of-a-kind devices, implants, scaffolds for tissue engineering, diagnostic platforms, and drug delivery systems. Recent explosion in public interest and access to affordable printers, there is renewed interest to combine stem cells with custom 3D scaffolds for personalized regenerative medicine. Before 3D Printing can be used routinely for the regeneration of complex tissues (e.g., bone, cartilage, muscles, vessels, nerves in the craniomaxillofacial complex), and complex organs with intricate 3D micro architecture (e.g., liver, lymphoid organs), several technological limitations must be addressed. In this discussion, the common 3D Printing technologies (Three- Dimensional Printing, Fused Deposition Modeling, Stereolithography, Selective Laser Sintering, and 3D Plotting/ Direct-Write/Bioprinting) are described. Advantages and*

*disadvantages are identified to motivate future research in this field of advanced manufacturing.*

***Keywords:*** *Computer-aided tissue engineering, 3D Printing, Stereolithography, Selective laser sintering, 3D plotting, Bioprinting*

## Introduction

Bone tissues exhibit an excellent ability to spontaneously regenerate and repair themselves from the surrounding osteoprogenitor cells without scarring. Spontaneous repair may not occur in patients with bone injuries beyond the extent of self-healing. This may lead to non-union, scar formation, and even long-term persistent bone defects. Replacement of extensive local bone loss is a significant clinical challenge.

There are a variety of techniques to manage this problem, each with their own advantages and disadvantages. Therapeutic strategies for bone regeneration mainly involve autologous, homologous, and xenologous bone grafting. The limitation of available clinical application of these grafting's caused by donor site morbidity and autogenous bone deficiency in autografts, infectious risks and rejection. Bone graft substitutes or novel approaches have been explored to treat large bone defects. Variety of synthetic bone graft substitutes has been developed with the aim to minimize these complications. The benefits of synthetic grafts include availability, sterility and reduced morbidity.

Bone tissue engineering is a novel technique that requires biology, engineering, and material science to create biological substitutes to reduce the known drawbacks of traditional grafts. The establishment of sufficient vascular system is crucial to satisfy the nutrient supplement and removal of waste during bone tissue regeneration. Bone tissue engineering scaffold should closely mimic the natural bone extracellular matrix that provides basic structure and correct microenvironment for bone tissue growth. Essential features required for scaffold include biomimetic 3D structure, excellent biocompatibility, adaptable biodegradability, pre- vascularized structure, osteoconductivity,

and less immunogenic responses.

**Introduction to 3D Printing**

The ability to design and fabricate complex, 3D Scaffolds is critical in tissue engineering. Applications for 3D scaffolds are restoration of 3D anatomic defects, the reconstruction of complex organs with intricate 3D micro architecture (liver, lymphoid organs), and scaffolds for stem cell differentiation.

In tissue engineering, scaffolds are critical to provide structure for cell infiltration and proliferation, space for extracellular matrix generation and remodeling, biochemical cues to direct cell behavior, and physical connections for injured tissue. When making scaffolds, design of the architecture on the macro, micro, and nano level is important for structural, nutrient transport, and cell-matrix interaction conditions.

The macro architecture is the overall shape of the device which can be complex (patient and organ specificity, anatomical features). The microarchitecture reflects the tissue architecture (pore size, shape, porosity, spatial distribution, and pore interconnection). The nanoarchitecture is surface modification (biomolecule attachment for cell adhesion, proliferation, and differentiation). Although an ideal scaffold will account for all these factors, challenges still exist with biomaterial selection and 3D shape specificity.

Biomaterials commonly used are polymers (synthetic and natural), ceramics, and metals. Each biomaterial has specific material and mechanical properties, processing methods, chemical properties, cell material interactions, and FDA approval. Common fabrication methods to produce porosity and a range of pores size are gas foaming, solvent casting with particle leaching, freeze-drying, and electrospinning.

While the microarchitecture in these methods is well-controlled and understood, the ability to control macro architecture with these methods is limited to 3D shapes and geometries determined by molds and manual processing. The ability to incorporate internal architecture or curved channels is also limited when using these methods. Solid free form

fabrication (SFF) has allowed for the design and fabrication of complex 3D structures which can be patient specific. The integration of computer aided design, advanced imaging techniques (magnetic resonance imaging and computer tomography), and rapid prototyping has advanced fabrication of objects with both macro and microarchitecture control.

Patient specific imaging can be used to customize builds for individuals. A type of rapid prototyping, SFF offers a method to control both the micro and macroarchitecture to create complex biomedical devices. Most surface modifications can be completed in post-processing. While conventional material processing techniques can be highly effective in scaffold engineering, SFF technologies offer exciting opportunities for tissue engineering of highly complex tissues.

However, each technology has its limitations. The selection of the fabrication technique depends upon the materials of interest, machine limitations, and the specific requirements of the final scaffold. The term “3D Printing” is being used to refer to all SFF technologies like Three-dimensional printing, Fused deposition modelling, Stereolithography, Selective laser sintering/melting, 3D Plotting/ Direct-write bioprinting.

The state-of-the-art 3D Printing, especially for the production of implantable scaffolds is severely limited by printable materials. Alternative material processing methods are required to work with materials that are not easily printed. While industrial 3D printers have reached extremely high resolution in the past few years, the advancements in machine capability have not translated to the use with biomaterials.

The cost of each of these technologies is currently difficult to compare since many advances are based on home-made setups or modification of commercial machines by creative engineers. Actual cost will be easier to compare when the materials become available for large scale adaptation for industrial 3D printers. That stage will also determine the ease of use for both printing and post-processing.

Even with current modeling materials, most printers require some type of sacrificial support materials that require careful removal SFF methods, particularly FDM, have recently exploded in popularity and gone viral. Machines are being developed specifically for home, school, and small business use with much lower pricepoints and less complexity than industrial grade machines. Low-cost consumer 3D scanners and free CAD software has allowed those interested in SFF to design and fabricate parts themselves at home.

**3D printing of tissue engineering scaffolds**

Most SFF methods build 3D biomedical devices in a layer-by-layer process. The general SFF process involves:

- Creating a 3D computer model (can be generated from medical imaging data such as CT scans or X-rays).
- Slicing the 3D computer model into a build file of 2D images with software.
- Fabricating the build by a computer-controlled layer-by-layer process.
- Finishing with any post processing such as surface modification for nano-architecture.

Complicated three-dimensional features such as internal voids, cantilevers, undercuts, and narrow tortuous paths are simply reduced to a stack of common two-dimensional features such as circles, lines, and points. Exempted from tooling path restrictions, these additive technologies offer much higher levels in shape complexity.

Although these SFF technologies were developed primarily for industrial applications, their flexibility in creating complex three-dimensional shapes make SFF technologies attractive candidates for biomedical engineering. Various SFF techniques were introduced to build objects with controlled macro-architecture as well as microstructures with biomedical and tissue engineering applications.

The freedom in form, combined with the appropriate material deposition technology offer control over the tissue

engineering triad by simultaneously directing the spatial distribution of cells, signals, and scaffolding substrates during fabrication. Furthermore, these technologies allow integration between digitized medical imaging data with computer-aided-design models.

The integration of SFF technologies with patient-specific medical imaging data enables the aseptic manufacturing of tissue engineering grafts that match precisely to a patient's contours can be produced by these technologies enable the fabrication of multi-functional scaffolds that meet the structural, mechanical, and nutritional requirements based on optimized models.

The five popular SFF technologies are Three-dimensional printing, Fused deposition modelling, Stereolithography, Selective laser sintering/melting, 3D Plotting/Direct-write bioprinting.

**Three-dimensional printing**

Three-dimensional printing was invented at the Massachusetts Institute of Technology. It fabricates 3D structures by inkjet printing liquid binder solution onto a powder bed. A wide range of materials has been utilized in printing since most biomaterials exist in either a solid or liquid state. The process begins by spreading a layer of fine powder material evenly across thepiston. TheX-Y positioning system and the print head are synchronized to print the desired 2 Dpattern by selective deposition of binder droplets on to the powder layer. The piston, powder bed, and part are lowered, and the next layer of powder is spread. The drop-spread-print cycle is repeated until the entire part is completed. Removal of the unbound powder reveals the fabricated part (Billiet *et al.,* 2012).

The local composition can be manipulated by specifying the appropriate print head to deposit the predetermined volume of the appropriate binder. The local microstructure can be controlled by altering the printing parameters during fabrication. The incorporation of micro-channels effectively distributed additional seeding surfaces throughout the interior of the device, increasing the effective seeding density and

uniformity. Patterned surface chemistry potentially offers spatial control over cell distribution of multiple cell type.

Disadvantage is the competing needs between print head reliability and feature resolution, as small nozzles can make finer features but are more prone to clogging. Fabrication of complex scaffolds such as internal channels or hanging features is easily achievable with this technique, since objects are being supported by surrounding unbounded powders.

Advantages include room temperature processing conditions allowing the incorporation of temperature sensitive materials such as pharmaceutical and biological agents into scaffolds.

Another advantage of this technology for tissue engineering is multi-color printing. This feature allows to simultaneously arranging multiple types of cells, deposit multiple extra cellular matrix materials, and exert point to- point control over bioactive agents for biological tissue manufacturing.

A wide range of biological agents such as peptides, proteins (fibrinogen, collagen), polysaccharides (hyaluronan, alginate), DNA plasmids, and living cells have been printed with 3DP. Deposition of these biological materials requires modification of industrial 3DP machines. Cells in particular must be kept in a proper environment with appropriate temperature, oxygenation, and nutrient supply.

Other materials used in direct 3DP include powder composed of a synthetic polymer (poly (ε-caprolactone), polylactide-coglycolide or poly (L-lactic acid)) with organic solvent as binder and natural polymer powder (starch, dextran and gelatin) with water as binder.

**Indirect 3DP**

Indirect 3DP prints a mold which is then cast with the final polymer and porogen materials. Materials used in indirect 3DP to print the mold include commercially available plaster powder (calcium sulfate hemihydrates plaster powder) and water-based binder. The mold is then cast with a slurry of biodegradable polymer dissolved in solvent mixed with porogen

(polylactide-coglycolide in chloroform mixed with NaCl).

Advantage of direct 3DP is direct control over both the microarchitecture (pore size) and macroarchitecture (overall shape). Prints which use porogen as the powder result in high pore interconnectivity, uniform porosity, and defined pore size after leaching. There are no limitations on the macroarchitecture and no need for demolding.

Disadvantage of direct 3DP is that organic solvents can dissolve polymers used in most print heads. To overcome this limitation, investigators used stencils to pattern polymer solutions on to porogen particles (NaCl) to fabricate scaffolds. The use of stencils prevents fabrication of highly complex shapes or small features. Organic solvent compatible, high precision print heads are available but they are optimized for a narrow range of polymeric solutions.

Another disadvantage of direct 3DP is that layer thickness must be greater than porogen particle size, and less than 150μm maximum thresholds to maintain inter layer connectivity and part strength during printing. To overcome this porogen size limitation, larger pores must be printed.

Another disadvantage of 3DP is a limited available pore size in the final constructs when porogens are incorporated into powders prior to fabrication. The shape complexity of scaffolds is also limited when the powder material is degradable polymer.

The 3DP approach for degradable polymer demands the use of organic solvents as liquid binders. Organic solvents can dissolve most commercially available drop-on-demand print head component.

Advantages of indirect 3DP: Molds are printed using commercially available modeling materials such as plaster, and biodegradable polymers are cast into the printed mold. Many different materials can be cast under the similar printing process parameters. The use of aqueous binder allows the use of consumer grade inkjet print heads, and eliminates the need for stencils. The porogen size is not limited since it is introduced in to the mold cavity after printing, and does not affect printing

resolution or layer interconnectivity. High materials flexibility with polymer-porogen combinations is possible due to independence from powder material properties.

Disadvantages of indirect 3DP are challenges in uniform, high density packing of porogen in complex features (intricate internal undercuts or intersecting channels) and restrictions on shape or feature design due to difficulty demolding. Incomplete packing will result in loss of uniform microarchitecture and desired microarchitecture.

Advantages of 3DP are the wide range of materials able to be used due to room temperature processing and the material used in powder form, ability to print overhangs and internal architecture, and microstructure control.

Disadvantages of 3D Printing are the limited use of organic solvents as binders due to dissolving of commercial print heads and difficulty in removing unbound powder from small or curved channels. 3DP materials include calcium polyphosphate and PVA, HA and TCP, TCP, HA with SrO and MgO doping, HA and apatite-wollastonite glass ceramic with water-based binder, HA with collagen in binder, PLGA, and Farringtonite powder (Mg3(PO4)2). Indirect 3DP materials: gelatin preforms replaced with PCL and chitosan.

**Clinical Application**

A study was undertaken by Jason A. Inzana *et al.* (2014) in critical sized murine femoral defects to assess the bone healing performance of 3D printed composite hydroxyapatite and collagen scaffolds. Collagen was dissolved into the binder solution to fabricate collagen-HA composites. The implants were confirmed to be osteoconductive, with new bone growth incorporating the degrading scaffold materials.

Tarafder *et al.* (2014) used direct three-dimensional printing (3DP) technology for fabricating SrO-and MgO-doped microwave sintered hydroxyapatite scaffolds. This 3DP scaffold possessed multi-scale porosity, 3D interconnected designed macro pores along with intrinsic micropores. A study reports the presence of SrO and MgO as dopants in HA scaffolds improves

mechanical and *in vivo* biological performance. These 3DP SrO-MgO- doped HA scaffolds have the potential for early wound healing through accelerated osteogenesis and vasculogenesis.

**Fused deposition modeling**

Fused deposition modeling (FDM) is the deposition of molten thermoplastic materials through two heated extrusion heads with a small orifice in a specific laydown pattern. One nozzle deposits the thermoplastic material and the second deposits temporary material to support cantilevers. In FDM, thermoplastic polymer is melted into a semi-liquid state and the head extrudes the material onto the build platform. The part is built in a layer-by-layer fashion where the layers are fused together. (Van *et al.,* 2012)

No restriction on compositional gradients in all three dimensions for FDM. The most important material selection criteria for FDM materials are heat transfer characteristics and rheology (behavior of liquid flow). Thermoplastics are commonly used due to the low melting temperature. PVC, nylon, ABS, and investment casting wax have been successfully used. For bio applications, PCL is commonly used due to its low melting temperature of ~60°C, low glass transition temperature of -60°C, and high thermal stability. PLGA has been used with FDM to create scaffolds, the high glass transition temperature of PLGA (40-60°C) makes processing PLGA challenging with a higher extrusion temperature required.

Rheological modifiers can be used but must be biocompatible. Controllable variables are raster thickness, raster gap width (space between rasters), raster angle, and layer thickness (dependent on extrusion tip diameter). This results in scaffolds with controlled pore size, morphology, and interconnectivity. The extruded molten liquid must be hot enough to rapidly induce fusion with previously extruded material and solidify quickly to minimize flow and feature size.

The viscosity of the material is critical to be both high enough to allow extrusion through a fine nozzle and low enough to Scaffolds with biocompatible materials have been made with

different pore morphology and channel sizes by controlling the x-y movement of the extrusion head.

Materials can also be combined in this technology such as poly (ethylene glycol) terephthalate/poly (butylene tere phthalate) or polypropylene/ TCP. Composites such as PCL/HA or PCL/TCP are used with FDM due to favorable mechanical and biochemical properties for bone regeneration.

The advantages of FDM are high porosity due to the laydown pattern and good mechanical strength.

Disadvantage for FDM is the limitation to thermoplastic materials with good melt viscosity properties which have high enough viscosity to build but low enough viscosity for extrusion. These properties have limited shape complexity for biological scaffolding materials and typically result in relatively regular structures. It should be noted that geometric complexity is not limited for FDM using industrial materials which are selected to have optimal thermal and rheological properties but lack biocompatibility. Another disadvantage for FDM is the inability to incorporate living cells or temperature sensitive biological agents during extrusion due to the high processing temperature.

Materials used in FDM to create scaffolds are PCL and bioactive glass composites, L- lactide/e-caprolactone, PLGA with collagen infiltration, PCL-HA with gentamicin, PCL/HA, PLGA-HA and coated with HA, PCLPLGA- HA, PLGA-PCL, PCL coated with gelatin, PCL, PMMA, and PLA. Applications include cartilage tissue engineering, antibiotic delivery system, osseous craniofacial defects in humans, and bone tissue engineering.

**Clinical Application**

A study was conducted by Shim *et al.* (2012) to investigate the healing capacity within an 8-mm rabbit calvarial defect using a polycaprolactone (PCL)/poly (lactic-co-glycolic acid) (PLGA) scaffold blended with hydroxyapatite (HA) that was constructed using fused deposition modeling technique. ThePCL/PLGA/HA scaffold showed a 37 % higher compressive strength and rougher surface than the PCL/PLGA scaffold. The PCL/PLGA/HA groups had greater neo tissue areas, greater bone

density at 4 and 8 weeks. Study suggest that the PCL/PLGA/HA scaffold fabricated using fused deposition modeling technology is useful for recovering and enhancing new bone formation in bony defects in rabbits.

Kim *et al.* (2012) fabricated scaffold composed of poly (D, L-lactideglycolide) (DL-PLGA) and β-tricalcium phosphate (β-TCP) nanocomposites by fused deposition modeling (FDM), scaffolds were then coated with hydroxyapatite (HA). Scaffolds were implanted into rabbit femoral unicortical bone defects. It was observed that all configurations of the scaffolds integrated with the host bone and were biocompatible.

**Stereolithography**

Stereolithography (SLA) is the first rapid prototyping process and was developed in the late 1980s. Stereolithography is the polymerization of photocurable resin by a bottom-up system with scanning laser or top-down setup with digital light projection. The original SLA rasters a HeCd-laser beam to spatially control the polymerization of photocurable resin in 2D patterns. After each layer is cured, the platform with the cured structure attached then lowers in the bottom-up approach and another layer of uncured liquid resin spreads over the top. The topmost layer is now ready to be patterned (Melchels *et al.* 2010).

Top-down approach, light is projected onto a transparent plate initially positioned near the bottom of the vessel holding the liquid resin. After a layer is patterned through the transparent plate, the cured structure is detached from the transparent plate. The cured structure is raised to allow uncured liquid resin to fill the space between the structure and transparent plate. The next layer is now ready to be patterned. Rastering a laser beam is slow, for large parts, the masked lamp technique was developed to cure an entire layer of photopolymers at a time. After the structure is built, the unpolymerized liquid resin is removed by draining. Post curing in a UV oven converts any unreacted groups and strengthens the part.

Kinetics of the curing reactions occurring during polymerization is critical. This affects the curing time and the thickness of the layer polymerized. The kinetics can be controlled by the power of the light source, the scanning speed and the chemistry and amount of the monomer and photo intiators. In addition, UV absorbers can be added to the resin to control the depth of polymerization. Materials must have photocurable moieties for photo crosslinking.

Typical materials used in STL include acrylics and epoxies. For tissue engineering applications, there are very few biodegradable and biocompatible biomaterials that are dimensionally stable during photopolymerization. Photo-cross-linkable poly (propylene fumarate) (PPF) is commonly used in SLA and has been used to fabricate complex 3D scaffolds with controlled microstructures. PPF requires a reactive diluent, diethyl fumarate or N-vinyl-2-pyrrolidone, to reduce the viscosity of the resin for proper processing conditions. These diluents introduce significant amounts of a non-degradable component.

Advantages of SLA are the ability to create complex shapes with internal architecture, ease of removal of unpolymerized resin, and extremely high feature resolution.

Disadvantage of SLA is the scarcity of biocompatible resins with proper SLA processing properties. Other challenges are the use of photointiators and radicals which may be cytotoxic (with long processing times), entrapment of unreacted monomer and residual photo initiator, and inability to create compositional gradients along horizontal planes. Photopolymerized resin also has poor mechanical properties that are needed for hard tissue engineering. Also, temporary support structures must be incorporated into the CAD model to fabricate unsupported features. Complete removal of support structures may be difficult. Novel macromers synthesized include segments of PCL (three-armed hydroxyl- terminated), Photo-curable poly (D, L-lactide) (PLLA), PPF-DEF, PPF-DEF with BMP-2 loaded PLGA microspheres, PPF-DEF with HA, Poly (trimethylene carbonate) Lee *et al.* (2009), Lee *et al.* (2011).

Applications of SLA include the fabrication of anatomical models for pre-surgical planning, and indirect fabrication of medical devices by using the SLA patterns for molds. Other large application of SLA is bone tissue engineering.

**Clinical Application**

Lee *et al.* (2009) fabricated a nano/microscale composite scaffold containing hydroxyapatite (HA) nano powder using stereolithography. The photopolymer [poly (propylene fumarate) (PPF)], viscosity reducer [diethyl fumarate (DEF)], photo initiator [bis-acylphosphine oxide (BAPO)] and 7% (w/w) HA were used in generation of the scaffold. *In vivo* rat study, PPF/DEF-HA scaffolds showed better cell adhesion and proliferation and was suggestive that scaffolds containing HA powder can be used in bone tissue regeneration.

Lee *et al.* (2009) developed a 3D scaffold using stereolithography. The scaffold consisted of BMP-2-loaded poly (DL-lactic-co-glycolic acid) (PLGA) microsphere suspension and a poly (propylene fumarate) (PPF)/diethyl fumarate (DEF) photopolymer with Hydroxyapatite. Scaffolds created by SLA were superior to traditional scaffolds produced using a particulate leaching/gas foaming method. Scaffold showed enhanced reconstruction quality in complex bony defects in rat model.

**Selective laser sintering/melting**

Selective laser sintering (SLS) was developed by the University of Texas in 1989. SLS binds together powder particles in thin layers using a CO2 laser beam. The laser scans the surface of the powdered polymer particles in a specific 2D pattern to sinter by heating them above the glass transition temperature. During sintering, molecular diffusion along the outermost surface of the particle lead to neck formation between neighboring particles. After one layer is created, the piston containing the part is lowered and a fresh layer of powder material is rolled across the top surface. The subsequent layer is formed and is bound to the previous layer. Unbound, loose powder is removed after the part is completed and is heat

treated to achieve full density. Temporary support structures are not needed, since unbound solid particles support any cantilever structures (Pattanayak *et al.,* 2011).

Since sintering does not result in complete melting of the powder particle, the porosity between the original particles can be preserved, and a wide range of pure and mixture of materials can be processed.

Melting is more easily accomplished if all powder has a single melting point, and is therefore more easily accomplished with pure metals than with alloys due to variation in liquid metal flow behavior, surface tension, and laser-material interactions. Therefore, the range of materials for SLM is more limited than SLS.

The resolution of features is determined by powder particle size, focused laser beam diameter and heat transfer in the powder bed. The limit to particle size is 10 μm due to poor spreading and sintering too quickly causing edge inaccuracies.

Materials commonly used are PCL and a combination of polyether ether ketone and hydroxyapatite. Previously coated ceramic powders and ther moplastics have been used in SLS. Intermediate binding materials are required because of an excessively high glass transition temperature and the melting point of ceramic powder. The intermediate binding materials would melt before the ceramic powder and fuse together the ceramic particles.

Advantage of SLS/SLM is the ability to directly make metallic implants that promote either bone ingrowth or regeneration for load-bearing applications in which high fracture toughness and mechanical strength is needed. Even for non-load bearing applications, polymers can be processed without the use of organic solvent. It is easy to achieve compositional gradients in SLS by spreading different powder between different vertical layers, but compositional gradients in the horizontal plane is very limited.

Disadvantages are limited materials which fuse but do not decompose under the laser beam (high temperatures) and

the post processing needed to remove trapped powder. Another limitation is the conduction and diffusion of laser heat causes unwanted fusion of neighboring powder particles, limiting the resolution of final features. Another limitation, smaller pore sizes are limited since the created pores depend on the particle size of the powder used. Powder particles too small cannot be used due to poor spreading from powder clumping.

SLS has the ability to produce lower stiffness scaffolds and higher resolution features. PCL scaffolds have been produced at lower stiffness. This lower stiffness allows for applications of soft tissue engineering such as cardiac tissue.

Common materials used in SLS are PCL and HA, PCL and β-TCP with collagen coating, Ca-P/PHBV and CHAp/PLLA, and PVA. Applications are bone tissue engineering and interbody cages for spinal fusions.

**Clinical Application**

In a preclinical large animal study, Kang *et al.* (2013) created Porous Biodegradable Lumbar Interbody Fusion Cage by selective Laser Sintering of poly (e-caprolactone) mixed with hydroxyapatite for management of lumbar compression in mini pigs. Compression tests revealed that the yield strength of optimized fusion cages was two times that of typical human lumbar spine loads.

Liao *et al.* (2013) fabricated three dimensional polycaprolactone (PCL), Polycaprolactone and Hydroxyapatite scaffolds via a selective laser-sintering technique (SLS). Collagen type I was further coated onto PCL–HA scaffolds to form PCL–HA–COL scaffolds. The compressive modulus was increased by adding 30% HA into a 70% PCL scaffold. No significant increase of mechanical strength was found by surface-coating with collagen type I. Hydrophilicity and swelling ratios showed elevation after collagen type I was coated onto the PCL–HA scaffolds. The nude mice experiments showed better woven bone and vascular tissue formation in the PCL–HA–COLgroup.

**3D Plotting/Direct-write bioprinting**

**3D Plotting**

3D plotting was developed at the Freiburg Materials Research Center in 2000. 3D plotting is based on extruding a viscous liquid material (a solution, paste, or dispersion) from a pressurized syringe into a liquid medium with matching density. The material is deposited in one long continuous strand or in individual dots from a nozzle or syringe to create a desired 3D shape of ceramics, polymers, or hydrogels. The process can be at room temperature or at elevated temperatures. This SFF method is particularly applicable for natural biomaterials to create hydrogels.

The Advantages are material flexibility and room temperature processing. Many of the other SFF technologies cannot use natural polymers due to processing conditions. The disadvantage is the difficulty in fabricating complex shapes with overhangs since a temporary, sacrificial material is needed. Hydrogels created in this method have low stiffness which may result in collapse of structures or limitations on complexity of shapes.

**Bioprinting**

Bioprinting is the fabrication of hydrogel structures with direct incorporation of cells. In bioprinting, small balls of bioink composed of cells and hydrogel materials (alginate or decellularized extracellular matrix) are printed in a desired shape. Cells are added during processing in cell printing strategies such as alginate-cell (bovine chondrocytes) solution extruded from a syringe, electrostatically driven inkjet printing of bovine vascular endothelial cells in culture medium, laser-guided direct writing of embryonic chick spinal cord cells, and laser-induced forward transfer of cells suspended in alginate. This technology provides a controlled spatial distribution of cell or growth factors as well as the scaffold structures.

This fabrication technique is generally limited to hydrogel materials such as alginate and fibrin, which may not be ideal for the implantation in biological environments that

require strong mechanical properties. This SFF method is especially good for low viscosity materials and the buoyancy due to the density matching of the extruded material to the liquid medium prevents collapse of the shape. The strand thickness can be varied by material viscosity, deposition speed, extrusion tip diameter, and applied pressure.

The advantages of bioprinting are the room temperature processing, direct incorporation of cells, and homogenous distribution of cells. The disadvantages are limited mechanical stiffness, critical timing of gelation time, specific matching of material and liquid medium densities to preserve shapes, and low resolution.

Bio plotting materials include PLGA, HA, collagen and chitosan, chitosan, collagen- alginate-silica composites coated with HA, soyprotein, andagarose with gelatin. Applications include bone tissue engineering and tissue regeneration.

Bioprinting materials are agarose with human umbilical vein smooth muscle cells (HUVSMCs) and human skin fibroblasts (rods), gelatin-HA-tetraPEG-DA with NIH 3T3s (rods), rat primary bladder smooth muscle cells in collagen droplets, human microvascular endothelial cells in fibrin (inkjet printer), and alginate droplets. Applications are mainly for vascular tissue engineering.

**Clinical Application**

A study was conducted in nine sheep by Haberstroh *et al.* (2009) aimed at investigating the osteogenic effect of cell-seeded 3D-bioplotted scaffolds in an ovine calvarial critical-size defect model. The scaffold-materials PLGA, HA/Col, and HYDR (HA/Col/chitosan) were cell-seeded with osteoblast-like cells whether gained from bone (OLB) or from periosteum (OLP). OLB- and OLP-seeded HYDR and OLB seeded HA/Col scaffolds significantly increased the amount of newly formed bone (NFB) at the defect bottom and OLP-seeded HYDR also within the scaffold area, whereas PLGA scaffolds showed lower rates. HA/Col had good stiffness to prepare complex structures by bio plotting but HYDR and PLGA were very soft. HYD R showed

appropriate biodegradation, HA/ Col and PLGA seemed to be nearly undegraded after 14 weeks.

A study was conducted in calverial critical size defects in rats by Lee *et al.* (2014) using biomimetic composite scaffolds prepared usinga mixture of collagen and alginate as a matrix material, and various silica coated HA weight fractions as a coating agent. Various levels of bone-like hydroxyapatite (HA) on the surface of the composite scaffolds developed in proportion to the increase in the silica content coating the scaffolds, indicating that the composite scaffolds have osteoinductive properties. The mechanical improvement of composite scaffold in compressive mode was noticed. Cell proliferation on the composite scaffold was significantly improved. Osteocalcin levels of the composite scaffold after 28 days were significantly enhanced.

**Conclusion and Future Prospects**

Progress for 3D Printing technologies is needed for increasing resolution without sacrificing shape, strength, and handability of scaffolds. Anatomical features and tissue architecture may have details on the scale of hundreds of microns.

Both SLS and 3DP is challenged with creating stronger structures without increasing dimensions. To create small features which survives the fabrication process, powder particles much be bound together tightly. By increasing the strength of the laser for SLS or amount of binder for 3DP, additional powder particles would bind and therefore increase the dimensions. Additional work is needed to move SLS and 3DP to resolutions below 400-500μm.

Unbound trapped powder is difficult to remove from small channels. Future work is needed to create powder that is easily removable with traditional methods of high-pressured air. One strategy is to create spherical powder particles which would facilitate removal in tight spaces.

SLA can reach extremely high resolutions, but there are a limited number of biodegradables, biocompatible resins. Advances have been made to synthesize new macromers with

biodegradable moieties. FDM, SLS, and 3DP are able to use polymers such as PLGA, PLLA, and PCL without chemical modification.

Work should focus on the nanoarchitecture (biochemical molecules). Due to harsh processing conditions of SFF methods (heat, organic solvent), biochemical molecules are not generally incorporated directly into the scaffold. While biochemical molecules can be coated onto structures in post-processing, there is a need for sustained growth factor release over time. Strategies to incorporate biochemical molecules directly into scaffolds for prolonged release will be needed.

Degradation kinetics and by-products of the materials are in fact a very significant problem in 3D scaffolds due to mass transport limitations within thick scaffolds. This is a moving boundary diffusion-reaction problem that even without biodegradable biomaterials can result in hypoxia and acidosis within the scaffolds. The release of acidic degradation products is expected to worsen the acidosis which may harm the seeded cells and/or the surrounding cells.

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# ULTRASONOGRAPHY OF OVARY IN BOVINES

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***ABSTRACT-***

*Exact identification of ovarian structures by rectal palpation is a real challenge to the veterinarian as these structures can present diverse morphological manifestations depending on the stage of development. Trans-rectal ultrasonography (TRUS) offered a much better break in the bovine reproductive studies, which enabled the exact evaluation of ovary in bovines like position, size and consistency of ovaries, ovarian function and ovarian pathology. Color Doppler ultrasonography, an advanced noninvasive technique for evaluating the vascularization of ovaries allowed the visualization of changes in blood flow within Corpus luteum (CL) or in the wall of preovulatory follicle.*

***Keywords****: Bovine, Ultrasonography, Ovary, Follicle, Corpus luteum*

## INTRODUCTION

The ultrasonic characteristic of a tissue depends on its ability to reflect sound waves. Liquids do not reflect sound waves (i.e. nonechogenic or anechoic) and are represented on the viewing screen as black. The ultrasonic images of liquid containing portions of structures such as ovarian follicles, embryonic vesicles appear black. Dense tissues (e.g. bone) reflect a large proportion of the transmitted sound waves (i.e. echogenic) and are represented on the viewing screen as light

grey or white. Various tissues and contents of the reproductive tract appear on the screen in varying shades of grey depending upon their echogenicity.

Follicular growth and regression in cattle occurs in a wave like pattern, starting at different stages of oestrous cycle. Sonography revealed that most of the bovine oestrous cycles are comprised of either two or three such waves, rarely one or four wave cycles also observed (Adams *et al*., 2008). Each wave consists of the contemporaneous emergence of a cohort of follicles 3-4 mm with a future dominant follicle of comparatively bigger size. Dominant and subordinate follicles pass through growing, static and regressing phases that have distinct morphological and biochemical characteristics; these changes are the basis of efforts focused on diagnosing and manipulating follicular status.

In two wave cycle, the dominant follicle of the first wave is anovulatory. It remains dominant for 4-5 days, and generally by day 11 or 12 of the oestrous cycle, it loses its dominance and begins to regress which lasts for 5-7 days. In the meantime, the second wave of follicles has been recruited and selection of the second wave dominant follicle has occurred, this dominant follicle goes on to ovulate. In a three wave cycle, however, this second dominant follicle regresses, making way for yet another group of follicles, with the third dominant follicle ovulating. Mihm *et al*. (2002), who observed that in animals with three waves per cycle, the follicle that dominated in the first wave was larger than the second wave, probably due to the FSH wave post ovulation and the maximum diameter of preovulatory follicle in the third wave was due to the ovulatory status in the third dominant follicle which offered the stimulus for the increased growth.

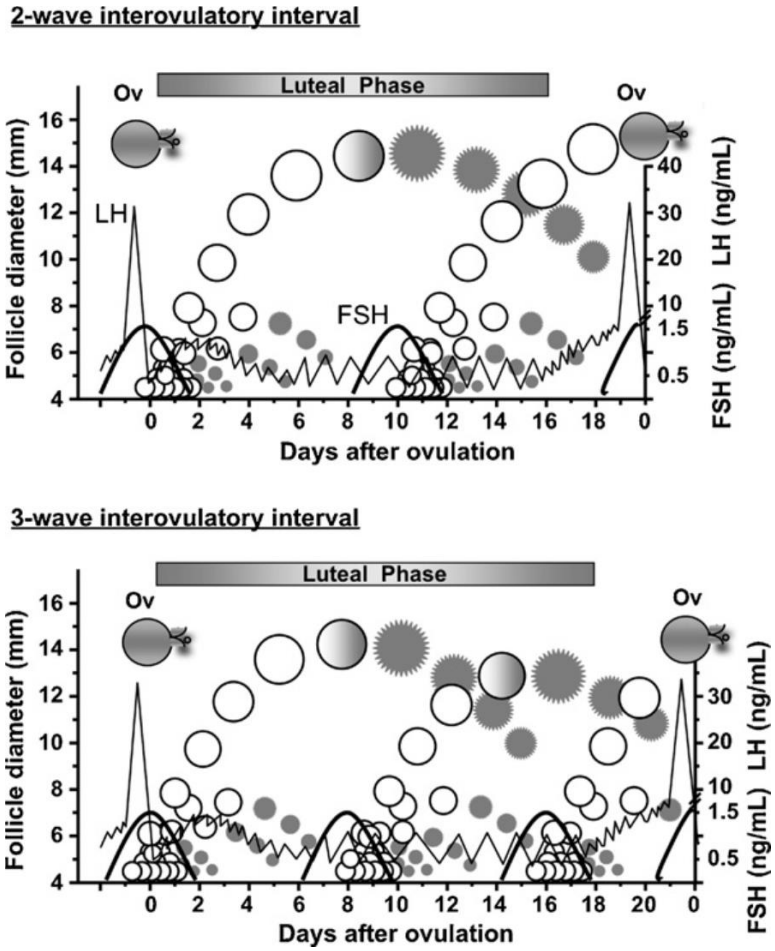



Fig. 1: Dynamics of ovarian follicular development and gonadotropin secretion during two- and three-wave oestrous cycles in cattle. Dominant and subordinate follicles are indicated as open (viable) or shaded (atretic) circles. A surge in circulating FSH concentrations (thick line) precedes emergence of each wave. A surge in circulating LH concentrations (thin line) precedes ovulation. The LH surge is preceded and succeeded by a period of high-LH pulse frequency as a result of low-circulating progesterone concentrations (i.e., period of luteolysis and luteogenesis, respectively)

**Sonographic imaging of corpus luteum (CL)**

Ovulation can be detected either by the disappearance of Graafian follicle or by the detection of corpus hemorrhagicum on the day of ovulation. Corpus hemorrhagicum appeared as a more hypoechoic, more dark area as against the ovarian stroma. Clear appearance of CL by TRUS was identified on third day of ovulation and sonographically imaged as poorly defined, uneven, greyish-black structure with echogenic spots within the ovary. The mature CL is hypoechoic (darker) compared to the ovarian stroma due to extensive vascularization (Ginther, 2014).

**Luteal blood flow characteristics**

Corpus luteum blood flow (CLBF) studies can be done with the help of Doppler ultrasonography. Luteal blood flow values are symbolized as extremely reliable predictors of luteal status better than luteal size particularly during luteal regression (Miyazaki *et al*., 1998).

A double fold increase in luteal blood flow can be observed during the growth phase of corpus luteum (7-8 days), thereafter a moderate increase or persistent as such in some other cows during stationary phase (8-16 days). A further raise of blood flow can be noticed on day 16 or 17 followed by a drastic reduction of blood flow in two wave cycle animals where as it may occur on day 18 or 19 in animals with three wave cycle: this is in correlation with the countercurrent blood flow from uterine endometrium to the ovary in order to supply endometrial prostaglandins during luteal regression (Herzog *et al*., 2007 and Adams *et al*., 2008). A drastic reduction in blood flow can be noticed during the phase of CL regression. Cows with three waves had an extended period of increased blood flow during stationary phase corresponding to increased functionality of CL and a decline in blood flow at a delayed period corresponding to delayed stage of luteal regression.

Luteal blood flow detected using color-flow Doppler ultrasound can be used to identify non- pregnant animals based on the decrease in CLBF associated with luteolysis. In case of non-pregnant animals, luteal blood flow reduced during

regression and is completely absent on the day of estrus whereas in pregnant animal, the luteal blood flow remains and will persist till the end of gestation (Beindorff *et al*., 2010).

**Ovarian pathological conditions**

Luteal and follicular cyst is the most frequently encountered pathological conditions in bovine ovaries. An ovary is usually considered cystic when it contains a hollow structure greater than 2-2.5cm that persists for more than 10 days. Cystic ovary disease or syndrome (COD) is commonly considered to be associated with negative energy balance and stress factors in dairy cows that are high milk producers. During normal proestrus, regression of the CL coincides with development of a selected follicle, while the growth of any additional follicles is inhibited. In animals developing COD, ovulation fails to occur and the dominant follicle continues to enlarge. Exaggerated growth of a non-ovulating follicle may lead to the creation of a follicular or luteal cyst. Accurate diagnosis and treatment of ovarian cysts are important aspect of proper reproductive management for any dairy herd. While the negative effects of ovarian cysts on normal estrous cyclicity, conception and prolonged calving intervals are evident; determining which type of cyst is on an ovary (follicular, luteal, or even a cystic corpus luteum) can be difficult. Diagnosis of each type is important as the proper treatment for follicular and luteal cysts differs, and use of ultrasound is the best tool for achieving this. The positive values for follicular cysts diagnosed by palpation or by ultrasonography are 66 and 74% respectively, and for luteal cysts, the values are 66 and 85%, respectively (Hanzen and Nguyen Kien, 2016).

**Follicular cyst**

A follicular cyst can be differentiated from a luteal cyst by its thin outer wall and uniformly anechoic follicular fluid extending to its outer edges (Ginther, 1997). Development of follicular cyst in cows occurs when a dominant follicle reaches the ovulatory size but fails to ovulate leaving a large persistent follicular structure on the ovary. It can be single or multiple structures in the ovary. The classical identifying feature of a

follicular cyst is a follicular structure on the ovary with a size ≥25mm if it is single or multiple structures with size ≥17mm each in the absence of any corpus luteum and it should persist at least a period of 10days.

**Luteal Cyst**

These are fluid filled ovarian structure with greater than 25mm diameter which persist for more than 10 days in the absence a CL. Walls are thicker (>3mm)than follicular cyst because of the luteal tissue lining. May be considered as an extension of follicular cyst such that non ovulatory follicle is partially luteinized spontaneously. Often progress into luteal cysts by forming a thicker wall of luteal tissue around their outer edges. Presence of cobwebs indicates luteinisation. On per rectal palpation, quiescent uterus of luteal phase of the estrous cycle. On per rectal examination luteal cysts are identified as smooth fluctuating domes protruding above the surface of the ovary, usually single in/ number.

**Conclusion**

Manual palpation or ultrasonographic examination of the cow's genital tract are currently used by veterinarians involved in reproductive management, but knowledge of the potential and the confines of both methods is significant to attain an optimal precision in the diagnosis of physiological and pathological ovarian structure. In bovine reproduction, identification of the presence of CL at different stages of oestrous cycle is very important as it is helpful in deciding to initiate a synchronized ovulation protocol (eg: Ovsynch) or to propose treatment with prostaglandin. Detection of CL in heifers confirms that it has attained puberty. Recognition of presence of CL on the left or right ovary can give indication to the veterinarian, in which uterine horn an embryo or foetus is present, which needs to be examined for pregnancy diagnosis.

**SONOGRAPHIC IMAGING OF OVARIAN STRUCTURES**

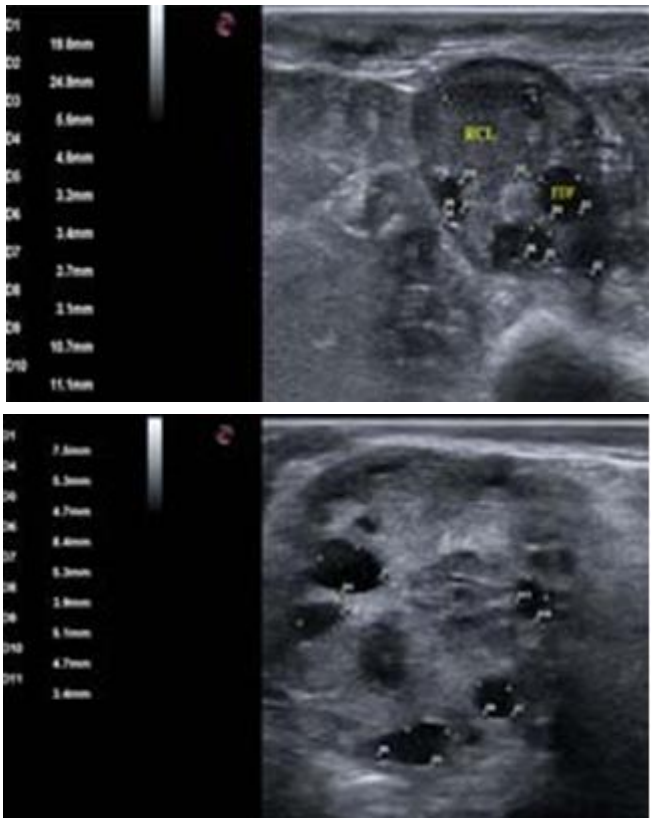
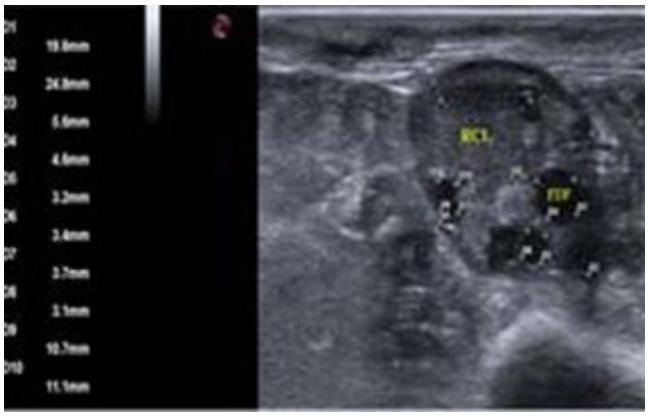



Fig. **1**. Day one after ovulation: appearance of new follicular wave with one follicle having larger diameter, future dominant follicle (FDF). RCL-regressed corpus luteum of previous cycle



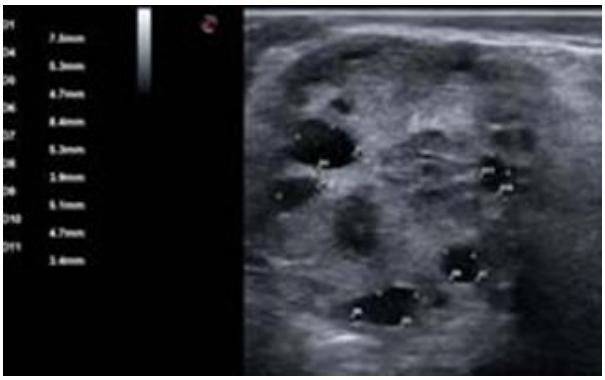


Fig. **2**. Day 5 after ovulation: after follicular emergence, all follicles grow at same growth rate until the day ofcdeviation (Day 5 or 6). There after the FDF continues to grow and subordinate follicle undergo either regression or retarded growth

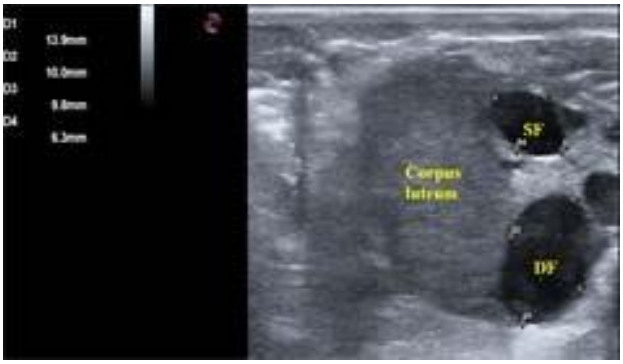



Fig. **3**. Day 9 after ovulation: DF- Dominant follicle (reach its maximum diameter by day 8- 11 then undergo regression), SF- Subordinate follicle

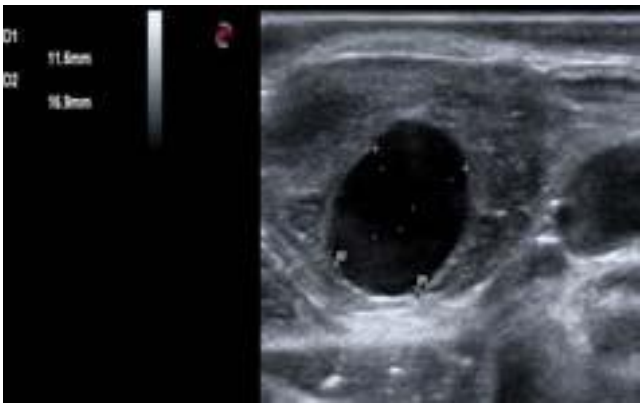


Fig. **4**. Day of oestrus: during day of oestrus the dominant follicle in the last follicular wave attain its maximum diameter (ranges from 12-20 mm) and become Graafian follicle (ovulatory follicle)

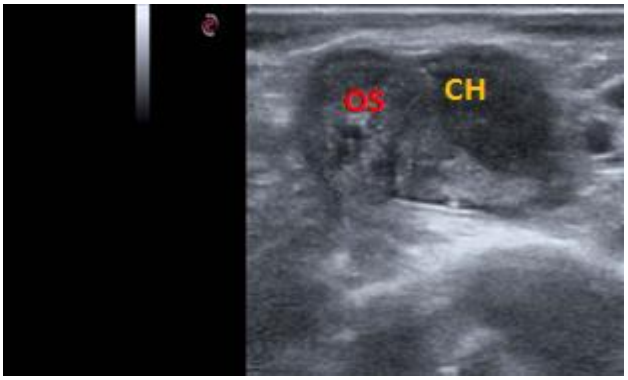



Fig. 5. CH- Corpus Hemorrhagicum, OS- Ovarian Stroma

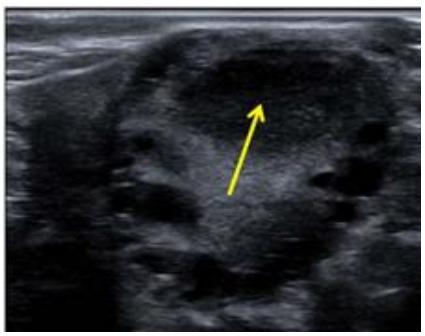


Fig. 6. Corpus luteum can be observed on third day of post ovulation by trans rectal ultrasonography and imaged as poorly defined, uneven, greyish- black structure with echogenic spots within the ovary.

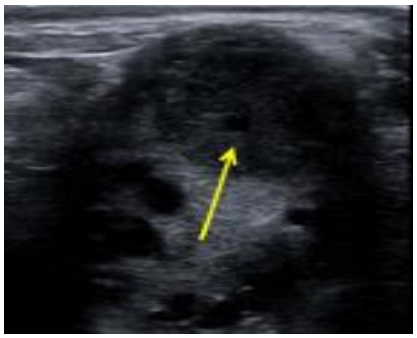


Fig. 7. Corpus luteum on 5th day after Ovulation: A definite shape for CL can be observed by day 4-5 after ovulation

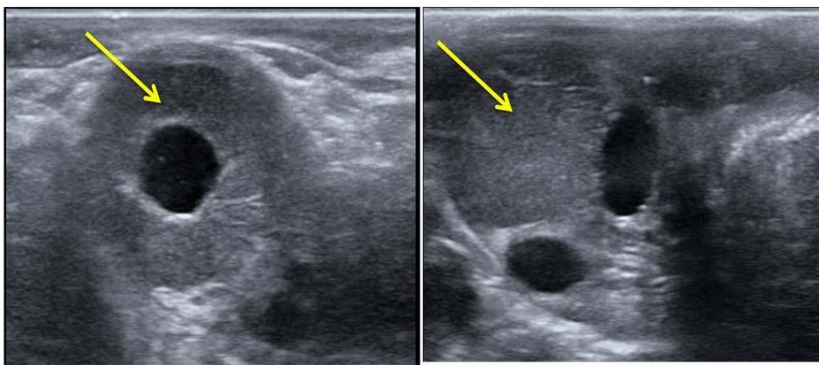


Fig **4**. (**A&B)** - Developed CL on day 10 A Cystic corpora lutea: corpus luteum with cavity B) Corpus luteum without cavity

**Imaging of Luteal blood flow characteristics using Doppler ultrasonography**

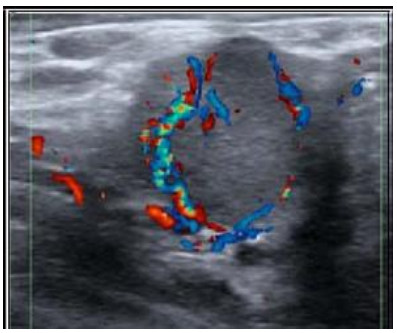


Fig.5. Luteal blood flow on day 8

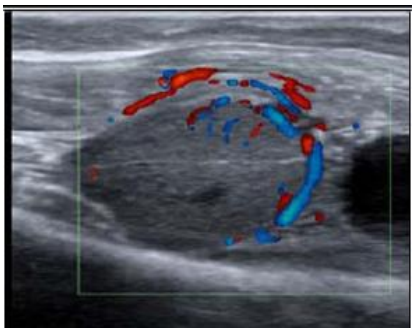


Fig.6. Luteal blood flow on day 10

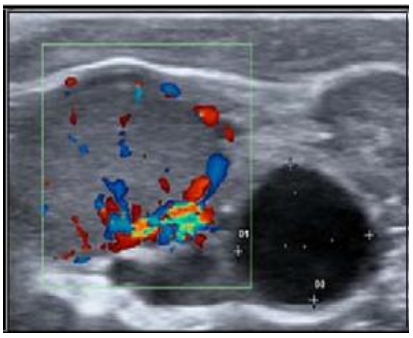


Fig.**7**. Luteal blood flow on day 12

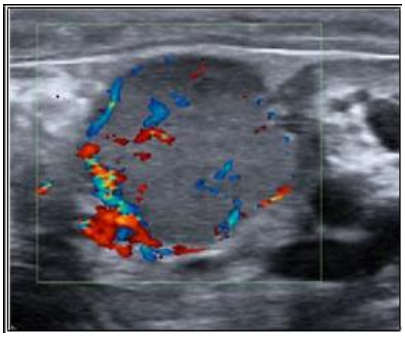


Fig. **8**. Luteal blood flow on day 14

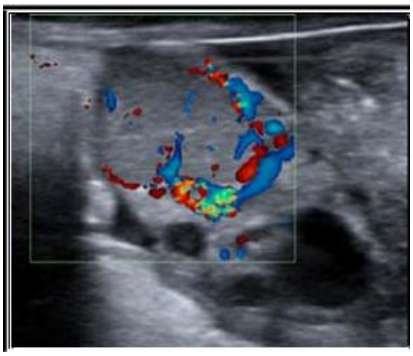


Fig. **9**. An increase in luteal blood flow on day 16

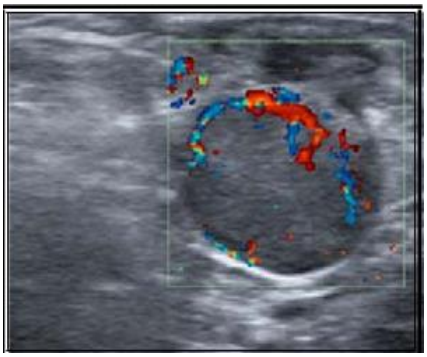


Fig. **10**. Decreased luteal blood flow on day 18

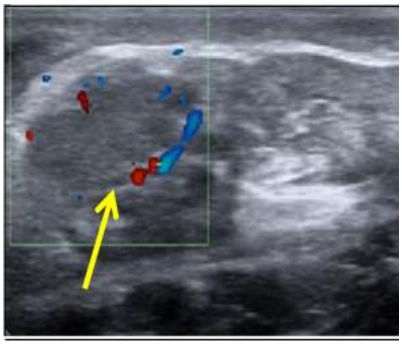


Fig. **11**. Decreased luteal blood flow on day 19

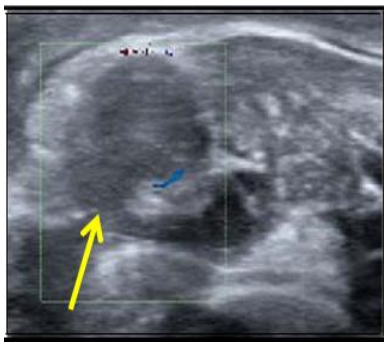


Fig. **12**.Absence of luteal blood flow on day 20 indicating completion of luteal regression

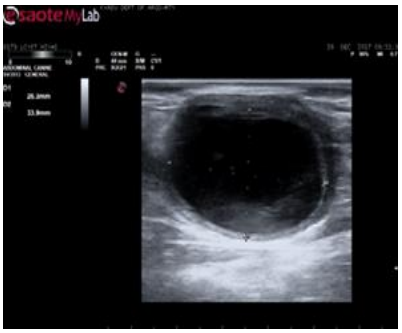


Fig. **13**. **Follicular cyst**: An anechoic structure similar to a follicle but of abnormally large size with wall thickness less than 3mm.

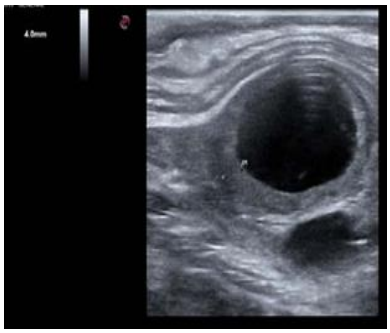


Fig. 14. **Luteal cyst**: Clear and thick luteinized wall can be seen. Usually the wall thickness will be greater than 4mm.

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# BIOMATERIALS AND ITS APPLICATIONS IN REGENERATIVE MEDICINE

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***ABSTRACT -***

*Biomaterials play central roles in modern strategies in regenerative medicine and tissue engineering. Biomaterials are non-viable materials that can be implanted to replace or repair missing tissues in man and animals. They may be of natural or synthetic origin. An ideal biomaterial should be durable, pliable, and resistant to infections and should possess adequate strength. It should not cause any hypersensitivity reaction, and should be reactive enough to induce a rapid fibroblastic reaction and must be biocompatible to facilitate tissue growth. Synthetic materials may result in complications during tissue repair like infections, fistula, increased chance of recurrence, repair failure etc. In addition, the high cost associated with synthetic material implants initiated the search for safe and cheap biodegradable material. Recently derived biomaterials of biological origin are collagenous in nature such as porcine intestinal submucosa, bovine pericardium, tunica vaginalis etc.*

***Key words****: Biomaterials, Regenerative medicine*

## INTRODUCTION

With the rapid improvement of standards of living and progress of society, people are facing social pressure which is

accompanied by increased rates of occurrence of various diseases and thus limiting their life. With the broad application and swift improvement of micro traumatic intervention treatment, the implantation of biomaterials is recognized to be one of the most efficient strategies to save and prolong the life of the human community.

Every day thousands of surgical procedures are performed to replace or repair tissues that have been damaged through disease or trauma. As per the 2021 data by the Ministry of Health and Family Welfare of Government of India, 70 lakhs burn injury cases occur annually, of which 1.4 lakh people die every year (Dhasmana *et al.,* 2018). The developing field of tissue engineering aims to regenerate damaged tissues by combining cells from the body with highly porous scaffold biomaterials, which act as templates for tissue regeneration, to guide the growth of new tissue. A biomaterial is any substance that has been engineered to interact with biological systems for a medical purpose-either a therapeutic or a diagnostic one.

In the first Consensus Conference of the European Society for Biomaterials (ESB) in 1976, a biomaterial was defined as a nonviable material used in a medical device, intended to interact with biological systems; however, the ESB's current definition is a material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body. In the area of biomaterial development, researchers are working to use biodegradable materials-both natural and synthetic-with appropriate mechanical properties that can be modified to incorporate biological activity, such as growth factors and structural adhesive proteins.

## 1. Properties of biomaterials

A biomaterial used for implant should possess some important properties in order to facilitate long-term usage in the body without rejection.

### 1.1 Host response

Boretos and Eden (1984) defined that host response is the local and systemic response of the host organism to the

implanted material or device. Host responses towards foreign implants may result in failure of the biomedical device. To solve this problem, first a better understanding of the biomaterial-induced host reactions including protein adsorption, leukocyte activation, inflammatory and fibrotic responses to biomaterials is required.

### 1.2 Biocompatibility

According to Williams (1987), biocompatibility indicates the biological performance of materials. Black and Hastings (1998) explained that biocompatibility is an ability of a material to perform with an appropriate host response, in a specific application. Biocompatibility is the ability to exist in contact with tissues of the human body without any harmful effects. Biomaterials should be non-carcinogenic, non-pyrogenic, non-toxic, nonallergenic, blood compatible, non-inflammatory.

### 1.3 Biofunctionality

Courtesy (2008) found that biofunctionality is playing a specific function in physical and mechanical terms. The material must satisfy its design requirements and should function as good as possible.

- Load bearing (mechanical, physical, chemical)
- Articulating (low wear and few wear debris)
- Control of blood and fluid flow
- Filling the volumes/space filling
- Transmission of light and sound
- Creating electrical stimuli
- Stimulation of the regeneration of tissue
- Sterilizable, storable and resorbable

### 1.4 Toxicity

A biomaterial should not be toxic, unless it is specifically engineered for such requirements. Toxicological manifestations, of an implanted biomaterial primarily results from the release of chemical constituents from the material leading to responses like

systemic toxicological response as well as allergic, carcinogenic, teratogenic and mutagenic responses.

### 1.5 Appropriate design and manufacturability

Biomaterials should be machinable, mouldable and extrudable. Finite element analysis is a powerful analytical tool used in the design of any implants.

### 1.6 Mechanical properties of biomaterials

Some of the most important properties of biomaterials that should be carefully studied and analysed in their applications are tensile strength, yield strength, elastic modulus, corrosion and fatigue resistance, surface finish, creep, and hardness. Physical properties are also taking in to account while selecting materials. The dialysis membrane has a specified permeability. The articular cup of the hip joint has high lubricity. The intraocular lens has clarity and refraction requirements.

### 1.7 Corrosion resistance

Corrosion is a major problem in selection of metallic biomaterials because the corrosion of metallic implants due to the corrosive body fluid is unavoidable. The implants release undesirable metal ions which are non-biocompatible. Corrosion can reduce the life of implant device and consequently may impose revision surgery.

## 2. Classification of biomaterials

Amogh *et al*. (2010) stated that a biomaterial is essentially a material that is used and adapted for a medical application. Based on source, biomaterials can be broadly classified as biological and synthetic materials (Kazim, 2009).

### 2.1 Biological biomaterials

Biological or natural biomaterials are any material taken from plants or animals and used to augment, replace, or repair body tissues and organs. And they can be further classified into soft and hard tissue types.

| Soft tissue biomaterials | Hard tissue biomaterials |
|---|---|
| Tendon, Skin, Cornea, Pericardium, Collagen | Dentine, Bone, Cuticle |

Based on animal source biological biomaterial can be grouped in to

1. Autograph-tissue transplanted from another part of the body of the same individual
2. 2. Allograft-from a donor of the same species
3. Xenograft-from a donor of a different species
4. 4. Isograft-from an identical twin

The advantages of natural biomaterials are that they mostly come from an *in vivo* source and hence large quantities are constantly available at a reasonable price.

### *2.1.1 Collagen*

Collagen is the most frequently used and studied biomaterial. There are rich *in vivo* sources, as all connective tissues of animals are rich in this ubiquitous protein. Collagen has a superior biocompatibility being a conserved protein. Additional benefit is that the immune system well tolerates collagen. It is capable of supporting a large spectrum cellular differentiation types. Therefore collagen is well preferred as scaffold. Sayani and Ronald (2014) stated that high biocompatibility and intrinsic biodegradability by endogenous collagenases make exogenous collagen ideal for use in biomedical applications.

**Preparation of collagen-based materials**

Natural collagen-based biomaterials can be classified into two categories based on the extent of their purification.

1. Decellularized collagen matrices
2. More refined scaffolds

***1. Decellularized collagen matrices***

Decellularization of collagen

Decellularized tissues and organs have been successfully used in a variety of tissue engineering or regenerative medicine applications. Common methods to produce the biomaterials comprise of physical (snap freezing or high pressure), chemical (acid or alkali chelation with EDTA, or treatment with detergents

or solutions of high osmolality) and enzymatic (digestion with trypsin) methods. The efficiency of cell removal from a tissue is dependent on the origin of the tissue and the specific physical, chemical, and enzymatic methods that are used. Each of these treatments affect the biochemical composition, tissue ultrastructure, and mechanical behaviour of the remaining extracellular matrix (ECM) scaffold, which in turn, affect the host response to the material.

Types of collagen based biomaterials

***a. Sponges***

Commercial collagen sponges are insoluble forms of the protein derived from animals like cows, horses, and pigs. The sponges are prepared by lyophilizing aqueous acid- or alkali swollen collagen solutions. Yannas (1989) observed that sponges are capable of absorbing large amounts of tissue exudate, adhere smoothly to a wet wound bed, and maintain a moist environment, while shielding against mechanical trauma and bacterial infections. So they can be used as a wound dressing for severe burns, pressure sores, donor sites, and leg ulcers, and in in vitro experiments.

***b. Injectables and hydrogels***

For several decades, dermatological defects have been treated with subcutaneous injections of collagen solutions. This application is a commercial success, particularly in the area of plastic and reconstructive surgery.

***c. Films and membranes***

Collagen films have been used in wound healing and tissue engineering, primarily as a barrier. Films of 0.1-0.5 mm thickness can be cast from collagen solutions and air-dried in a manner similar to ophthalmological shields. The loaded films afford easy sterilization and become pliable after hydration, without compromise to their mechanical strength.

Collagen membranes have been used for wound dressings, dural closures, reinforcement of compromised tissues, and guided tissue regeneration.

Applications of collagen

1. Shields in ophthalmology
2. Sponges for burns and wounds
3. Mini-pellets for protein delivery and drug delivery
4. Controlling material for transdermal delivery
5. Nanoparticles for gene delivery
6. Drug delivery formulations for tissue infections
7. Cell culture based biological studies
8. Gene therapy

Apart from these, it also finds its application in tissue engineering including skin replacement, bone substitutes, and as artificial blood vessels and valves.

***2. Bioscaffolds***

Bio scaffolding is the use of biocompatible and bioresorbable materials to construct a three dimensional porous structure comparable to the implant tissue area, in order to promote tissue regeneration and injury recovery. Bioscaffold is mainly utilized in bone and cartilage regeneration. However, it is also successfully implemented in areas of skin and muscle regeneration and possibilities for organ regeneration are being researched.

Scaffold design and fabrication are major areas of biomaterial research, and they are also important subjects for tissue engineering and regenerative medicine research. Scaffold plays a unique role in tissue regeneration and repair. During the past two decades, many works have been done to develop potentially applicable scaffold materials for tissue engineering.

**Functions of scaffolds**

1. Promote cell-biomaterial interactions, cell adhesion and ECM deposition
2. Permit sufficient transportof gases, nutrients and regulatory factors to allow cell survival, proliferation and differentiation

3. Biodegrade at a controllable rate that approximates the rate of tissue regeneration under the culture conditions of interest.
4. Provoke a minimal degree of inflammation or toxicity *in vivo*

### *2.1.2 Fibrinogen*

Fibrinogen is obtained from blood plasma. Although in its uncleaved form it occurs as a soluble protein, upon cleavage with thrombin, fibrinogen sets as a gel and forms a three dimensional meshwork which is 100% biocompatible. It is often used as a biological glue when cells to be seeded onto scaffolds. Recent applications of fibrin include cardiovascular, cartilage, bone and neuronal tissue engineering.

### *2.1.3 Silk*

Silk is a protein produced within specialized glands of silk worms. It has a special tertiary structure consisting of repeating amino acid motifs. Silk consists of two different protein components, namely fibroin and sericin. Sericin forms the outer layer on the fibroin core making it slippery and elastic. Fibroin that is biocompatible and possesses excellent mechanical properties is also used in bone, cartilage and ligament engineering.

### *2.1.4 Polysaccharide-based biomaterials*

Polysaccharide-based biomaterials are polymers consisting of sugar monomers which can be either of plant or animal origin. Some of the polysaccharides may trigger unwanted immune reactions so a careful selection is advised. Polysaccharides are most frequently used as three dimensional hydrogel injections. They can be dispensed directly to the site of injury so that it supports wound healing and also cell growth and differentiation.

#### *a. Agarose*

Agarose is the most frequently used polysaccharide scaffold consisting of a galactose based backbone. It is immunologically inert, so no immune response is triggered. One of its great advantages lies in its versatility. Thus it has been used

for scaffolding cartilage, heart, nerve tissues and it also supports stem cell differentiation.

***b. Alginate***

Alginate is the polysaccharide component of the cell walls of brown algae. It is an acidic compound, so in tissue engineering various cationic alginate salts are used. Sodium-alginate is a frequently used food additive and its use is also widespread in gastronomy. Besides of gastronomic applications, sodium alginate is used in industry as a heavy metal-binding or fatbinding agent.

***c. Hyaluronan***

Hyaluronan, also termed as hyaluronic acid, is an animal-derived polysaccharide which is extensively used as a scaffold material in tissue engineering. Hyaluronan has an important role in wound healing and tissue repair. Moreover, it supports embryonic stem cell differentiation, survival and proliferation. Like other polysaccharides, hyaluronan is used as a gel in nerve, cartilage and skin tissue engineering.

***d. Chitosan***

Chitosan is derived from the deacetylation of chitin, which is the main component of the arthropod exoskeleton. Chitosan is commercially derived from sea-dwelling crustaceans.

It is widely used for wound dressing, due to its ability to enhance blood clotting.

**2.2 Synthetic biomaterials**

In tissue engineering a large scale of synthetic biomaterials are used besides those of natural origin. They show high reproducibility, availability on demand and constant quality supporting industrial-scale production over natural biomaterials. Synthetic materials are further classified into: a) Metals b) Polymers c) Ceramics and d) Composite biomaterials.

***2.2.1 Metals***

Metals are widely used as biomaterials due to their strength and toughness. They have been used almost exclusively for load-bearing implants, such as hip and knee prostheses and

fracture fixation wires, pins, screws, and plates. The main considerations in selecting metals and alloys for biomedical applications are their excellent electrical and thermal conductivity, biocompatibility, appropriate mechanical properties, corrosion resistance and reasonable cost. Although pure metals are sometimes used, alloys like stainless steel, cobalt-chromium molybdenum alloy, and titanium and titanium alloys are commonly used and they frequently provide improvement in material properties, such as strength and corrosion resistance. Sometimes metallic implants may cause immunological reactions like metal allergy in sensitive individuals and are not biodegradable.

**a. Stainless steel**

The stainless steels, especially 316L type is the most used metallic biomaterials for biomedical applications due to their good biocompatibility, low price, excellent corrosion resistance, availability, easy processing and high strength. Due to these favourable properties 316L stainless steel has become the most attractive biomaterial for dental implants, stents and orthopaedic implants.

**b. Cobalt-Chromium alloys**

Cobalt- Chromium alloys have been extensively used for making various orthopaedic, dental, and cardiovascular implants and devices. These alloys possess superior mechanical properties with high resistance to corrosion, wear, and fatigue. The elemental composition of this alloy includes cobalt, chromium and molybdenum as the major elements. Cobalt provides continuous phase for basic properties. Chromium provides corrosion resistance whereas Molybdenum provides strength and bulk corrosion resistance.

**c. Titanium and Titanium alloys**

Titanium has a good record of being used successfully as an implant material and the success with titanium implants is credited to its excellent biocompatibility due to the formation of stable oxide layer on its surface. Titanium reacts with several other elements to form alloys. Increased use of titanium and its

alloys as biomaterials comes from their superior biocompatibility and excellent corrosion resistance because of the thin surface oxide layer, and good mechanical properties.

### *2.2.3 Polymeric biomaterials*

Polymers are organic materials that form large chains made up of many repeating units. Their unique properties are: flexibility, resistance to biochemical attack, good biocompatibility, lightweight, availability in a wide variety of compositions with adequate physical and mechanical properties and due to the fact that they can be easily manufactured into products with the desired shape. eg. Nylon, silicon rubber, polyester, PTFE, etc. They have been widely used in medical disposable supplies, prosthetic materials, dental materials, implants, dressings, extracorporeal devices, encapsulants, polymeric drug delivery systems, tissue engineered products and orthodoses.

### *2.2.4 Ceramics*

Ceramics are polycrystalline materials. The main characteristics of ceramic materials are hardness and brittleness, great strength and stiffness, resistance to corrosion and wear, and low density. Ceramics are typically electrical and thermal insulators and are used in several different fields such as dentistry, orthopaedics and as medical sensors. Ceramics typically fail with little, if any; plastic deformation and they are sensitive to the presence of cracks or other defects. eg. Aluminium oxide and Calcium Phosphates including hydroxyapatite carbons.

**a. Alumina (Al2O3)**

High density, high purity alumina was the first ceramic widely used clinically. It is used in load-bearing hip prostheses and dental implants, because of its combination of excellent corrosion resistance, good biocompatibility as well as high wear resistance, and high strength.

**b. Zirconia ($ZrO_2$)**

Zirconia is a biomaterial that has a bright future because Zirconia ceramics have several advantages over other ceramic materials due to its high mechanical strength and fracture toughness. Today's main application of Zirconia ceramics is in total hip replacement ball heads.

**c. Pyrolytic carbon**

Unlike metals, polymers and other ceramics, these carbonaceous materials do not suffer from fatigue. However, their intrinsic brittleness and low tensile strength limits their use in major load bearing applications.

**d. Bioglass and glass ceramic**

This material has been widely used for filling bone defects. The porosity of bioglass is beneficial for resorption and bioactivity. Bioglasses are degraded by hydrolytic breakdown in the body and replaced by regenerating natural tissue.

**e. Calcium Phosphate ceramics**

Calcium phosphate biomaterials are available in various physical forms. One of their main characteristics is their porosity. The ideal pore size for bioceramic is similar to that of spongy bone.

In general, ceramics show high resistance to corrosion and low electrical and thermal conductivities. These characteristics make them very suitable for implants. A significant influence in bone tissue regeneration is given to phosphate salts because their physical, chemical and structural properties are very similar to those of bone tissue.

### *2.2.5 Composite biomaterials*

The term “composite” is usually reserved for those materials in which the distinct phases are separated on a scale larger than the atomic, and in which properties such as the elastic modulus are significantly altered in comparison with those of a homogeneous material. The most successful composite biomaterials used in the field of dentistry are restorative materials and dental cements.

## 2.3 Classification of biomaterials based on material-tissue interactions

When a synthetic material is placed within the body, tissue reacts towards the implant in a variety of ways depending on the material type. The mechanism of tissue interaction, if any, depends on the tissue response to the implant surface. According to Ben-Nissan and Heness (2004) biomaterials are classified in to three groups based on the tissue responses.

These are bioinert, bioresorbable, and bioactive biomaterials.

***a. Bioinert materials***

Bioinert materials have minimal interaction with its surrounding tissue. This group includes stainless steel, titanium, alumina, partially stabilized zirconia (PSZ) and ultra high molecular weight polyethylene (UHMWPE).

***b. Bioactive materials***

Bioactive material interacts with the surrounding bone and in some cases, even soft tissue. This occurs through a time dependent kinetic modification of the surface, triggered by their implantation within the living bone. Examples of these materials are synthetic hydroxyapatite, glass ceramic and bioglass.

***c. Bioresorbable materials***

Bioresorbable materials will be resorbed and slowly replaced by advancing tissue. Common examples of bioresorbable materials are tricalcium phosphate and polylactic polyglycolic acid. Biomaterials are used to copolymers.

**Applications of biomaterials**

Make devices to replace a part or a function of the body in a safe, reliable, economic and physiologically acceptable manner. There are many applications of biomaterials in plastic surgery. One can think of joint reconstruction, osteosynthesis (e.g. plates and screws), reconstruction and creation of the shapes of bones and soft tissues (e.g. breast reconstruction and augmentation), glues, suture materials, and injectables. Increasing the sophistication and diversity of biomaterial products that are

available have contributed to their expanding role in various facets of medical field in recent years.

**a. Functions of biomaterials with examples**

| Problem Area | Examples |
|---|---|
| Replacement of diseased or damaged part | Artificial hip joint |
| Assist in healing | Sutures, bone plates and screws |
| Improve function | Cardiac pacemaker and intraocular lens |
| Aid to diagnosis | Probes and catheters |
| Correct functional abnormality | Cardiac pacemaker |
| Correct cosmetic problem | Augmentation mammoplasty |
| Aid to treatment | Catheters, drains |

**b. Biomaterials used in body systems**

| Body System | Biomaterials |
|---|---|
| Nervous System | Hydrocephalus drain, nerve stimulator |
| Musculo-Skeletal System | Bone plate, total joint replacements, muscular sutures, muscle stimulator |
| Circulatory System | Pacemaker |
| Endocrine System | Microencapsulated pancreatic islet cells |
| Reproductive System | Augmentation mammoplasty, other cosmetic replacements |

**c. Biomaterials used in organs**

| Organ | Material |
|---|---|
| Lung | Oxygenator machine |
| Eye | Contact lens, intraocular lens |
| Ear | Artificial stapes, cochlea implant |
| Heart | Cardiac pacemaker, artificial heart valve, total artificial heart, blood vessels |
| Kidney | Catheters, stent, Kidney dialysis machine |
| Bladder | Catheter and stent |
| Bone | Bone plate, intramedullary rod, screws |

**Future prospects**

Biomaterials have made a great impact on medicine. However, numerous challenges remain. Recently, biological scaffolds have been introduced as important players in surgical strategies of tissue reconstruction. There is a huge advancement

in the area of scaffold fabrication which has improved the potentiality of tissue engineering. Most emerging scaffolds for tissue engineering are hydrogels and cryogels. A large panel of extracellular matrices cultured with different cell populations have been used and promising results were reported in complex tissue loss repair including bone, muscles, nerves, blood vessels and skin defects. Future research is mandatory to standardize the bioengineered structures, in order to get the best results. Together with biomaterials, stem cell technology is also being used to improve the existing healthcare facilities. These concepts and technologies are being used for the treatment of different diseases like cardiac failure, fractures, deep skin injuries, etc. Introduction of nanomaterials on the other hand is becoming a big hope for a better and an affordable healthcare. Technological advancements are underway for the development of continuous monitoring and regulating glucose levels by the implantation of sensor chips. Other area which can improve the tomorrow's healthcare is drug delivery. Micro-needles have the potential to overcome the limitations of conventional needles and are being studied for the delivery of drugs at different location in human body.

For instance, biomaterials for targeted delivery of chemotherapeutic agents will allow effective treatment of lung cancer along with several other cancers. Polymer-based vaccine adjuvants may help in preventing the spread of HIV/AIDS and numerous other infectious diseases. Biomaterials for nerve regeneration will have clinical applications that extend well beyond stroke, to various additional neurodegenerative diseases. With further developments we expect these technologies to hit the market in near future which can immensely improve the healthcare facilities.

**Conclusion**

Biomaterial is defined as any material of natural or of synthetic origin that comes in contact with tissue, blood or biological fluids, and intended for use in prosthetic, diagnostic, therapeutic or storage application without adversely affecting

the living organism and its components. Innovative biomaterials have revolutionized the areas like bioengineering and tissue engineering for the development of novel strategies to combat life threatening diseases like cardiac failure. In addition to the use of biomaterials as implant devices they have also shown applicability in other healthcare related areas like disposable medical devices, diagnostic kits, polymeric therapeutics, etc. Research is being performed to improve the existing methods and for the development of new approaches. The selected biomimetic approach involves the design of a biomaterial to which the host-biological system could respond in a more favourable and effective manner, providing an exciting new era for the research and development of biomaterials.

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# INCREASING PRODUCTION OF DAIRY ANIMALS THROUGH NATURAL BYPASS NUTRIENTS AND ORGANIC MINERALS

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***ABSTRACT-***

*Production systems of present day warrant minimum use of antibiotics, hormones and chemicals to improve production and productivity in dairy cattle. Bypass proteins, bypass fats of plant origin and organic minerals play a major role in this regard. Natural by pass protein is formulated in a way that it escapes rumen degradation which avoids loss of valuable protein by decomposition for protein synthesis and methane emission. They are formulated by different techniques like heat treatment and binding with forage tannins. Bypass fat overrules negative energy balance in high producing dairy cattle. Bypass fat comprises of fatty acids associated with calcium ions instead of glycerol so it is rumen inert. It has 46-49 percent of palmitic acid and 36-38 percent of oleic acid. Organic minerals provide natural protection and are more likely to be absorbed and do not interact with antagonists or vitamins unlike inorganic trace minerals. Area specific mineral mixtures supplement the required quantity of specific minerals based on the requirements in specific agro climatic zone. Adoption of these techniques will improve production and productivity in in dairy cattle and will not only reduce cost of production but provide safe milk to society.*

***Key words*****:** *Dairy animals, Productivity, Bypass protein, Bypass fat, Organic minerals*

## INTRODUCTION

Livestock production accounts for 40% of the gross value of the global agricultural production (FAO, 2012). India's bovine population accounts to about 19.2 per cent of World's and 51.0 per cent of Asia's population with annual production of about 132.40 million tons of milk. As the demand for livestock products is increasing rapidly with the increase in buying power and rapid urbanization, people and governments have started to give importance to increase the livestock production apart from rearing animals just to generate the daily family income in the rural households. Agrochemicals, veterinary drugs, antibiotics and improved feeds have been used in this context in various livestock production systems around the world, to increase the food supply and minimize the production costs. The after effect of this is an increase in the number of quality-conscious consumers who seek environmentally safe, chemical residue free livestock products, along with product traceability and a high standard of animal welfare, which is ensured in organic farming.

Most organic markets and consumers are affluent urban dwellers, residing mostly in developed countries, who are prepared to pay a premium for organic products. This makes organic farming a niche area with excellent prospects for exports. Developing country like India has started to become important suppliers of organic foods, since organic practices tend to suit their farming conditions. It is important to note that organic livestock production not only offers good potential export market, but also huge challenges along with it. Organic agriculture is practised by almost 1.8 million producers in various countries and it continues to increase steadily by 15% per year. There has been a drive to improve production by improving the productivity of the animals rather than increasing the heads of bovine population. Adoption of newer technologies such as protected nutrient technology (Rumen bypass technology) is an essential requirement to achieve this goal. However, to increase the production of animals without compromising the quality parameters pertaining to the organic production has been a constant problem of dairy farmers.

In organic milk production systems, consumers expect organic milk and dairy products to come from farms that have been inspected and verified that they meet the stipulated standards, which includes the use of organic feed, avoiding usage of prophylactic antibiotics and give animals access to the outdoors, fresh air and sunlight. To maintain the milk production status of the country and to ensure the availability of milk, it is very much essential to maintain high producing animals. These animals should be fed with special diet to ensure such a status. Rumen protected proteins, fats and starch help in the delivery of nutrients directly to the small intestine of the animal. Feeding protected protein in diets containing supplemental fat may alleviate the decrease in milk protein percentage associated with fat supplementation. Therefore, negative energy balance during early lactation and increase in the production of milk with desirable composition will have far reaching benefits on their reproductive performance by supplementation of protected nutrients in the ration of medium and high yielding lactating animals.

Many chemical methods like formaldehyde treatment and alkali treatments are employed for imparting rumen protection effect. But employing these in organic production systems is disputed. This paper discusses in brief, the various natural methods and feed materials that can be employed to ensure rumen bypass effect for high yielding animals.

**Protected nutrient technology**

Protected nutrient technology is done through passive rumen manipulation, by which the dietary nutrients (fat, starch and proteins) are protected from hydrolysis, allowing these nutrients to bypass rumen and get digested and absorbed from the lower digestive tract (small intestine) of the animal. The protected nutrients mainly include protected fat and protein and they is also called as bypass nutrients. The other protected nutrients are protected starch, chelated minerals and vitamins. The use of these supplements of protein, starch and lipids to provide nutrients for milk production above those obtained,

should be used in a wider range after optimization of the utilisation of the basal feed.

**NATURAL BYPASS PROTEINS/PROTECTED PROTEINS**

Proteins, especially its soluble fraction are degraded by microbial enzymes in the rumen to give the end-products *i.e.* VFA, $CO_2$, $CH_4$ and ammonia. The microbial fermentation of soluble protein in the rumen is an unavoidable consequence of the ruminant mode of digestion. In the absence of other forms of N, it ensures a supply of ammonia nitrogen for micro-organisms from which they synthesize the protein in their cells. It is a wasteful process, as high quality proteins are broken down to ammonia, absorbed as such, converted to urea in the liver and this is excreted in the urine. In order to increase the efficiency of protein utilization from these highly degradable cakes, these proteins need to be protected from excessive ruminal degradation and can be used as protected protein, so that the amino acids from these protein feeds are absorbed intact from the intestines of the animal for tissue protein synthesis as well as for the process of gluconeogenesis in liver ().

By-pass proteins are defined as those dietary proteins that pass, intact, from the rumen to the lower digestive tract. Digestible by-pass protein is that portion of the by-pass protein that is enzymatically hydrolysed in and absorbed as amino acids from the small intestine. Only a few feeds are good sources of naturally occurring protected protein viz., maize gluten meal, cottonseed cake, fish meal, coconut cake and maize grain. Feeds like linseed cake, deoiled rice bran, soybean meal and *Leuceana* leaf meal are of medium protein degradability, while Mustard Cake (MC) and Groundnut Cake (GNC) are highly degradable cakes. While the proteins of lower protein degradability do not need any protection, highly degradable cakes like MC, GNC and sunflower seed cake need protection against attack of ruminal proteolytic enzymes, for improving their utilization by ruminants.

Appropriate physical methods such as temperature, pressure or a combination of both, for the proteinous feeds and

their by-products can be employed before their inclusion in the rations of livestock for improving productivity. There are several technologies adopted for giving natural bypass effect. They are:

Dietary proteins can escape the rumen for digestion in the lower alimentary tract when the protein meal has been made insoluble by heat treatment. found that heating GNC and soybean cake at 150°C for 2 h protected its protein from ruminal degradation. Partial heat treatment ensures their un-degradability in the rumen, as microbial enzymes fail to act on them. These proteins, on reaching the small intestine, are enzymatically digested by the animal. This has been traditionally used by the rural farmers in India. Both dry cooking and moist cooking may be employed for this purpose. Dry cooking involves baking in sun or frying in a vat. Moist cooking is done by boiling the cakes in water and feeding it directly to the animal after cooling it to ambient temperature. Salt or molasses may be added to the cooked meal to increase the palatability.

**2. Binding with forage tannins**

When the protein meal contains tannins, (2-4%) they bind to make an insoluble tannin-protein complex (Barry, 1985), which is not degraded in the rumen but is degraded in the abomasum/small intestine. Tree forages contain high amounts of tannins. However, these forages contain 16-53% of total N in the form of acid detergent insoluble nitrogen. This is because of the presence of tannins, particularly the condensed tannins which bind the proteins irreversibly and if fed to animals, are capable of corroding the epithelial lining of the gastrointestinal tract. So, tree forages with considerably lesser amounts of tannins or after employing simple de-tannification methods may be mixed with the protein cakes and fed to the animals for imparting bypass effect. The method of de-tannification of tree leaves employed may be biological or biotechnological approaches such as water soaking, chopping or chopping and water soaking (Ajith *et al*., 2014).

### 3. Feeding high amounts of proteins

This is a relatively simpler technique. When a relatively soluble protein meal is fed in very high quantities, some of the protein escapes the rumen because of the rapid movement of digesta out of the rumen. The amount of by-pass protein can be as high as 30% of the total protein in the feed if this is highly digestible (Nolan and Leng, 1989). But this method is not widely recommended, as the cost of production per Kg. of milk produced will remain on a higher side.

### Effects of supplementation of by-pass protein

Supplementing a diet of crop residues fed to cattle with a by-pass protein improves the Protein:Energy ratio in the nutrients absorbed. This has a large influence not only on the level of production but also on the efficiency of feed utilisation (i.e. the amount of feed required per unit of milk production or growth, is lowered). Thus, essentially the feeding of protected protein increases the efficiency of protein and energy utilization within the ruminant system. Positive results have been obtained from various numbers of studies conducted on feeding of naturally occurring protected protein like cottonseed cake and maize gluten-meal to lactating ruminants.

### NATURAL BYPASS FATS/PROTECTED FAT

During early lactation, high producing dairy animals remain in considerable negative energy balance leading to metabolic stress and high milk production. Addition of concentrates at higher level in ration of high producing dairy animals as a strategy for enhancing energy density of ration decreases fiber digestibility and leads to acidosis. Although, dietary fat has great potential to enhance energy density of the ration, there are various factors which limit its use in large amounts in ration. The extent of hydrolysis of these dietary FFA in rumen is very high (85-95%), which causes reduction of the fiber digestibility. It may be due to coating of the fibrous portion of the diet with the lipids thereby preventing attack by the microorganisms.

Role of bypass fats in the rations of the high producing dairy animals is very crucial for enhancing the energy density of ration (NRC, 2001). Dietary fat, that resists lipolysis and bio hydrogenation in rumen by rumen micro organisms, but gets digested in lower digestive tract, is known as bypass fat or rumen protected fat or inert fat.

**Natural bypass fats**

Whole oil seeds, when fed without processing except drying have natural bypass fat properties due to their hard outer seed coat, which protects the internal fatty acids from lipolysis and bio-hydrogenation in rumen. However, during mastication by animals there is physical breakdown of seed coat, which gives poor result of rumen inertness. Important whole oil seeds commonly used in the ration of dairy animals are cotton, roasted soybeans, sun flower and canola. Further, feed ingredients containing saturated fatty acids are less toxic to the ruminal micro organisms and minimize the adverse effects of the fat supplementation as they react more readily with the metal ions forming insoluble salts in rumen and do not go for further ruminal bio-hydrogenation. The main sources of Short Chain Fatty Acids (SCFA) are cottonseed oil and palm oil.

**Methods of fat protection:**

**Crystallisation or prilled fatty acids**

Prilled fats are non-hydrogenated vegetable oil and contain more than 85% palmitic acid with high melting point. It does not melt at low pH, by pass rumen degradation and is digested in small intestine by lipase enzymes. It is prepared by liquefying mixture of fatty acid by spraying it under pressure into a cooled atmosphere. Prillfat remain inert in the rumen and resist hydrolysis and association with the bacterial cells of feed particles. Thus total supplemented energy in diet of a lactating animal will be available for the productive process. Crystalline or prilled fatty acids can be made by liquifying and spraying the saturated fatty acids under pressure into cooled atmosphere, so that melting point of the fatty acids is increased and do not melt at ruminal temperature, thus resisting rumen hydrolysis and association with bacterial cells or feed particles.

**Performance of dairy animals fed with bypass fat**

Study in lactating dairy animals showed significant increase in milk yield and milk fat content by feeding rumen protected fat. Bypass fat in the form of calcium salts of fatty acids (Palm oil and others) has been known to increase energy density of the ration without adversely affecting the DM intake and digestibility (Naik *et al*. 2009) and also help to increase milk yield (2) and milk fat percentage or both. The positive effect of feeding prilled fats was more evident at the early lactation in buffaloes.

**Organic mineral mixtures (OMM)**

Trace minerals plays a significant role in enhancing the milk production and reproductive status of an animal. These inorganic elements play critical roles in the proper functioning of enzymes, hormones and cells. Deficiencies can result in performance issues such as compromised rates of gain, milk production and reproduction. The use of trace minerals as such in organic farming is disputed. Providing high-quality wholesome products that meet stringent consumer demand is a consistent goal among dairy producers. Dairy producers producing milk under certified-organic management practices require products manufactured under stringent requirements to meet this objective. But an important fact need to be known is that, by organic farming, it implies an ecological production management system that promotes and enhances biodiversity, biological cycles and soil biological activity. The principal guidelines for organic production are to use materials and practices that enhance the ecological balance of natural systems and that integrate the parts of the farming system into an ecological whole.

The organic trace minerals are manufactured in such a way as to provide natural protection and are more likely to be absorbed and used. They do not interact with antagonists or vitamins. Inorganic trace minerals, however, can readily interact. These interactions leave inorganic trace minerals bound and unavailable to the animal. Also, organic trace mineral

supplementation could improve production and reproduction in lactating dairy cows.

**Area specific organic mineral mixtures (ASOMM)**

ASOMM supplementation is the cost effective way of supplementing minerals to animals, based on the deficiency of minerals in different agro-climatic zones. Studies have shown that organic minerals improve the bioavailability and absorbance of a particular mineral than the inorganic ones. Blindly feeding the animals with common type of mineral mixtures could sometimes lead to deleterious effects, as some of the minerals may be present in excess amounts in traditional mineral mixtures. Supplementing animals with the most deficient minerals in organic form through ASOMM not only increases the level of production, but also helps to complies with the norms stipulated for organic milk production.

It reduces the volume of mineral mixtures to be fed to the animal. This approach has been found to improve the reproductive efficiency of crossbred cattle under field conditions. Supplementing the limiting micronutrients in various feed and fodder by ASOMM not only allows the farmer to have flexibility in feeding, but also improves the usability of the same.

**Conclusion**

Dairying is acknowledged as the major instrument in bringing about socio-economic transformation of rural poor in India. This can bring about a socio-economic transformation of rural poor in the country. Rapid urbanization Adoption of newer technologies is an essential requirement to achieve this goal. Farmers in resource-constrained countries traditionally use few external inputs, such as allopathic medicines and antibiotics, and follow grazing-based extensive or semi-intensive production systems. In many ways, they are thus closer to organic farming systems, though largely by default. Organic livestock farming is still evolving and it will take some time to become sustainable on its own, using organic methods without depending on the artificial or chemical products used in conventional livestock production. This underscores the need for more research on

organic alternatives, including medications, feeds and feeding practices that are compatible with organic management practices and standards.

Most of the methods described are traditionally used by many rural farmers, either to increase the digestibility or palatability of the feed resources available locally. However, a lack of appropriate agro-ecological knowledge means that they fail to gain most of the environmental, social and economic benefits of organic management, which translate into ecological intensification. The fact that most organic markets and consumers are in developed countries and are prepared to pay a premium for organic products makes organic farming a niche area with excellent prospects for exports. In developing countries like India, supplementation of protected organic fat and protein is beneficial to medium and high yielding cows and buffaloes but the cost effectiveness of the same needs to be kept in mind. Fodder resources management and livestock management are mutually complementary and should go hand in hand for livestock development so that maximum advantage can be derived from minimum amount of resources available. These organic feeding strategies, not only helps the farmers to gain valuable income, but also improves the quality of milk and health of the animal.

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