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INTERACTIONS BETWEEN ANGIOTENSIN II AND PPAR-γ IN CARDIAC MYOFIBROBLASTS

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ABSTRACT

Alterations in Angiotensin II (Ang II) and Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ) affect cardiovascular diseases, diabetes and metabolic disorders, suggesting that these two systems intersect at some common biochemical pathways. Elucidating common cellular signaling factors between these two systems are important for better design of combinatorial drugs for treating patients with these disorders. Inhibitors of Ang II and agonists of PPAR-γ appear to have similar and beneficial effects. Ang II, a pro-fibrotic factor, enhances collagen synthesis and contributes to adverse remodeling in case of hypertensive heart diseases that damage tissue structure and alter cardiac function. In contrast, increased PPAR-γ activity inhibits collagen synthesis, reduces fibrosis and adverse remodeling of the cardiac tissue. Treatments with Ang II blockers or with PPAR-γ agonists improve cardiac function. These effects are evident in cardiac myofibroblasts, which appear at the site of myocardial infarction. Cardiac myofibroblasts are the major cell types involved in tissue repair and remodeling. In this article, we review Ang II and PPAR-γ pathways operative in cardiac myofibroblasts and provide evidence for interactions between these two systems.

INTRODUCTION

Cardiac remodeling generally refers to alterations in the structure and architecture of left ventricular heart under pathological conditions [1]. The process is maladaptive as in the case of hypertension, myocardial infarction (MI) and heart failure, which subsequently leads to death. At cellular level, cardiac remodeling is characterized by hypertrophy of cardiac myocytes and excessive production of extracellular matrix proteins (ECM) by cardiac

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fibroblasts and myofibroblasts. Historically, the role of cardiac myocytes, the muscular compartment of the heart, has been extensively studied both *in vitro* and in animal models. In contrast, the role of cardiac fibroblasts, major non-muscular compartment of the heart, has been appreciated only in recent years [2-5]. Cardiac fibroblasts account for two thirds of the cells in the heart. Until recently, they were considered quiescent, monotonous and uniform cells, but considerable heterogeneity in fibroblast population is now being recognized. Cardiac fibroblasts secrete cytokines, growth factors and ECM proteins. They are mainly responsible for ECM homeostasis, maintenance of structural integrity and physiological environment in which cardiac myocytes function. They provide a mechanical scaffold for cardiomyocytes and coordinate the pump function of the heart. In pathological conditions, cardiac fibroblasts play an important role in regulating cardiac function. In response to injury, cardiac fibroblasts differentiate into a myofibroblast phenotype. Cardiac myofibroblasts acquire smooth muscle cell markers such as α-smooth muscle actin and become contractile [6-8]. They act as both paracrine and autocrine cells, producing growth factors, cytokines, pro-inflammatory and pro-remodeling factors. Cardiac myofibroblasts are proliferative, invasive and possess contractile and motile properties, allowing them to migrate to the site of injury and repair tissue damage. Under normal circumstances, cardiac myofibroblasts undergo apoptosis and disappear from the normal functioning heart. However, under pathological conditions due to continued stress on the heart, they fail to undergo apoptosis, producing excessive collagens and other ECM proteins [3, 5]. Thus, remodeling becomes excessive, maladaptive and causes considerable alterations in the structure and physiology of heart. These changes profoundly influence the function of cardiac myocytes, leading to heart failure and death. Consistent with the central role cardiac fibroblasts play in cardiac remodeling, several classes of drugs, which exert profound effects on cardiac fibroblasts are currently prescribed for treatment of cardiovascular diseases [3]. These include drugs that target Angiotensin II (Ang II) and peroxisome proliferator-activated receptor-γ (PPAR-γ), among others. Our understanding of various biochemical pathways affected by these drug targets largely comes from studies carried out with *in vitro* cultured cardiac fibroblasts and myofibroblasts. In this article, we will summarize recent findings on cross-regulatory interactions between Ang II and PPAR-γ pathways in cardiac fibroblasts and myofibroblasts.

Angiotensin-II (Ang II)

Ang II is the main mediator of the renin-angiotensin-aldosterone system (RAAS) [9, 10]. Ang II is first produced as biologically inactive Angiotensinogen (Ao), which is converted to Ang I by the action of aspertyl or serine proteases. Angiotensin converting enzyme (ACE), a membrane bound ectoenzyme, cleaves Ang I to biologically active Ang II peptide ([11-13]. A second enzyme, ACE2, further cleaves Ang II to a heptapeptide referred to as Ang-(1-7). Ang-(1-7) may antagonize effects of Ang II on cardiac remodeling and thus play a regulatory role. The effect of Ang II and Ang-(1-7) peptides are mediated through specific G-protein coupled receptors (GPCR). Ang II acts through Angiotensin II type 1 (AT1) and type 2 (AT2) receptors; whereas, Ang-(1-7) acts through another GPCR receptor, referred to as Mas, but its biological effects are unclear [14, 15]. Although Ang II, Ang-(1-7) and their receptors have been detected in fibroblasts at the site of fibrosis, most of the effects of RAAS system are mediated by Ang II through the AT1 receptor mediated signaling pathways.

Local RAAS systems are integral to the orderly and sequential nature of the repair process occurring in response to tissue injury [16]. Several lines of evidence suggest a central role for Ang II in the initiation and progression of cardiac remodeling [10, 17]. *De novo* production of Ang II significantly influences the post-infarction remodeling of the heart [9]. Drugs that inhibit RAAS activation have beneficial effects on the remodeling heart [18-19]. Clinical studies have shown that ACE inhibitors and angiotensin II type 1 receptor blockers (ARB's) reduce mortality and morbidity in patients with post-MI dysfunction and heart failure [6-8, 18, 20]. Ang II appears to have multiple, direct cytotoxic effects on myocytes including apoptosis, promoting cell hypertrophy and stimulating myocardial fibrosis [21].

Many of the effects of Ang II on cardiac remodeling are mediated through cardiac fibroblasts and myofibroblasts. Although activated macrophages and endothelial cells also produce Ang II and contribute to the initial process of remodeling, cardiac fibroblasts remain a major target cell type for Ang II mediated biological actions associated with cardiac remodeling [10, 17]. Consistent with this view, the distribution of AT1 receptors is predominant among cardiac fibroblasts compared to other cell types, including cardiomyocytes. The levels of cardiac fibroblast Ang II and AT1 receptors are elevated in post-MI hearts. Myofibroblasts remain the major cells expressing ACE and AT1 receptors in the fibrogenic stage of cardiac repair following MI [22, 23]. Both Ang II and AT1 receptor are expressed in cardiac myofibroblasts isolated from the site of MI [24, 25]. Myofibroblasts associated with the healing of infarct scars show increased production of Ang II and AT1 receptor [24, 26-29]. The levels of ACE are also elevated at the site of MI, co-localized with the myofibroblast population [11, 23, 30, 31].

Ang II influences multiple biochemical pathways in cardiac fibroblasts and myofibroblasts during remodeling (Figure 1). Ang II induces proliferation of myofibroblasts, which in turn participate in collagen synthesis [25, 32-35]. In a rat model of MI, Ang II induced hypertrophy of myofibroblasts at the infarct site. In sham operated control and non-infarct myofibroblasts, mitogenic activity of Ang II treatment was also detected [36]. There are several discrepancies in regards to the proliferative effects of Ang II, but a general consensus is that perhaps some of these effects are secondary due to the induced secretion of other growth factors, including TGF-β1, FGF2 and platelet derived growth factor-AA [10, 37-38].

Ang II induces differentiation of both neonatal and adult cardiac fibroblasts into myofibroblasts by activating TGF-β1 [28, 39-41]. Treatment of cardiac myofibroblasts *in vitro* with Ang II results in rapid accumulation of TGF-β1 mRNA and protein [28, 42-45]. Chronic administration of Ang II induces cardiac expression of TGF-β1 *in vivo* [46-48]. In contrast, blockade of AT1 receptor by antagonists reverses cardiac TGF-β1 expression, cardiac hypertrophy and fibrosis [48-51]. Ang II fails to induce cardiac hypertrophy and fibrosis in mice lacking in functional TGF-β1 [52]. Most of the effects of Ang II on cardiac function are likely mediated through TGF-β1 signaling [41]. Treatment of cardiac fibroblasts with anti-TGF-β1 antibodies reduces Ang II induced collagen I levels [52]. The mechanism by which Ang II induces TGF-β1 expression in cardiac myofibroblasts is not completely understood. However, studies in cardiomyocytes of hypertrophied left ventricle indicate that Ang II regulates TGF-β1 expression at the level of transcription through nuclear activating protein 1(AP1) binding activity [48]. Ang II mediates its effect through AT1 receptor, which in turn activates NAD(P)H oxidase, protein kinase C (PKC), p38 MAP kinase and finally AP1 protein [41, 53].

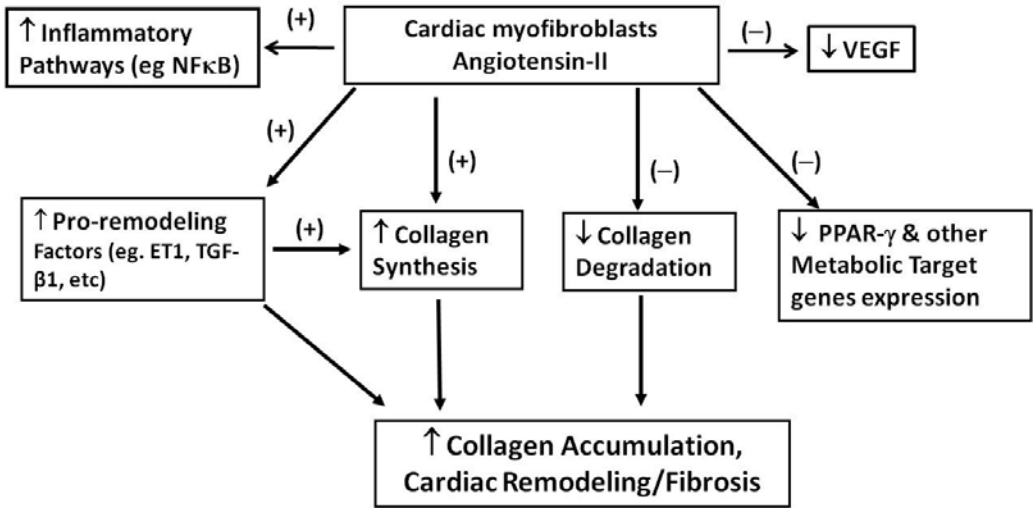

Figure 1. Effect of Ang II on various pathways operative in cardiac myofibroblasts.

Besides inducing TGF-β1, Ang II also induces secretion of other bioactive molecules such as endothelin-1, ET-1 [15, 40, 54, 55], leukemia inhibitory factor, LIF [15], osteopontin [56] vascular endothelial growth factor, VEGF [57], natriuretic peptides [53, 57, 58], and interleukin-6 [60].

Ang II promotes differentiation and proliferation of cardiac fibroblasts. It accelerates pro-fibrotic responses as indicated by increased production of ECM proteins. Ang II treatment increases synthesis of collagens I and II, fibronectin and integrin [15, 36, 42, 61-66]. Ang II is also known to increase the synthesis of tissue inhibitor of metalloproteinase (TIMP) and reduce metalloproteinase (MMP) synthesis thereby inhibiting degradation of ECM proteins (65, 66, 67-69]. Many of these effects are probably indirect and mediated by induction of other pro-remodeling factors, such as ET1 and TFG-β1.

Ang II, also a potent pro-inflammatory agent, influences all stages of the inflammatory response. Through interaction with its AT1 receptor, Ang II activates NADPH oxidase and production of reactive oxygen species. This leads to activation of Ets-1, early growth response 1 and nuclear factor-κB(NF-κB). Some of the inflammatory responses by Ang II are mediated by the caspase-recruitment domain-9 (CARD-9) protein [70]. Ang II also induces known mediators of inflammatory responses such as interferon-γ, INF-γ, and tumor necrosis factor-α, TNF-α [71].

In summary, cardiac myofibroblasts elaborate Ang II mediated multiple signaling pathways at the site of MI. These Ang II induced pathways result in pro-inflammatory and pro-fibrotic effects leading to increased synthesis and accumulation of collagen, a hallmark of fibrosis in MI and resulting heart failure.

PPAR-γ

In recent years, emerging evidence from different experimental systems suggest interactions between a xenobiotic activated transcription factor, peroxisome proliferators-activated receptor, PPAR-γ and various molecules that contribute to the remodeling process.

Developing tools and reagents to manipulate PPAR-γ activity may represent a new paradigm in our understanding of cardiac remodeling. PPARs are implicated in the regulation of pathways that interface between intermediate metabolism and inflammation [72]. PPAR-γ plays a critical role in adipocyte differentiation, cellular energy homeostasis, insulin signaling, obesity and inflammation [72-75]. PPAR-γ agonists, such as thiazolidinediones (TZDs) that sensitize cells to insulin are used for their antidiabetic effects in liver, adipose tissue and skeletal muscle [73]. Diabetic patients treated with PPAR-γ agonists also show lower risks for CVD, demonstrating a potential beneficial role for PPAR-γ in modulating cardiac function. Consistent with this hypothesis, treatment with PPAR-γ agonists results in reduced infarct size [76-77], decreased inflammation [74,75], improved vasodialation [76] and decreased left ventricular hypertrophy [78,79]. Thus, PPAR-γ agonists are likely to promote several cellular responses relevant to tissue repair and remodeling.

PPAR-γ agonists could modulate several aspects of cardiac remodeling by affecting the activities of key players, especially in cardiac myofibroblasts (Figure 2). PPAR-γ agonist's show marked influences on collagen synthesis at several levels.

When cultured myofibroblasts are treated with rosiglitazone, a decrease in collagen expression is observed [80]. The molecular mechanism, by which collagen synthesis in cardiac myofibroblasts is reduced, is yet to be elucidated. The effect of PPAR-γ on collagen synthesis could be mediated by decreasing the activities of pro-fibrotic factors including Ang II, ET1, and TGF-β1.

When cultured cardiac fibroblasts and myofibroblasts are treated with PPAR-γ agonists such as rosiglitazone or pioglitazone, there is a decrease in both AT1 receptor and the type I collagen expression [80, 81-83]. Rosiglitazone has been shown to significantly attenuate Ang II induced proliferation of cardiac fibroblasts [83]. The mechanism by which PPAR-γ agonists decrease AT1 receptor mediated collagen synthesis is unknown. Ang II and AT1 receptor induce oxidative stress in mouse cardiac fibroblasts. The inhibitory effect of pioglitazone on collagen synthesis is perhaps due to its anti-oxidant effect [82]. In vascular smooth muscle cells, PPAR-γ has been shown to suppress AT1 receptor gene transcription by the inhibition of the Sp1 binding to GC-box related element in the AT1 receptor cis-regulatory/promoter region [84]. Whether a similar mechanism is operative in cardiac myofibroblasts remains to be determined.

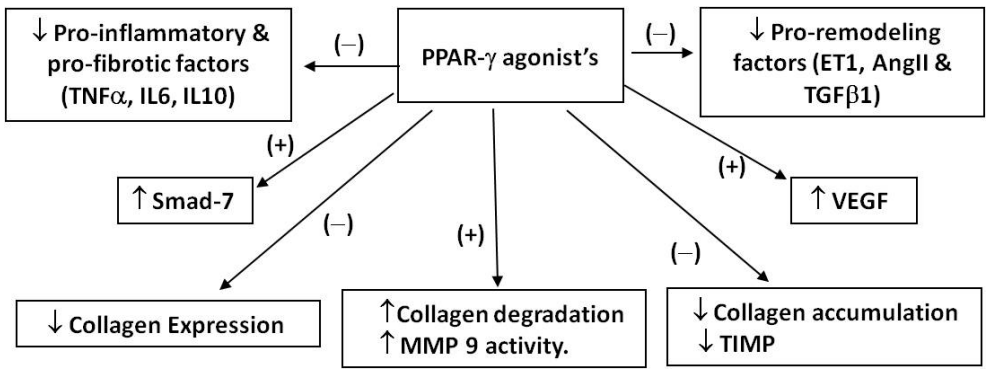

Figure 2. Effect of PPAR-γ agonists on cardiac collagen turn over, pro-angiogenic and pro-fibrotic factors.

Rosiglitazone reduces ET1 expression and collagen synthesis in cultured cardiac myofibroblasts derived from the site of MI (Katwa unpublished observations). The mechanism by which PPAR-γ inhibits ET1 activity in cardiac myofibroblasts remains to be studied. However, in cardiac myocytes, both PPAR-γ overexpression and treatment with rosiglitazone have been shown to inhibit ET1 induced cardiac hypertrophy [85]. Interaction between PPAR-γ and calcineurin/ nuclear factor of activated T cells (NFAT) has been implicated in the inhibition of ET1 activity. PPAR-γ has also been suggested to mediate conjugated linoleic acid mediated inhibition of ET1 activity in reducing cardiac hypertrophy [86]. Treatment of cardiac myofibroblasts with rosiglitazone also results in a decrease in TGF-β1 levels (Katwa, unpublished observations). The effect of rosiglitazone on TGF-β1, however, may not be direct but mediated through its effect on Ang II [6, 80, 83]. The effect of TGF-β1 on collagen synthesis is mediated through the canonical Smad pathway [87]. Smads interact with transcriptional coactivator and histone acetyltransferase, p300, in the regulation of transcriptional activity of collagen promoter [88, 89]. PPAR-γ abrogates Smad-dependent pathways linked to stimulation of collagen synthesis by preventing p300 recruitment and histone H4 hyperacetylation in dermal fibroblasts [88]. In hepatic stellate cells, PPAR-γ inhibits p300 facilitated NF-1 binding to response elements present in the collagen promoter [90]. Although p300 is known to play a role in inflammation as well as cardiac hypertrophy, its role in the regulation of collagen synthesis in cardiac myofibroblasts is yet to be described.

PPAR-γ is known to antagonize TGF-β1stimulated type 1 collagen synthesis in other fibroblast systems by antagonizing the effects of interferon-γ (IFN-γ). PPAR-γ could also increase activities of matrix metalloproteinases, which promote collagen degradation. PPAR-γ agonists appear to cause a decrease in the levels of MMPs in various experimental systems [81, 92, 93]. A similar decrease in the levels of MMPs and TIMPs was observed in cardiac myofibroblasts isolated from site of MI treated with rosiglitazone (Katwa, unpublished observation).

INTERACTIONS OR CROSSTALK BETWEEN ANG II AND PPAR SYSTEMS

Both Ang II and PPAR systems are implicated in metabolic syndrome and heart diseases, where they seem to have opposing effects. Increased frequency of left ventricular dysfunction and structural abnormalities are often observed in patients with metabolic syndrome [94-96]. In contrast, animal models of metabolic syndrome often show cardiac fibrosis [94, 97, 98]. Clinical strategies for treating both metabolic syndromes and cardiac failures often employ ACE inhibitors, ARB and PPAR agonists. Although molecular mechanisms that link fibrosis and metabolic syndrome are yet to be elucidated, several studies suggest a link between Ang II and PPAR systems [70, 80, 94, 99, 100]. Ang II and PPAR-γ mediated pathways intersect and modulate each other's activities in regulating cardiac collagen expression (Figure 3).

In cardiac myofibroblasts, treatment with Ang II leads to decrease in PPAR-γ expression and activity, whereas treatment with ARB (losartan) leads to increase in PPAR-γ expression and activity (Figure 4). In contrast, treatment with PPAR-γ agonist's leads to a decrease in AT1 receptor expression and activities in cardiac myofibroblasts isolated from the site of MI [80].

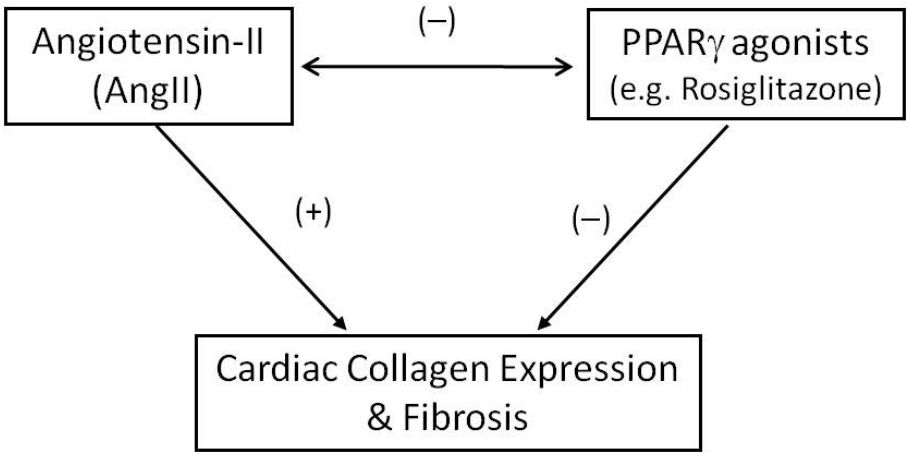

Figure 3. Cross talk between Ang II and PPAR-γ in post-MI cardiac collagen expression and fibrosis.

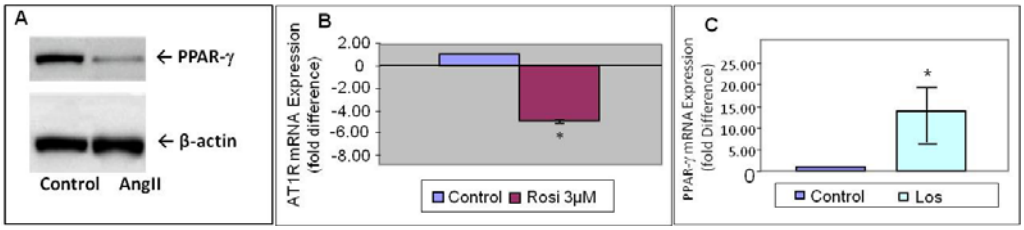

Figure 4. Cross talk between Ang II and PPAR-γ in cardiac myofibroblasts. Myofibroblasts (passage 4, 90% confluent) were treated in serum free DMEM medium with; (A) Ang II (10^{-7}M) for 24 hours and isolated protein (30μg/well) was analyzed by western blot resulted in the down regulation of PPAR-γ expression. Panel B &C are real-time PCR data of myofibroblasts treated with PPAR-γ agonist Rosiglitazone (3 μM). (B) demonstrates down regulation of AT1 receptor expression by PPAR-γ agonists and (C) results show effects of ARB (Los=Losartan) on myofibroblasts PPAR-γ expression
(*p> 0.05).

By mechanisms yet to be elucidated, these interactions affect collagen expression in cardiac myofibroblasts either directly or indirectly. Pro-inflammatory and pro-fibrotic factors could mediate some of the effects of Ang II and PPAR systems. Ang II decreases PPAR-γ expression with a concomitant increase in the expression of pro-inflammatory and pro-fibrotic agents in diabetics with MI and cardiac myofibroblasts [5, 6, 101, 102]. In contrast, PPAR-γ agonists appear to decrease expression of pro-inflammatory and pro-fibrotic agents, including Ang II in cardiac myofibroblasts, and in diabetic heart tissue with MI [80,103, Katwa et al., unpublished data]

Combination of drugs such as PPAR-γ agonists and ARBs have shown beneficial effects in diabetes and CVD by decreasing infarct size and increasing metabolic activities of the cardiac tissue [104, 101,102]. Cardiac myofibroblasts treated with both PPAR-γ agonists and ARBs demonstrated significant decrease in not only collagen expression but also decreased levels of pro-inflammatory and pro-fibrotic agents. Our recent experimental findings on the

effects of combination treatment with ARB (e.g. Lasortan) and PPAR-γ agonists (e.g. Rosiglitazone or Pioglitazone) demonstrated an increased PPAR-γ expression and decreased AT1 receptor expression and activity, as well as a significant reduction in collagen expression in cultured rat cardiac myofibroblasts isolated from the site of MI [80].

PPAR-γ agonists have recently been used to treat patients with diabetes and MI. These studies have revealed increased cardiovascular risks and mortality [95, 97, 98, 105]. The clinical relevance of combination therapy of ARBs and PPAR-γ agonists has yet to be determined. Since last decade, the experimental evidence from animal and clinical studies suggest that some of the drugs that interrupt the Ang II system may improve glucose and lipid metabolism and decrease the risk for type 2 diabetes [101,102, 104, 106, 107]. Some of the anti-diabetic properties of ARBs may be mediated by their ability to block adverse effects of Ang II on carbohydrate and lipid metabolism. The beneficial metabolic effects of certain ARBs may go well beyond just simple interruption of the Ang II action, as ARBs have been found to effectively activate PPAR-γ, a well-known target for insulin-sensitizing, antidiabetic drugs [102, 104]. Thus, the identification of ARBs selective PPAR-γ modulating ability suggests new opportunities for developing third-generation ARBs and PPAR-γ activators with enhanced potential for treating hypertension, diabetes and the metabolic syndrome.

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DNA REPAIR AS THE PRIMARY ADAPTIVE FUNCTION OF SEX IN BACTERIA AND EUKARYOTES

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ABSTRACT

The essential feature of sex common to both bacteria and eukaryotes is information exchange (recombination) between two genomic DNA molecules derived from different individuals. In bacteria a naturally occurring sexual process termed transformation is characterized by transfer of DNA from one bacterium to another, followed by recombination between the resident DNA and the incoming DNA. In eukaryotes, the sexual cycle involves recombination between paired DNA molecules (chromosomes) in which the two DNA molecules are derived from two different parents. This process occurs in diploid cells during meiosis, and is followed by formation of haploid gametes. The fusion of gametes from different individuals to generate diploid progeny completes the eukaryotic sexual cycle.

DNA damage appears to be a fundamental problem for life. Here, we present evidence for the view that the enzymatic machinery for carrying out recombination, during transformation in bacteria and meiosis in eukaryotes, is an adaptation for DNA repair. Both bacterial and eukaryotic cells, generally, contain repair enzymes that are adept at removing DNA damages. However there are limits to the capability of cells during ordinary cell divisions to repair their DNA. Damages causing loss of information in both DNA strands (double-strand damages) are particularly difficult to handle since they require information from a second homologous chromosome. A repair process referred to as homologous recombinational repair (HRR) appears designed to carry out repair of such double-strand damages.

Evidence is presented for the hypothesis that sexual processes (i.e. transformation in bacteria and meiosis in eukaryotes) are maintained as evolutionary adaptations to facilitate HRR. In bacteria and microbial eukaryotes the problem of DNA damages is particularly acute during periods of stress, especially oxidative stress. It is during such stressful conditions that sex (which facilitates HRR) tends to occur in these facultatively sexual organisms. In multicellular eukaryotes, meiosis facilitates HRR and appears to be designed to protect against damages to the DNA of gametes, thus minimizing infertility and defective progeny.

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Another consequence of recombination during sex is increased genetic variation among progeny. Increased variation appears to have significant consequences at the population level over the long-term and is sometimes considered the primary adaptive function of sex. However, we present evidence that the machinery for sexual recombination is maintained primarily by the strong short-term advantage of passing relatively undamaged DNA from one generation to the next. The genetic variation that is generated appears to be a byproduct of HRR.

INTRODUCTION

The adaptive function of sex in bacteria and eukaryotes is widely regarded as a major unsolved problem in biology. However, many investigators in this field consider that understanding the benefit of the exchange of information (recombination) during the sexual process is the key to understanding the adaptive function of sex.

In this chapter, we present evidence from diverse fields (i.e. molecular and cellular biology, microbiology, and ecology and evolutionary biology) bearing on the adaptive function of sex in bacteria and eukaryotes. On the basis of this evidence, particularly recent findings, we propose that DNA repair is the primary adaptive function of sex.

DNA DAMAGE: A BASIC PROBLEM FOR LIFE

DNA damage appears to be a ubiquitous problem for organisms with a DNA genome. As observed by Haynes (1988), DNA is made up of rather ordinary molecular subunits that are not endowed with any peculiar kind of quantum mechanical stability. He noted that its very "chemical vulgarity" makes DNA vulnerable to all the "chemical horrors" that might befall any such molecule in a warm aqueous medium. These chemical horrors are DNA damages, such as a break in a DNA strand or an adduct molecule added to one of the DNA bases. DNA damages are distinct from mutations. DNA damages are structural and chemical alterations in the DNA, whereas mutations ordinarily involve the normal four bases in new arrangements. Mutations can be replicated when the DNA replicates, in contrast to DNA damages that are altered structures that cannot be replicated.

DNA damages are subject to several different repair processes, but those DNA damages that escape repair can have detrimental consequences. DNA damages may block replication or transcription, leading to cell death. Alternatively, during DNA replication, when a DNA polymerase uses a template DNA strand containing a damaged site, it may bypass the damage and in the process generate a mutation.

In bacteria and eukaryotes, the DNA genome is assaulted both by reactive chemicals naturally present in the intracellular environment and by agents from external sources. A major internal source of DNA damage in both bacteria and eukaryotes is reactive oxygen species (ROS) produced as byproducts of normal aerobic metabolism. For eukaryotes, oxidative reactions appear to be a major source of DNA damage (for review of oxidative damage in humans, see Sedelnikova et al., 2010). It is estimated that about 10,000 oxidative DNA damages occur per cell per day in humans (Ames et al., 1993). In the rat, which has a higher metabolic rate than humans, the number of oxidative DNA damages occurring per cell per day is about 100,000. ROS appear to be an important source of DNA damage in

aerobically growing bacteria, as indicated by the finding that 89% of spontaneously occurring base substitution mutations are caused by ROS-induced damages and then inaccurate replication past the damaged bases (Sakai et al., 2006). Most oxidative DNA damages affect only one of the DNA strands, but a fraction, about 1-2%, are double-strand damages such as double-strand breaks (DSBs) (Massie et al., 1972). In humans, it is estimated that the average number of endogenous DNA DSBs per cell occurring at each cell generation is about 50 (Vilenchik and Knudson, 2003). This rate of formation of DSBs likely reflects a natural level of damages, such as may be caused by ROS produced by active metabolism.

There are five major pathways employed in repairing different types of DNA damages. These are base excision repair, nucleotide excision repair, mismatch repair, non-homologous end joining and homologous recombinational repair (HRR) [see Bernstein et al. (2002) for review]). However, only one of these pathways, HRR, can accurately repair double-strand damages, such as double-strand breaks (DSBs). The HRR pathway requires the availability of a second homologous chromosome for recovering the information lost by the first chromosome due to the double-strand damage (Figure 1). As described below, in both haploid and diploid organisms, sex promotes the conditions needed for highly effective HRR of double-strand damages. In diploid organisms sex promotes effective repair without the expression or accumulation of recessive or nearly recessive deleterious mutations.

BACTERIAL TRANSFORMATION IS AN ADAPTIVE SEXUAL PROCESS

In haploid organisms, such as bacteria, sex is usually required for the presence in the cell of a second homologous template. Homologous gene transfer followed by recombination (sex) can occur by three recognized processes in bacteria. These processes are (1) bacterial virus (bacteriophage) mediated transduction, (2) plasmid-mediated conjugation and (3) natural bacterial transformation. Bacteriophage mediated transduction of bacterial genes appears to reflect an occasional mistake in assembly of virus particles, rather than an adaptation of the host bacteria. The transfer process is controlled by the bacteriophage's genes rather than by the host's genes. Plasmid-mediated conjugation is controlled by plasmid genes, and is an adaptation for distributing copies of the plasmid from one bacterium to another. Infrequently, during this process, the plasmid may become integrated into the host bacterial chromosome, and subsequently transfer part of the host chromosome to another bacterium. Plasmid mediated transfer of host bacterial DNA also appears to generally be an accidental process, rather than a bacterial adaptation.

Natural bacterial transformation involves the transfer of DNA from one bacterium to another through the surrounding medium. In contrast to the first two processes, transformation is clearly a bacterial adaptation for DNA transfer, since it depends on the expression of numerous bacterial genes whose products appear to be specifically designed to carry out this process (Chen and Dubnau, 2004). Transformation is a complex, energy requiring, developmental process. In order for a bacterium to bind, take up and recombine exogenous DNA into its chromosome, it must enter a special physiological state referred to as competence. In *Bacillus subtilis*, expression of about 40 genes is required for development of competence (Solomon and Grossman, 1996). Ordinarily, but with rare exceptions, the DNA integrated into the host bacterial chromosome is from another bacterium of the same species, and thus is homologous to the resident chromosome.

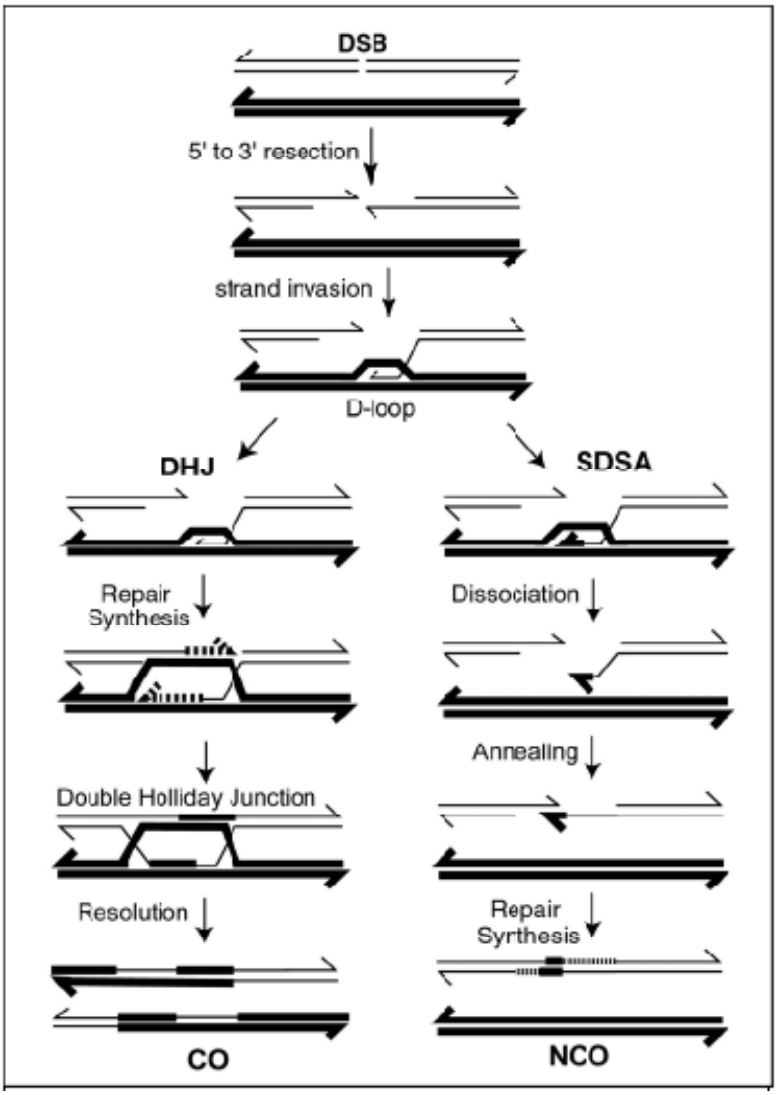

Figure 1. The initiating event in current models of meiotic recombination is formation of a double-strand break (DSB) or gap. This is followed by pairing of the damaged chromosome with a homologous chromosome, and then strand invasion to form a displacement loop (D-loop). Repair of the gap allows restoration of the lost information and can result in a crossover (CO) or non-crossover (NCO) of the flanking regions. CO recombination is considered to arise by the Double Holliday Junction (DHJ) model, shown on the left. NCO recombination is considered to arise mainly by the Synthesis Dependent Strand Annealing (SDSA) model shown on the right. The majority of recombination events during meiosis are likely of the SDSA type.

The amount of DNA transferred during a transformation event can be a substantial portion of the bacterial chromosome. In *B. subtilis* the transferred DNA can be as large as a third to the whole chromosome (Akamatsu and Taguchi, 2001; Saito et al., 2006). Transformation appears to be common in the bacterial world, and thus far 67 prokaryote species (in seven different phyla) are known to be naturally competent for transformation (Johnsborg et al., 2007).

COMPETENCE DEVELOPMENT IS USUALLY ASSOCIATED WITH STRESSFUL CONDITIONS

During logarithmic growth there can be two or more copies of any particular region of the chromosome in a bacterial cell, as cell division and DNA replication are not precisely matched. During this phase, HRR can occur between recently replicated sister chromosome regions. However, once cells approach stationary phase they typically have just one copy of the chromosome and HRR requires input of homologous template from outside the cell by transformation. Therefore it is no surprise that competence for transformation is typically induced by high cell density and/or nutritional limitation, conditions associated with stationary phase. In *Haemophilus influenzae*, transformation occurs most efficiently at the end of exponential growth as the bacteria approach stationary phase (Goodgal and Herriott, 1961). In *Streptococcus mutans*, as well as many other streptococci, development of competence for transformation is associated with high cell density and biofilm formation (Aspiras et al., 2004). In *Bacillus subtilis*, competence is induced toward the end of logarithmic growth, especially under conditions of amino acid limitation (Anagnostopoulos and Spizizen, 1961).

BACTERIAL TRANSFORMATION, AS AN ADAPTATION FOR DNA REPAIR

In this section we review evidence that competence is specifically induced by DNA damaging conditions, and that transformation functions as a DNA repair process employing HRR. The DNA damaging agents mitomycin C (a crosslinking agent) and fluoroquinolone (a topoisomerase inhibitor) induce transformation in *Streptococcus pneumoniae* (Claverys et al., 2006). Transformation in *B. subtilis* is enhanced by ultraviolet light, a DNA-damaging agent (Michod et al., 1988). In *Helicobacter pylori*, ciprofloxacin, which binds DNA gyrase and causes double-strand breaks in DNA, induces expression of competence genes, thus increasing transformation frequency (Dorer et al., 2010). Charpentier et al. (2011) tested 64 toxic molecules to determine which of these induce competence for natural transformation in *Legionella pneumophila*. Of these 64 compounds, only six, all DNA damaging agents, caused strong induction. These agents were mitomycin C (which causes DNA inter-strand crosslinks), norfloxacin, ofloxacin and nalidixic acid [each of which inhibits DNA gyrase leading to DSBs (Albertini et al., 1995)], bicyclomycin [which causes single- and double-strand breaks (Washburn and Gottesman, 2011; Tran et al., 2011)], and hydroxyurea [which induces DNA base oxidation (Sakano et al., 2001)]. Among the toxic molecules that did not induce competence were antibiotics that block cell wall or protein synthesis. UV light, another DNA damaging agent, was also tested and proved to induce competence in *L. pneumophila*. Charpentier et al. (2011) suggested that competence likely evolved as a DNA damage response.

To test whether the adaptive function of transformation is repair of DNA damages, a series of experiments was conducted using *B. subtilis* irradiated by UV light (Hoelzer and Michod, 1991; Michod et al., 1988; Michod and Wojciechowski, 1994; Wojciechowski et al., 1989; and reviewed in Michod et al., 2008). The experimental approach was to administer transforming DNA to cultures grown to competence either before exposing the cells to the

DNA damaging effects of UV radiation ("DNA-UV") or after UV radiation ("UV-DNA"). The basic result obtained was that after UV irradiation, survival of those cells that were transformed was greater than survival of the majority of cells in the culture that were not transformed. The enhanced survival of the transformed cells was only observed in the UV-DNA experiments in which the donor DNA was administered after the UV treatment (Michod et al., 1988). These results suggest that the transforming DNA acts to repair DNA damages introduced by UV in the recipient DNA, but is of no benefit for survival when the damages are introduced after transformation is completed. The increase in survival in the UV-DNA experiments occurred even if the transforming DNA was irradiated with UV at levels comparable to that given to the recipient cells (Hoelzer and Michod, 1991). This finding suggested that DNA damages introduced more-or-less at random in the donor DNA did not often correspond to the exact locations of the damages in the recipient DNA, and thus the transforming DNA could provide locally intact template for repair of most recipient DNA damages. In other experiments, H_2O_2, which causes oxidative DNA damage, was used and similar results to that obtained with UV were observed (unpublished results).

The results with *B. subtilis*, described above, are reasonably interpreted in terms of homologous recombinational repair (HRR), a well-established repair process in both bacterial and eukaryotic cells (described in Figure 1, and discussed further, below). HRR is unique among DNA repair processes in that it can accurately repair damages causing loss of information in both DNA strands [double-strand damages, such as double-strand breaks (DSBs) and inter-strand crosslinks]. HRR requires, in addition to the damaged DNA molecule, a second homologous DNA molecule containing an intact sequence corresponding to the damaged sequence in the first DNA molecule, so that information can be exchanged between the two. As already mentioned, *B. subtilis* cells typically become competent in late-log phase, at which time most cells are haploid (presumably because maintaining a second chromosome would be costly under conditions approaching starvation). Haploid, non-replicating cells thus lack a second homolog for HRR. However, homologous template DNA for HRR can be provided by transforming DNA. Thus the association of competence (required for bacterial sex) with haploidy is adaptive in terms of the need for DNA template for use in HRR.

SEX WAS PRESENT EARLY IN THE EVOLUTION OF EUKARYOTES

The evidence reviewed in this section indicates that facultative sex was present early in the evolution of eukaryotes. Since the earliest ancestor of eukaryotes had bacterial antecedents, continuity in evolution from bacterial sex to eukaryotic sex is a reasonable possibility. Evidence that eukaryotic meiosis is derived from bacterial transformation was reviewed in Bernstein and Bernstein (2010).

Eukaryotes emerged in evolution more than 1.5 billion years ago. Even though sexual reproduction is widespread among extant eukaryotes, it seemed unlikely, until recently, that sex is a primordial and fundamental characteristic of eukaryotes. The reason for this view was that sexual reproduction appeared to be lacking in early observations on certain human pathogenic single-celled eukaryotes that diverged from early ancestors in the eukaryotic lineage. However, several of these organisms are now known to be capable of, or to recently have had, the capability for meiosis and hence sexual reproduction. As one example, the

common intestinal parasite *Giardia intestinalis* had been thought to be a descendant of a lineage that predated the emergence of meiosis and sex. Contradicting this view, *G. intestinalis* was recently found to have a core set of meiotic genes, including five meiosis specific genes (Ramash et al., 2005). More recently, evidence for meiotic recombination, indicative of sexual reproduction, was found in *G. intestinalis* (Cooper et al., 2007). In a second example, parasitic protozoa of the genus *Leishmania*, which cause human diseases, have recently also been shown to have a sexual cycle (Akopyants et al., 2009).

Trichomonas vaginalis, a parasitic unicellular eukaryote is not known to undergo meiosis. However, when Malik et al. (2008) tested for the presence of 29 meiotic genes in *T. vaginalis*, they found 27, including 8 of 9 genes specific to meiosis in model organisms, suggesting that that capability for meiosis may have been present in its recent ancestors. Twenty-one of the 27 genes were also present in *G. intestinalis*, indicating that most of these meiotic genes were present in a common ancestor of these two species. Because these species are descendants of lineages that are highly divergent among eukaryotes, Malik et al. (2008) suggested that each of these meiotic genes were likely present in a common ancestor of all eukaryotes. Dacks and Roger (1999), based on a phylogenetic analysis, also proposed that facultative sex was present in the common ancestor of eukaryotes. A study of amoebae by Lahr et al. (2011) lent further support to this view. Although amoebae are generally regarded as asexual, these authors presented evidence that most amoeboid lineages are likely anciently sexual, and that the majority of asexual groups probably arose recently and independently.

THE SEXUAL CYCLE IN EUKARYOTIC MICROORGANISMS OCCURS UNDER STRESSFUL CONDITIONS

In eukaryotic microorganisms, as in bacteria, sex occurs under stressful conditions. Among extant unicellular or simple multicellular eukaryotes, sex is usually facultative. These organisms generally reproduce asexually under favorable environmental conditions, but reproduce sexually under stressful conditions. Starvation, desiccation, mechanical damage and heat shock are all known to induce sex in these organisms. For instance, the paramecium tetrahymena can be induced to undergo mating (conjugation) by starvation conditions brought about by washing (Elliott and Hayes, 1953). When the unicellular green alga *Chlamydomonas reinhardi* is grown in a medium depleted of a nitrogen source, vegetative cells differentiate into gametes (Sager and Granick, 1954). These gametes can then fuse, form zygotes and undergo meiosis. The haploid fission yeast *Schizosaccharomyces pombe* is a facultatively sexual microorganism that undergoes mating when nutrients become limiting (Davey et al., 1998).

THE SEXUAL CYCLE IN UNICELLULAR OR SIMPLE MULTICELLULAR EUKARYOTES AS A RESPONSE TO DNA DAMAGING CONDITIONS

We reviewed evidence, above, that sex was present early in the evolution of eukaryotes, raising the likelihood of continuity in the evolution of bacterial to eukaryotic sex. We also reviewed evidence, above, that bacterial sex (transformation) is an adaptation for repair of

DNA damage. In this section we cite evidence that, in simple eukaryotes, sex is induced under conditions of oxidative stress and may be an adaptation for repairing oxidative DNA damage. Thus sex may have been retained through the prokaryotic to eukaryotic evolutionary transition as an adaptation for repairing DNA damage, especially DSBs.

Life depends on a delicate balance of reduction and oxidation reactions. Various metabolic or environmental stress factors can upset this balance, leading to an increase in the cellular levels of reduced and highly reactive molecules, including a series of oxygen-containing compounds collectively termed reactive oxygen species or ROS. At high concentrations, ROS can be damaging to biological systems (i.e., oxidative stress), and cause DNA damage. A variety of adaptive responses have evolved to cope with the DNA-damaging effects of oxidative stress, including cell cycle arrest (CCA, allowing time for DNA repair) and programmed cell death (PCD, allowing for the elimination of damaged cells). It also has been known for some time that stress can trigger the sexual cycle in facultatively sexual species (Williams, 1975). As many forms of stress (temperature, drought, UV, pathogens) trigger an increase in cellular levels of ROS, it is reasonable to expect that the sex-inducing stress in facultatively sexual species might also result in ROS overproduction, and that sex in such species is an additional response to oxidative stress and its consequences such as DNA damage.

Oxidative stress causes a variety of DNA damages including modified bases, single-strand breaks and DSBs (Slupphang et al., 2003). To test whether oxidative stress is implicated the induction of sex in the yeast *S. pombe*, Bernstein and Johns (1989) treated late exponential phase *S. pombe* cells with hydrogen peroxide, which produces oxidative stress. This treatment increased the frequency of mating and formation of meiotic spores by 4 to 18-fold. *Phytophthora cinnamoni*, an oomycete fungus, also can be induced to undergo sexual reproduction by exposure to hydrogen peroxide (Reeves and Jackson, 1974). *Volvox carteri*, a simple, haploid, multicellular, facultatively sexual green algae, can be induced to reproduce sexually by heat shock (Kirk and Kirk, 1986). This induction can be inhibited by antioxidants, indicating that oxidative stress mediates the induction of sex by heat shock (Nedelcu and Michod, 2003). An inhibitor of the mitochondrial electron transport chain that induces oxidative stress also induced sex in *V. carteri* (Nedelcu et al., 2004).

During investigations of the role of ROS in stress-induced sex in the multicellular *V. carteri,* Nedelcu and Michod (2003) noted that the decline in the proportion of sexual progeny with increasing oxidative stress is paralleled by an increase in the proportion of gonidia that arrest and delay cleavage (i.e., the fast successive round of cell divisions that result in formation of the embryo). Many of these arrested gonidia develop into asexual rather than sexual embryos (Nedelcu and Michod, 2003). This pattern suggests the hypothesis that at higher doses of ROS cell-cycle arrest replaces sex as a stress-response. It was also noted that temperatures slightly higher than the one used for inducing sex, increased duration of the heat-stress (3 hours), or the addition of chemicals known to interfere with p53 (a main player in responses to DNA damage – including CCA and PCD in mammals), all result in a PCD-like response in gonidia (Nedelcu, 2006). This relationship between sex and two known responses to DNA damage (CCA and PCD), suggest a connection between sex and DNA damage.

Nedelcu and Michod (2003) and Nedelcu (2006) previously reported three kinds of data suggesting DNA damage is the cause of sex induction by ROS in *V. carteri.* (a) There is a bi-modal response pattern of sex to increasing stress (Nedelcu, 2006) similar to the pattern

observed for cellular responses to increasing doses of DNA-damaging H_2O_2. (b) An iron-chelator is effective at reducing the sex response, as is predicted by the DNA damaging effects of the iron-based Fenton reaction (Imlay et al., 1988). (c) The relationship between sex and two known responses to DNA damage, namely, CCA and PCD, imply a connection between sex and DNA damage. Nedelcu (2006) showed in *V. carteri* that sex-inducing stress, when performed in the presence of PFTα (a compound that interferes with p53 activity) triggers PCD in gonidia, which further suggests that DNA damage is involved in sexual induction (Achanta and Huang, 2004; Chlamygenome, 2007). In addition to these data indicating that DNA damage is the basis of ROS' sex-inducing capacity in *V. carteri*, known DNA damaging agents such as glutaraldehyde, formaldehyde and UV have been shown to induce sex in *V. carteri* (Loshon et al., 1999; Zeiger et al., 2005; Starr and Jaenicke, 1988).

The budding yeast *Saccharomyces cerevisiae* divides by mitosis when nutrients are plentiful, but when starved becomes arrested in the G1/G0 phase of the cell cycle. Under this condition oxidative stress increases and DNA DSBs and apurinic/apyrimidinic sites accumulate (Steinboeck et al., 2010). Cells arrested in the G1 phase of the cell cycle undergo meiosis to form haploid spores (Herskowitz, 1988). Since starvation causes oxidative stress and DNA damage in *S. cerevisiae*, the induction of sex by starvation may be mediated by these factors.

BACTERIAL TRANSFORMATION AND EUKARYOTIC MEIOSIS HAVE FUNDAMENTAL SIMILARITIES

Although transformation in bacteria and meiosis in eukaryotes are complex adaptations encoded by numerous genes, they share their central molecular processes and these processes are carried out by homologous enzymes. Bacterial transformation involves three major steps: (1) the entry of DNA from one "parent" into the cell of another parent; (2) the alignment and genetic recombination between non-sister homologous chromosomes (or parts of chromosomes) that are derived from the different parental bacteria; (3) the recombined chromosome is passed on to progeny bacteria. Meiosis occurs in diploid eukaryotic cells by three steps as well.

The key sequential steps in meiosis are (1) pre-meiotic DNA replication of chromosomes that originated from gametes from different parents (chromosomes inherited from the "mother" and the "father"; (2) pairing of non-sister homologs and recombination between them; and (3) two successive cell divisions leading to haploid gametes. When one gamete unites with another gamete (syngamy/fertilization), a diploid progeny is formed. As in transformation, in meiosis the central step (step 2) is the interaction of non-sister homologous chromosomes. This interaction in meiosis, as in transformation, involves the alignment of non-sister homologs followed by genetic recombination. In bacteria and eukaryotes, central to the exchange of genetic information between non-sister homologs is the RecA protein (in bacteria), and its orthologs (e.g. Rad51 and Dmc1) in eukaryotes. In the next section we discuss the key role of the RecA protein in bacterial transformation, and in the section after that we describe the similar central role of RecA orthologs in meiotic recombination.

ROLE OF THE RECA PROTEIN IN TRANSFORMATION

In *B. subtilis, S. pneumoniae* and *Neisseria gonorrhoeae* the RecA protein is essential for transformation (Dubnau et al., 1973; Martin et al., 1992; Claverys et al., 2009; Stohl et al., 2011). The RecA protein is induced during development of competence for transformation in *B. subtilis* (Cheo et al., 1992) and *S. pneumoniae* (Mortier-Barriere et al., 1998).

In *B. subtilis*, the RecA protein interacts with several competence proteins to form a multiprotein assembly. This assembly accepts incoming single-stranded DNA (ssDNA) at a cell pole, where the DNA is prepared for recombination with the resident chromosome (Kidane and Graumann, 2005). The incoming ssDNA interacts with the RecA protein to form striking filamentous structures. These filamentous structures emanate from the pole containing the competence machinery and extend into the cytosol. These RecA/ssDNA filamentous threads are considered to represent dynamic nucleofilaments that scan the chromosome for regions of homology. This brings incoming DNA to the corresponding site in the bacterial chromosome where informational exchange occurs (Kidane and Graumann, 2005).

Cox (1991; 1993; 2002) analyzed the molecular interactions of bacterial RecA protein with DNA. This analysis indicated that the RecA protein evolved as the central component of an HRR system for dealing with DNA damage, and that "DNA repair is the most important function of homologous genetic recombination". This conclusion suggests that, in bacterial transformation, the function of RecA is to facilitate repair of damaged DNA. We noted, above, that cells typically become competent in late log phase, when most cells are haploid. Thus the only available source of a second homologous DNA molecule for HRR is from another bacterium. During rapid log phase growth, after DNA replication but before cell division, a bacterial cell may have two homologs present, and thus be able to carry out HRR between sister homologs.

ROLE OF RECA ORTHOLOGS IN MEIOSIS OF UNICELLULAR EUKARYOTES

The HRR machinery appears to be highly conserved. Genes *rad51* and *dmc1* of the unicellular yeasts *S. cerevisiae* and *S. pombe* are orthologs of the bacterial *recA* gene. The *dmc1* gene has also been found in the unicellular eukaryotic microorganisms *Giardia*, *Trypanosoma*, *Leishmania*, *Entamoeba*, and *Plasmodium* (Ramesh et al., 2005). The Rad51 and Dmc1 proteins of yeast interact with ssDNA to form a filamentous intermediate, referred to as a presynaptic filament, that initiates recombination (Sauvageau et al., 2005; San Filippo et al., 2008). This filament is similar to the RecA/ssDNA nucleofilaments formed during bacterial transformation (described above). The Rad51 recombinase acts both during meiosis and mitosis, whereas Dmc1 acts only during meiosis (Bugreev et al., 2011). The tertiary structure of the yeast Dmc1 recombinase is, overall, similar to the bacterial RecA recombinase (Story et al., 1993). Like the bacterial RecA recombinase, the yeast Rad51 recombinase catalyzes ATP-dependent homologous DNA pairing and strand exchange (Sung, 1994). Thus the recombinases Rad51 and Dmc1 that act during meiosis in unicellular eukaryotes appear to be functionally and structurally similar to the RecA bacterial

recombinase that acts in transformation. This correspondence suggests continuity in the evolution of the sexual process from bacteria to unicellular eukaryotes.

ROLE OF RECA ORTHOLOGS IN MEIOSIS OF COMPLEX MULTICELLULAR EUKARYOTES

The evidence reviewed in this section indicates that orthologs of bacterial RecA play an important role in meiotic recombination in multicellular animals and plants, as they do in unicellular eukaryotes (previous section). Thus there appears to be evolutionary continuity of the central role of recA orthologs in sexual recombination in bacteria, unicellular eukaryotes and multicellular eukaryotes.

As in unicellular eukaryotes, Dmc1 and Rad51 play a key role in HRR during meiosis of complex multicellular eukaryotes. Orthologs of the RecA protein function in meiosis of animals (e.g. nematodes, mice and humans) and of plants (e.g. *Arabidopsis*, rice and lilies). In the nematode *Caenorhabditis elegans*, resistance to X-ray induced DNA damage in meiotic pachytene nuclei depends on the *rad51* gene that is strongly expressed in meiotic cells (Takenami et al., 2000), indicating the repair function of *rad51*. An ortholog of the *recA* gene is expressed at a high level in the testis and ovary of mice, suggesting that this RecA is involved in meiotic recombination (Shinohara et al., 1993). When the mouse *dcm1* gene is mutationally defective, the mice fail to undergo intimate pairing of homologous chromosomes, are unable to complete meiosis and are sterile (Pittman et al., 1998; Yoshida et al., 1998). In humans, Dmc1, the meiosis-specific recombinase, forms nucleoprotein complexes on ssDNA that promote a search for homology and informational exchange, the crucial steps of genetic recombination (Sehorn et al., 2004; Bugreev et al., 2005).

In the plant *Arabidopsis thaliana*, the functions of Rad51 (Li et al., 2004) and Dmc1 (Couteau et al., 1999) are required for meiosis. In the lily, proteins Rad51 and Lim15 (an ortholog of Dmc1) co-localize on meiotic prophase I chromosomes, forming discrete foci (Terasawa et al., 1995). The proteins of these foci are considered to cooperate in the search for, and the pairing of, homologous DNA sequences. In rice, an ortholog of Dmc1 plays a key role in the pairing of homologous chromosomes and is necessary for meiosis (Deng and Wang, 2007).

DNA DAMAGE INCREASES MEIOTIC RECOMBINATION

Classically, meiotic recombination has been identified and measured by exchange of allelic markers in genetic crosses. When measured in this way, the term "allelic recombination" is often used. A test of whether meiotic allelic recombination reflects the repair process of HRR would be to determine whether allelic recombination increases after exposure to DNA damaging agents. As would be expected if allelic recombination is a reflection of HRR, X-irradiation increases meiotic allelic recombination in *S. cerevisiae* (Kelly et al., 1983) and in the nematode *C. elegans* (Kim and Rose, 1987). In the fruit fly *Drosophila melanogaster,* recombination is increased by UV light (Prudhommeau and Proust, 1973), gamma-rays (Suzuki and Parry, 1964) and mitomycin C (Schewe et al., 1971). Even *D. melanogaster* males, which ordinarily do not display meiotic recombination (Chovnick et

al., 1970), can be induced to undergo recombination by X-rays (Hannah-Alava, 1964). That male *D. melanogaster* do not ordinarily undergo recombination, except when exposed to an exogenous DNA damaging agent, is somewhat puzzling. This may reflect a preference for removal of spermatocytes with spontaneous DNA damage by programmed cell death (apoptosis), rather than removal of the DNA damages by HRR; or it may reflect a low level of spontaneous DNA damage requiring HRR during spermatogenesis. In general, these observations show that exposure to DNA damaging agents increase meiotic recombination and are consistent with the view that meiotic recombination is an adaptation for DNA repair.

CROSSOVER AND NON-CROSSOVER RECOMBINATION: IMPLICATIONS FOR VARIATION THEORIES

Recombination involving exchange of genetic information between between non-sister homologs (i.e. homologs originating from different parents) is a near universal feature of meiosis. A double-strand break (DSB) in one of the two DNA homologs is considered to be a general early step in the recombination process (see Figure 1). Succeeding steps lead to the accurate repair of the DSB. When the chromosome arms on opposite sides of a DSB exchange partners during recombination, the event is called a crossover (CO). When the original configuration of the chromosome arms is maintained, the event is referred to as a non-crossover (NCO). CO recombination events produce genetic variation, whereas NCO recombination events have little effect on linkage disequilibrium (the statistical association of genes at different loci). Thus the relative occurrence of CO compared to NCO recombination events provides a test of the plausibility of evolutionary theories of sex based on the assumption that producing genetic variation is the principle function of sex. Variation theories predict that CO recombination should be the preferred product of the complex set of events involved in the recombination process.

Certain fungi are favorable systems for studying the products of individual meiotic events. Tetrad analyses from several species of fungi indicates that about 2/3 of recombination events during meiosis are NCOs [summaries of data from *S. cerevisiae*, *Podospora anserina*, *Sordaria fimicola* and *Sordaria brevicollis* were presented by Whitehouse (1982, Tables 19 and 38)].

In *D. melanogaster*, the ratio of NCOs to COs is about 3:1 (Mehrotra and McKim, 2006). Moens et al. (2002) presented evidence that during meiosis in the mouse there are $\geq$10-fold more DSBs than CO recombinants, and suggested that most DSBs are repaired by NCO recombination. These observations suggest that most recombination events are NCOs. Thus theories for the adaptive function of meiosis and sex that focus exclusively on CO recombination are inadequate to explain most recombination events. This includes theories that attribute the benefit of sex to the generation of genetic diversity.

THE FUNCTION OF NCO RECOMBINATION

A succession of molecular models for genetic recombination has been proposed over the years based on information from relevant genetic and biochemical experiments. A model proposed by Szostak et al. (1983), referred to as the Double-Strand Break Repair model, was

recognized at the time as providing a good explanation of experimental findings. According to this model, during each recombination event, two Holliday Junctions (see Figure 1) are formed to either side of a DSB, and resolution of these junctions leads to completion of the recombination event. Because of these features, the Double-Strand Break Repair model is also often referred to as the Double-Holliday Junction (DHJ) model (Figure 1). Originally, the DHJ model was thought to explain both CO and NCO types of recombination. Although the DHJ model is still considered to explain how CO recombinants are formed, evidence presented by Allers and Lichten (2001) indicated that NCO recombinants are formed by a different pathway that acts earlier in meiosis. Several groups (Allers and Lichten, 2001; McMahil et al., 2007; Andersen and Sekelsky, 2010) have reported evidence that NCO recombinants arise during meiosis by an HRR process which they call "Synthesis-Dependent Strand Annealing" or SDSA (Figure 1). In SDSA, an early step is the occurrence of a DSB, as in the DHJ model. This is followed by 5' to 3' resections of a single strand on each side of the break. Next, a single-strand extending from one end of the break invades the homologous chromosome with which it is paired, forming a displacement loop (D-loop). This is followed by continued synthesis of the invading strand using the undamaged complementary strand as a template. Next the invading strand is displaced from its template and anneals with a single strand at the other side of the break. Repair synthesis fills in any remaining gaps. By this process, the information lost in the region of the DSB is accurately restored by copying from the paired intact homolog, without a need for physical exchange of DNA. The process for forming NCO recombinants is one of informational exchange over a small region, rather than physical exchange. By contrast, the process for forming CO recombinants (the DHJ model) does involve physical exchange of DNA segments (Figure 1). Although the SDSA model of recombination includes a DSB as an early step, this model may also apply to other types of double-strand damages such as an interstrand-crosslink or single-strand break opposite an altered base in the other strand. In principle, these types of double-strand damage could be converted by nuclease action to a DSB that could then be repaired by SDSA to form a NCO recombination event.

CO and NCO recombination appear to have different functional consequences. The physical exchanges associated with CO recombination appear to be necessary to promote the intimate pairing of homologous chromosomes during meiosis in some organisms.

NCO recombination is not thought to have a role in synapsis since there is no physical exchange of DNA by the SDSA mechanism. Furthermore, CO recombination generates allelic recombination that contributes to genetic diversity among progeny, and this may have long-term benefits. NCO recombination produces relatively little allelic genetic variation. Thus NCO recombination, the most frequent type of recombination during meiosis, appears to be primarily for DNA repair, whereas CO recombination functions in DNA repair, synapsis, and in causing genetic diversity.

MEIOTIC HRR IS ESSENTIAL FOR FERTILITY AND EARLY EMBRYONIC SURVIVAL

One test of the idea that repair of DNA damages during meiosis is adaptively important is to study the effect on fertility of defects in enzymes known to be essential for HRR during

mitosis. About 15% of all couples in the US are infertile. An important cause of male infertility is oxidative stress during gametogenesis (Makker et al., 2009). As discussed above, oxidative stress causes DNA damage (Slupphaug et al., 2003). During mouse spermatogenesis, after meiosis is completed and mature sperm are being formed, DNA repair capability declines allowing accumulation of DNA damage in sperm (Marchetti and Wyrobek, 2008). Failure of HRR during meiosis would be expected to exacerbate the problem of DNA damage and increase infertility. Increased DNA damage in the germ line of men is associated with poor semen quality, low fertilization rate, impaired pre-implantation development, increased abortion, and more frequent disease in progeny including childhood cancer (reviewed in Lewis and Aitken, 2005). These authors also commented that the natural causes of this increased DNA damage are unclear, but the principal candidate is oxidative stress.

As expected on the hypothesis that meiosis is an adaptation for DNA repair, inherited mutations in human and mouse genes that encode proteins necessary for mitotic HRR are observed to cause infertility (Table 1). This finding indicates that formation of functional gametes depends on HRR. Genes *atm*, *brca1* and *mlh1* are essential for DNA repair during mitosis and are expressed at a higher level during meiosis than mitosis. In summary, it appears that increased damages during meiosis cause infertility, and that HRR of DNA damages during meiosis protects against infertility.

Meiosis is restricted to the cell division preceding gamete formation. This placement of meiosis likely reflects the high cost of producing nonfunctional gametes due to unrepaired DNA damages. A parent provides only one gamete to each progeny, and this gamete must be as free of damage as possible to result in a viable progeny. No other cell in a multicellular organism has as great an influence on reproductive success. Even though DNA repair enzymes involved in HRR are at higher levels during meiosis than during mitosis (Table 1) and thus HRR presumably occurs at a higher level during meiosis, attrition of fertilized eggs and miscarriages are still rather high. Austin (1972) pointed out that about half of fertilized eggs in humans fail to produce surviving embryos, as is generally typical of mammals.

Table 1. Mutations in genes encoding proteins necessary for HRR cause infertility in humans and/or mice

Species	Gene	Fold-increased expression in testes vs. somatic cells	Infertility in mutant females and /or males	References
human, mouse	atm	4×	Females and males in humans and mice are infertile	Galetzka et al., 2007; Barlow et al., 1998
Mouse	brca1	3×	Few surviving male mice are infertile	Galetzka et al., 2007; Cressman et al., 1999
Mouse	mlh1	1.7×	Female and male mice are infertile	Galetzka et al., 2007; Wei et al., 2002

Roberts and Lowe (1975) estimated a 78% loss of human conceptions, most of these occurring before the first missed period. Wilcox et al. (1988) concluded that 31% of embryos miscarry after implantation, often before a woman realizes she is pregnant. Although the causes of these malfunctions have not been determined, DNA damage is likely to be an important contributing factor. These observations point to an essential role for DNA repair during meiosis in mammals.

In plants, it is common for fertilized ovules to fail to mature as seeds (Bawa and Webb, 1984). During plant seed dormancy, often associated with unpredictable and stressful environments, DNA damages accumulate (Cheah and Osborne, 1978; Koppen and Verschaeve, 2001; Bray and West, 2005). Thus in plants, as in mammals, DNA damages appear to be a problem for survival, and HRR during meiosis may be necessary for viability of gametes and progeny.

DNA DAMAGES ARE REPAIRED BY HRR DURING MEIOSIS AND MITOSIS

In this section, we review findings indicating that DNA damages caused by different exogenous agents are repaired by HRR during mitosis, and that gene products required for mitotic HRR are also required for DNA repair during meiosis. In the next section, we present evidence that during meiosis HRR likely removes a class of DNA damages that cannot be accurately removed during mitosis.

During mitosis, HRR is largely limited to interaction between neighboring sister chromosomes after replication has occurred and before cell division. During mitosis, the frequency of recombination between non-sister homologs is about 1% of that between sister chromosomes (Moynahan and Jasin, 2010). On the other hand, during meiosis, in which there is systematic synapsis between non-sister homologous chromosomes, recombination occurs frequently between these non-sister homologs. Furthermore, while mitotic HRR only rarely leads to CO recombination, meiotic HRR leads to CO far more frequently (Virgin et al., 2001). This difference may reflect the function of CO recombination in promoting synapsis of non-sister homologs during meiosis, but not during mitosis. In spite of these differences concerning which chromosomes are involved, there appears to be close similarity of the recombination machinery used in meiosis and the HRR machinery used in mitosis. There are many common gene products essential to both processes. The apparent parallels in the machinery of recombination across meiosis and mitosis in eukaryotes, and even extending to the mechanism of transformation in bacteria suggests similarity of function, and this is what we seek to explain through the DNA repair hypothesis of meiosis.

In the yeast *S. cerevisiae*, mutations in a number of genes confer increased sensitivity to radiation and/or genotoxic chemicals, and also cause deficiency in meiotic recombination (Haynes and Kunz, 1981). As an example, studies of *rad52* mutants indicate that the *rad52* gene is necessary for meiotic recombination (Game et al., 1980), and for mitotic recombination (Malone and Esposito, 1980). Mutants defective in *rad52* have increased sensitivity to killing by X-rays, MMS and the crosslinking agent 8-methoxypsoralen-plus-UV light, and in addition have reduced meiotic recombination (Haynes and Kunz, 1981; Henriques and Moustacchi, 1980; Game et al., 1980).

In the nematode *Caenorhabditis elegans*, oocyte nuclei in the pachytene stage of meiosis (the stage in which recombination occurs) are hyper-resistant to X-rays compared to oocytes in diakinesis (a later stage of meiosis) or early embryonic cells after fertilization (Takanami et al., 2000). This pachytene hyper-resistance requires expression of gene *ce-rdh-51*, which is a homolog of yeast *rad51*, a key player in HRR. Furthermore, nuclei in the pachytene stage of meiosis have greater resistance to heavy ion particle irradiation than nuclei in the later diplotene and diakinesis stages and early embryonic cells (Takanami et al., 2003). Resistance during pachytene to heavy ion particle irradiation, like resistance to X-ray irradiation, depends on gene *ce-rdh-51*. This resistance also depends on gene *ce-atl-1*, which is related to gene *atm*, a gene necessary for repair of DSBs by HRR in mammals and for fertility in humans and mice (Table 1). Pachytene nuclei thus appear to effectively repair X-ray and heavy ion-induced DNA damage by meiotic HRR.

In the fruit fly *Drosophila melanogaster*, mutants defective in genes essential to the machinery of recombination (i.e. genes *mei-41*, *mei-9*, *hdm*, *spn* and *brca2*) have increased sensitivity to killing by a variety of DNA damaging agents, compared to wild-type flies. This sensitivity implies a reduced ability of somatic cells to carry out recombinational repair of the DNA damages caused by these agents.

These same mutants have reduced recombination during meiosis compared to wild-type flies, indicating that they are deficient in recombination (presumably HRR) during meiosis. Specifically, mutations in the "meiotic genes" *mei-41* and *mei-9* cause increased sensitivity to several DNA damaging agents [X-rays, UV, methylmethane sulfonate (MMS), nitrogen mustard, benzo(s)pyrene and 2-acetyl-aminofluorene], and also have deficient meiotic recombination (Baker et al., 1976; Boyd, 1978; Rasmussen, 1984). Also, *D. melanogaster* mutants defective in genes needed for mitotic HRR (i.e. *hdm*, *DmRad51*/*spnA* and *brca2*) have increased sensitivity to X-rays and/or MMS, and are also deficient in meiotic recombination (Joyce et al., 2009; Staeva-Viera et al., 2003; Klovstad et al., 2008).

The repair of double-strand damages after γ-irradiation of rat spermatogenic cells was measured in sequential stages of formation of germ cells (Coogan and Rosenblum, 1988). These sequential stages were: spermatagonia and preleptotene spermatocytes, pachytene spermatocytes and spermatid spermatocytes.

Pachytene spermatocytes, the meiotic stage in which recombination occurs, showed the greatest repair capability. Thus it appears that a function of meiosis (expressed specifically during the pachytene stage) is to repair double strand damages. The source of DNA damage that most likely provides a natural challenge to gamete formation is reactive (ROS) generated by active metabolism. Fisher and Aitkin (1997) showed that several germ cell stages in mammals, including pachytene spermatocytes, produce levels of ROSs sufficient to cause oxidative stress.

In the plant *A. thaliana*, *rad51* mutants are hypersensitive to the DNA cross-linking agent mitomycin C and are defective in meiotic recombination (Bleuyard and White, 2004; Bleuyard et al., 2005). In maize, mutants defective in orthologs of Rad51 produce embryos that are extremely susceptible to radiation induced DSBs, and are defective in meiosis (Li et al., 2007).

DURING MEIOSIS HRR LIKELY REMOVES A CLASS OF DNA DAMAGES THAT CANNOT BE ACCURATELY REMOVED DURING MITOSIS

During meiosis, HRR potentially provides a unique advantage compared to HRR during mitosis. During meiosis, non-sister homologs systematically pair and undergo HRR, whereas during mitosis HRR is largely between sister-homologs and occurs between non-sister homologs in only about 1 in 100 HRR events (Moynahan and Jasin, 2010).

During mitosis, HRR largely involves interaction between the duplicate sister homologs resulting from DNA replication. Consequently, during mitosis, HRR is limited to the portions of the cell cycle in which DNA replication is occurring (S phase) and after DNA replication is complete (G2/M phase) so that a closely adjacent homologous chromosome would be available. Thus double-strand damages occurring during or after DNA replication may be accurately removed by HRR between sister homologs (Tichy et al., 2010).

During the portion of the mitotic cell cycle after cell division but prior to DNA replication (G1 phase), double-strand damages, such as DSBs, are ordinarily not repaired by HRR, and are either repaired by an inaccurate process [non-homologous end-joining (NHEJ) that generates mutation] or the DSBs cause cell death.

In contrast, during meiosis HRR can likely remove, in an accurate manner, double-strand damages that arise at any stage of the cell cycle because of systematic pairing of non-sister homologs. Cells in the meiotic G1 phase are more resistant to the lethal effects of X-rays than mitotic G1 cells (Kelley et al., 1983). This observation suggests that the types of DNA damage caused by X-rays, which includes DSBs, are more effectively repaired during meiotic G1 than mitotic G1. Thus repair capability in meiosis is likely more effective than during mitosis, since during meiosis HRR proteins are available at an increased level (Table 1) and there is more access to a homolog for the source of needed information to copy.

At each cell division in humans, 30,000 to 50,000 DNA replication origins are activated (Mechali et al., 2010).

Consequently, the chromosome is replicated in segments. During meiosis, any segment containing a double-strand damage may fail to complete its replication until the damage is repaired. Such a temporary and limited blockage of replication may result directly from the damage itself or result from signaling by proteins that specifically bind to damaged regions of DNA. Once damage is recognized, HRR can remove the damage during the subsequent prophase I stage of meiosis, when the blocked segment becomes paired with a non-sister homolog. When the damage is removed, replication of the segment can be completed**.** Hence meiosis provides a means of accurately repairing a class of DNA damages that cannot be accurately repaired during mitosis.

WHAT ARE THE CONSEQUENCES OF DNA DAMAGES IN THE MITOTIC DIVISIONS PRECEDING MEIOSIS IN THE GERM LINE OF MULTICELLULAR ORGANISMS?

Typically, in multicellular eukaryotes, there are numerous mitotic divisions in the germ line preceding meiosis and the formation of gametes. Double-strand damages occurring during or after DNA replication in the mitotic cells of the germ line can be repaired by HRR involving the two sister-chromosomes. However, because the non-sister homologs present in a diploid cell ordinarily do not pair and undergo recombination during mitosis, double-strand damages occurring prior to replication are unlikely to be removed by HRR. Consequently when double-strand damages occur before DNA replication during the mitotic divisions in the germ line the result will either be increased mutation or increased apoptotic cell death.

It is likely that the preferred strategy for dealing with pre-replication double-strand damages during the mitotic divisions of the germ line will be apoptosis, since this avoids germ line mutations that could be passed on to progeny. This explanation is suggested by analogy to the behavior of mammalian embryonic stem (ES) cells. These cells use an apoptotic strategy for dealing with DNA damages occurring prior to DNA replication (Tichy et al., 2010). Clonal expansion of ES cells gives rise to all of the mammalian cell types. Since mutations occurring in early embryonic cells are passed on to all clonal descendents, they can be seriously deleterious to the organism as a whole. Therefore, in ES cells, robust mechanisms are employed for reducing DNA damages and eliminating cells with DNA damage, in order to avoid mutations. Mouse ES cells mainly use high fidelity HRR to repair DSBs, in contrast to differentiated somatic cells that mainly use inaccurate NHEJ (Tichy et al., 2010). In addition, ES cells, upon receiving DNA damage prior to replication (G1 phase) do not undergo cell cycle arrest, but rather undergo apoptosis (Aladjem et al., 1998). Mouse ES cells have a mutation frequency about 100-fold less than that of isogenic somatic cells (Cervantes et al., 2002). The lower mutation frequency likely reflects, in part, the apoptotic removal of G1 phase cells with DNA damage that would otherwise cause mutation. Thus, increased accuracy likely comes at the cost of somatic selection against cells with unrepaired DNA damages that arose prior to DNA replication. We consider it likely that a similar strategy is used during the mitotic divisions of the germ line preceding meiosis. In this case, the strategy of increased apoptosis in response to damage would substantially reduce transmission of mutations to gametes and progeny.

CROSSOVER HOTSPOTS IN MEIOSIS

A statistical analysis of genetic variation in the human genome has identified about 25,000 hotspots for meiotic CO (Myers et al., 2006). On average, about one cross-over event occurs per 1,300 meioses per hotspot. The nature of hotspots is not well understood. Hotspots may represent sites vulnerable to DNA DSBs and/or DNA sequences that are recognized by a protein that specifically targets the sequence for CO. The number of endogenous DSBs present prior to meiosis is unknown, but Vilenchik and Knudson (2003) have estimated that the average number of endogenous DNA DSBs per somatic cell occurring at each cell generation is about 50, so that a number in this range may apply to pre-meiotic cells.

Hochwagen and Marals (2010) have described a DNA-binding protein, PRDM9, which recognizes certain 13-mer sequences characteristic of some hotspots and may determine CO location. In the DHJ model the site of the actual CO (resolution of a Holliday junction) can be distant from the site of the initiating DSB [as indicated by the size of gene conversion tracts during meiosis which are hundreds to thousands of nucleotide pairs long (Judd and Petes, 1988)]. Thus hotspots may occur upstream or downstream of DSBs at sequences that favor Holliday junction resolution.

EVOLUTIONARY FORCES MAINTAINING SEX

In this section we present evidence that it is the short-term benefit of DNA repair, rather than the potential long-term benefits of genetic variation, that maintains sex. Explanations for the evolutionary maintenance of sex are often based on the assumption that the main adaptive advantage of sex is the production of genetic variation. This is an active area of research, and a variety of models and reviews have been presented based on this assumption (e.g. Barton and Charlesworth, 1998; Otto and Gerstein, 2006; Agrawal, 2006). However, it has also been noted by Otto and Gerstein (2006) that individuals surviving to reproductive age in a fairly stable environment have genomes that function well in that environment. They ask, why should such individuals risk shuffling their genes with those of another individual? This question and other similar considerations, have led many investigators to doubt that production of genetic diversity is the principal adaptive benefit of sex.

In contrast to the conventional view that sex increases genetic diversity, Heng (2007) and Gorelick and Heng (2011) have reviewed evidence that sex actually decreases most genetic variation. These authors consider that sex acts as a coarse filter, weeding out major genetic changes, such as chromosomal rearrangements, but permitting minor variation, such as changes at the nucleotide or gene level (that often are neutral) to pass through the sexual sieve. Thus they consider that sex acts as a constraint on genomic variation.

It is generally acknowledged that any explanation for the adaptive benefit of sex, to be plausible, must provide benefits that balance the costs of sex. Such costs vary from species to species, depending on the life-style of the organism. However, certain general large costs are widely recognized. These include the “cost of males” (Maynard Smith, 1978; Williams, 1975), the “recombinational load” that is a consequence of the randomization of genetic information during sex and break-up of coadapted gene complexes (Shields, 1982), the cost of finding a mate (Bernstein et al., 1985b), and the cost of sexually transmitted disease (Michod et al., 2008). These costs apply most strongly to diploid, obligate sexual species. HRR during meiosis, we think, provides an appropriate explanation for the adaptive maintenance of sex (and meiosis), since the benefits are large enough (removal of DNA damages that would otherwise be deleterious/lethal to gametes or progeny) to plausibly balance the large immediate costs of sex.

Furthermore, the explanation that meiosis is an adaptation for repair of DNA damage can be consistently applied to all organisms that undergo meiosis including facultative sexual organisms (as described above), or to species that undergo meiosis but have little or no outcrossing (see below in this section). A number of authors have proposed that the genetic variation produced by sex increases the rate of adaptation (Colegrave et al., 2002; Kaltz and Bell, 2002; Peters and Otto, 2003; Goddard et al., 2005; Cooper et al., 2005; deVisser and

Elena, 2007). We consider, however, that genetic variation is a byproduct of HRR and that any long-term benefit of variation would supplement the benefit of DNA repair. We think that, in the short-term, the benefit of variation, by itself, generally would be inadequate to maintain sex in the face of the large costs identified for sex.

Organisms that undergo meiosis, but experience little, or no, outcrossing sex are common in nature. In such cases, recombination produces little, if any, genetic variation among progeny. The budding yeast *S. cerevisiae* appears to outcross very infrequently in nature, although inbreeding sex is frequent. Ruderfer et al. (2006) analyzed the ancestry of natural *S. cerevisiae* strains and concluded that outcrossing occurs about once every 50,000 cell divisions. In contrast, mating between closely related yeast cells is much more common in nature. Mating can happen when haploid yeast cells of opposite mating types, MATa and MATα, contact each other. Ruderfer et al. (2006) and Zeyl and Otto (2007) noted that there are two reasons why mating between closely related *S. cerevisiae* cells is frequent in nature. First, cells of opposite mating type from the same ascus (the sac that holds the cells produced from a single meiosis) are in close proximity. Second, *S. cerevisiae* is capable of homothallism, the ability of haploid cells of one mating type to produce daughter cells of the opposite mating type. Thus, under natural conditions, meiotic events in *S. cerevisiae* that produce little or no recombinational variation are likely far more common than meiotic events that do produce recombinational variation. The relative rarity of outcrossing sex is consistent with the concept that the main adaptive function of meiosis in *S. cerevisiae* is HRR of DNA damages, since this benefit is realized whether meiosis results from inbreeding or outcrossing. If, as is often assumed, the primary adaptive benefit of meiosis is to produce genetic variation, it is difficult to understand how meiosis, a complex process, could be selectively maintained during the 50,000 generations of *S. cerevisiae* in which outcrossing is absent.

Outcrossing sexual reproduction is the most frequent type of reproduction in higher plants. However about 15% of plants undergo meiosis, but are principally self-fertilizing (Bernstein and Bernstein, 1991).

Among vertebrates, one extreme but well studied example of inbreeding is the Mangrove Killifish, *Kryptolebias marmoratus*. This fish occupies brackish water mangrove habitats from Florida to Brazil. It produces eggs and sperm by meiosis and reproduces routinely by self-fertilization. Normally each hermaphroditic individual fertilizes itself when an egg and sperm that are formed by an internal organ unite inside the body of the fish (Sakakura et al., 2006; see Avise, 2008, for review).

The examples reviewed in this section indicate that meiosis often occurs in circumstances that generate little, if any, genetic variation, suggesting that meiosis is maintained by another adaptive benefit, such as the benefit of DNA repair.

BENEFITS OF OUTCROSSING SEX

The essential feature of sex common to both bacteria and eukaryotes is information exchange between two genomic DNA molecules derived from different individuals. These different individuals (parents) can be closely or distantly related to each other. Above, we discussed some examples of mating between closely related individuals (inbreeding). These examples were discussed to illustrate our argument that meiosis provides a benefit (accurate DNA repair) that is independent of whether significant recombinational variation is also

generated. However, meiosis is frequently associated with mating between unrelated or distantly related individuals (outcrossing). We now consider when outcrossing and inbreeding are advantageous.

In predominantly haploid organisms such as bacteria and many unicellular eukaryotes, a simple explanation can be offered for why mating occurs. It serves to create the transient diploid (or partially diploid) state necessary for HRR. It is unclear how frequently outcrossing (vs. inbreeding) occurs in nature in predominantly haploid sexual organisms. In the case of *S. cerevisiae*, as discussed above, evidence indicates that outcrossing is uncommon, whereas mating between related individuals is common (Ruderfer et al., 2006). This is probably also the case for bacteria, where transformation in nature likely most often occurs between clonally related individuals.

Outcrossing sex appears to be preferred in organisms in which diploid cells are the predominant feature of their life cycle. A benefit of diploidy, in contrast to haploidy, is that diploidy allows the masking of recessive deleterious mutations. This masking effect is referred to as "genetic complementation." Once outcrossing is employed because of the benefit of complementation, switching to inbreeding becomes disadvantageous because it leads to expression of the (previously masked) deleterious recessive mutations, resulting in inbreeding depression. This idea has a long history. Charles Darwin (1889), in his book "The Effects of Cross and Self-Fertilization in the Vegetable Kingdom", arrived at clear and definite conclusions concerning the adaptive benefit of outcrossing sex. For example, on page 462 he stated that "the offspring from the union of two distinct individuals, especially if their progenitors have been subjected to very different conditions, have an immense advantage in height, weight, constitutional vigor and fertility over the self-fertilizing offspring from either one of the same parents." He thought that this fact was amply sufficient to account for outcrossing sexual reproduction.

Another possible beneficial consequence proposed for outcrossing is the generation of new genetic variants that may provide a long-term advantage (e.g. Colegrave et al., 2002; Kaltz and Bell, 2002; Peters and Otto, 2003; Goddard et al., 2005; Cooper et al., 2005; deVisser and Elena, 2007).

An analysis of the consequences of genetic complementation indicated that complementation provides a sufficient benefit to maintain outcrossing (Bernstein et al., 1985a; 1987; Michod, 1994). However, recently, more explicit population genetic models have raised some issues concerning this idea. From a population genetics perspective, the basic consequence of outcrossing is to bring populations to Hardy-Weinberg (HW) equilibrium. Consequently, outcrossing can be beneficial under conditions where it is advantageous for a population to go closer to HW equilibrium, and if there is another condition pushing the population away from HW equilibrium, i.e. generating either a deficit or an excess of heterozygotes. Roze and Michod (2010) considered gene conversion to be a potential force pushing away from HW equilibrium, since gene conversion can generate homozygosity.

Gene conversion can result from mitotic HRR between non-sister chromosomes. It was concluded that, if deleterious alleles tend to be partially recessive, outcrossing is advantageous in the short-term because it masks deleterious recessive alleles, but disadvantageous in the long-term because purging of deleterious recessive alleles is less efficient. The rate of loss of heterozygosity from gene conversion events during development was a critical parameter in the model determining the outcome of selection on outcrossing.

Unfortunately, there is not much data available, but the data reviewed by Roze and Michod (2010) suggests that the loss of heterozygosity may be low which means weak selection for outcrossing.

AGING, SEX AND REJUVENATION

Many, if not all, eukaryotes age, yet age-associated changes are not passed on to progeny. Over a century ago, Weismann (1892) commented that among his contemporaries, a widely held view was that the adaptive benefit of sex is rejuvenation (although he did not agree with this view himself). Supporting the rejuvenation concept was the observation that, in some protozoans, vitality declines during the course of successive asexual cell divisions by binary fission, but following sexual interaction (conjugation) vitality is restored. Here we review evidence that lifespan is reset from one generation to the next (rejuvenation) at the time of meiosis, and that this rejuvenation is a consequence, in part, of removal of DNA damages.

The ciliate protozoan *Paramecium tetraurelia* has a diploid micronucleus and a polyploid macronucleus that contains about 800 to 1500 copies of the genome. The micronucleus contains the germline DNA, whereas the macronuclear DNA expresses cellular functions. *P. tetraurelia* is facultatively sexual and can reproduce either by binary fission or by a meiotic process. For *P. tetraurelia* there are two kinds of meiotic process. The first is conjugation, a kind of outcrossing sex. The second is automixis, similar to self-fertilization. In the asexual fission phase, during which cell divisions occur by mitosis rather than meiosis, there is clonal aging, a gradual loss of vitality. An asexual line of clonally aging paramecia will lose vitality and die out after about 200 fissions if it fails to undergo automixis or conjugation.

The cause of clonal aging in *P. tetraurelia* was clarified by transplantation experiments (Aufderheide, 1987). When macronuclei of clonally young paramecia were injected into paramecia of a standard clonal age, the lifespan of the recipient was prolonged. In contrast, transfer of cytoplasm from young paramecia did not prolong the lifespan of the recipient. These experiments suggested that the macronucleus, rather than the cytoplasm, governs clonal aging. Further experiments by Smith-Sonneborn (1979), Holmes and Holmes (1986) and Gilley and Blackburn (1994) showed that, during clonal aging, DNA damage increases dramatically. When clonally aged paramecia are permitted to undergo meiosis, in association with either conjugation or automixis, the progeny are rejuvenated. Furthermore, during these processes, the old macronucleus disintegrates and a new macronucleus is formed by replication of the micronuclear DNA, the DNA that had recently undergone meiosis. There is little, if any, DNA damage in the new macronucleus. These observations suggest that clonal aging is due, in large part, to a progressive increase in DNA damage; and rejuvenation is due to repair of these DNA damages in the micronucleus during meiosis and the reconstitution of the macronucleus by replication of the repaired micronuclear DNA.

In cells of the budding yeast *S. cerevisiae*, mother cells experience replicative aging and death. However, when these cells undergo meiosis and gametogenesis, lifespan is reset (Unal et al., 2011). Gametes (spores) formed by aged cells show the same replicative potential as gametes generated by young cells, and age-associated damage is no longer detectable in the gametes. However in this system, it has not yet been determined if removal of DNA damage during meiosis contributes to the observed rejuvenation.

PARTHENOGENESIS

Parthenogenesis is the development of an individual from an egg without fertilization. In the animal kingdom there are over a million sexual species compared to less than a thousand parthenogenetic species (White, 1973). That is, less than 0.1% of animal species are parthenogenetic, suggesting that parthenogenesis is not a generally successful strategy. The relatively brief evolutionary persistence of most parthenogenic groups is apparent from their lack of high taxonomic rank, and from the low DNA sequence divergence from their nearest sexual relatives.

Among vertebrates, some of the most successful and conspicuous animal parthenogens undergo a process called "endomitosis." By this process, there are two sequential pre-meiotic chromosome replications, followed by an apparently normal meiosis resulting in production of diploid eggs (Avise, 2008). The extra chromosome duplication is designated "endomitotic" doubling. Recombination at meiosis in these parthenogens involves pairing between chromosomes derived by replication from the same progenitor chromosome. Unlike meiosis in a sexual species, recombination between non-sister chromosomes is unavailable. One well-studied organism that employs endomitosis is a common type of whiptail lizard (genus *Aspidoscells*) found in the southwestern United States. Other examples of endomitosis have been described among taxa of fish, salamanders, grasshoppers, earthworms, planarians and beetles. Because recombination between non-sister homologous chromosomes is lacking, the maternal genome is passed on intact to daughters. Parthenogenesis by endomitosis might appear to be an ideal strategy, since it provides the benefit of meiotic HRR, while avoiding expression of deleterious recessive alleles. This consideration raises the question of why parthenogenesis, involving endomitosis, does not predominate over sexual reproduction. The answer may be that double-strand damages occurring prior to the first pre-meiotic replication (G1 phase) would be difficult to accurately repair in this form of parthenogenesis. All pairing partners (closely adjacent sister chromosomes) are derived from the same initial chromosome, and if this chromosome had a double-strand damage, there is little opportunity for it to pair with an intact closely neighboring template corresponding to the damaged site. This problem does not occur in ordinary meiosis because systematic pairing and HRR occur between non-sister chromosomes. Thus if double-strand damages are common prior to replication, during the G1 phase of meiosis, parthenogenesis by endomitosis is an unsatisfactory option.

Avise (2008), in reviewing the comparative ecologies of vertebrate parthenogens and sexual reproducers, concluded that some parthenogenetic biotypes can be highly successful in the short or moderate term, but such ecological success does not continue indefinitely, as judged by the fact that no parthenogenetic lineage in vertebrates is demonstrably ancient. This lack of long-term success may reflect the observed reduced fecundity of parthenogens compared to their sexual relatives, in many cases less than 50% (Lynch, 1984).

Another parthenogenetic strategy, employed in plants and invertebrates, is automixis, which involves meiosis followed by fusion of haploid nuclei from the same parent. This is genetically similar to self-fertilization and allows expression of deleterious recessive alleles. Still another common parthenogenetic strategy, especially in plants, is apomixis, an oogenic process that is entirely mitotic, that is, without hints or vestiges of meiosis-type events. An analysis of advantages versus costs of switching from outcrossing sexual reproduction to the various forms of parthenogenesis (endomitosis, automixis and apomixis) led to the conclusion

that these alternatives will not generally be favored, except when the costs of outcrossing sex are high (Bernstein et al., 1985a; 1987; 1989). Comparisons of sexual species to related parthenogens, with respect to geographical and ecological correlates, indicate that parthenogens tend to occur where population densities are low (and finding a mate would be very costly) (reviewed in Bernstein et al., 1985b).

Among invertebrates, one exceptional parthenogentic class, the bdelloid rotifers, are thought to have remained asexual for more than 40 million years (Mark Welch and Meselson, 2000). Bdelloid rotifers produce eggs by mitosis, rather than meiosis. Bdelloid rotifers are extraordinarily resistant to ionizing radiation (Gladyshev and Meselson, 2008). This resistance is likely a consequence of an evolutionary adaptation to survive desiccation in temporary pools of water. Desiccation of the rotifers causes considerable DNA breakage, which they can repair. Bdelloid primary oocytes are in the G1 phase of the cell cycle so that sister chromosomes are lacking. MarkWelch et al. (2008) proposed that interaction of non-sister co-linear chromosome pairs provides a mechanism of DNA repair; and that these co-linear chromosome pairs are maintained by natural selection as templates for repair of DSBs caused by frequent desiccation and rehydration. Although lacking sex and meiosis, Bdelloid rotifers appear to retain an essential feature of meiosis, HRR between non-sister homologs.

One can ask why is this not a general strategy for life on earth, and why instead is sex the general strategy with its accompanying costs of outcrossing? We offer the following speculation. Since bdelloid rotifers typically inhabit small transient pools of water, they may often find themselves in pools with no other, or only a few other individuals. Thus finding a mate may often be impossible or very difficult and costly. This condition would tend to select for a parthenogenetic strategy. Furthermore, because bdelloid rotifers are "degenerate tetraploids" (Mark Welch et al., 2008), they may be able to avoid a major cost of producing eggs by mitosis instead of meiosis. If bdelloid cells were only diploid, then recombination between non-sister homologs during the mitotic divisions that produce eggs would generate homozygosity, and hence expression of deleterious recessive alleles. Such a strategy would select against this mode of parthenogenesis and favor a switch to sex. However, because bdelloid rotifers are not diploid, but rather degenerate tetraploids, recombination between non-sister chromosomes leading to homozygosity for one pair of homologs may not ordinarily lead to expression of deleterious recessive mutations due to masking by the second pair of homologs. Thus parthenogenesis in bdelloid rotifers may be a uniquely successful parthenogenetic strategy because of two features specific to the species, a small transient habitat causing a high cost of mating, and degenerate tetraploidy allowing more effective masking of deleterious recessive alleles. This strategy may not be generally advantageous as an alternative to sex because of the costs of maintaining tetraploidy compared to the costs of maintaining diploidy.

CONCLUSION

DNA damage, particularly oxidative DNA damage, appears to be a ubiquitous problem for organisms. We have summarized evidence bearing on the hypothesis that sex is an adaptation for repairing DNA damage that if unrepaired would cause mutation or inviability. We also reviewed evidence that sex has been maintained by this adaptive benefit throughout evolution in bacteria, simple unicellular eukaryotes and complex multicellular eukaryotes. In

bacteria, transformation involves transfer of DNA from one bacterial cell to another and appears to be a primitive form of sex. Since competence for transformation is a complex developmental process dependent on expression of many bacterial genes, it appears to be an adaptation that strongly benefits the bacteria. Development of competence for transformation ordinarily occurs under stressful environmental conditions such as high cell density and nutritional limitation. These are conditions in which DNA damage may be expected. Experiments with several different bacterial transformation systems indicate that transformation is a response to DNA damage, and that it serves to repair DNA by the process of homologous recombinational repair (HRR).

Eukaryotes evolved from bacteria (prokaryotes). As indicated by recent evidence, sexual reproduction was present very early in the evolution of eukaryotes, and sex in eukaryotes likely evolved from ancestral prokaryotic sex. The sexual cycle in eukaryotic microorganisms is facultative and tends to occur under stressful environmental conditions, similar to the conditions that induce sex (competence for transformation) in bacteria. Experiments with a wide variety of different facultatively sexual unicellular or simple multicellular eukaryotes suggest that the sexual cycle is induced by DNA damage. Thus sex may have been retained through the prokaryotic to eukaryotic transition as an adaptation for repairing DNA damage. There are fundamental similarities in the processes employed in bacterial and eukaryotic sex. Both involve the coming together of genomes from two individuals (parents) in a common cytoplasm, the formation of a DNA-RecA (or ortholog) nucleofilament, pairing of homologous regions of the two parental genomes, exchange of information between the genomes (recombination), and passage of the new recombined genomes to progeny.

A characteristic key molecular feature common to recombination in prokaryotes and eukaryotes is the role of the RecA protein and its orthologs in catalyzing the strand invasion step of recombination during transformation and meiosis. RecA protein interacts with DNA to form striking nucleoprotein filaments that scan the partner chromosome for regions of homology. This brings the invading DNA strand to the corresponding site in the partner chromosome where informational exchange occurs. Analysis of the molecular interactions of RecA protein indicates that RecA is the central component of an HRR system for dealing with DNA damage. This basic function of RecA and its orthologs appears to have been maintained through the evolutionary transitions from transformation in bacteria to meiosis in unicellular and simple multicellular eukaryotes, and then to meiosis in higher animals and plants. Experimental exposure of several different eukaryotes to a variety of DNA damaging agents was observed to increase meiotic recombination, consistent with the idea that meiotic recombination is a DNA repair process.

Current molecular models of meiotic recombination assume that an early event is a double-strand break or gap in one of the two pairing chromosomes. The double-strand break may be a direct result of action of a DNA damaging agent, or it may be formed secondarily (e.g. as part of the process of removing another type of damage such as an inter-strand crosslink). During the repair process, two types of recombination event may arise; one in which the chromosome arms flanking the break are exchanged between pairing partners [called a crossover (CO)], and one in which the flanking arms are not exchanged [called a non-crossover (NCO)]. Both the CO and NCO types of recombination are effective repair processes. Evidence indicates that during meiosis the NCO type of recombination is more frequent than the CO type. This observation seems inconsistent with the hypothesis that meiotic recombination is primarily an adaptation for generating genetic diversity, since the

NCO type of recombination generates little genetic variation compared to the CO type. The CO and NCO types of recombination arise by different pathways. The CO type arises by a pathway involving two Holliday junctions and the physical exchange of DNA segments. This pathway is referred to as the Double-Holliday Junction (DHJ) pathway. The NCO type arises by a pathway involving transfer of sequence information without physical exchange of DNA. The latter pathway is referred to as the Synthesis Dependent Strand Annealing (SDSA) pathway. The DHJ pathway appears to function during meiosis in three processes: DNA repair, promotion of pairing between non-sister homologous chromosomes, and in causing genetic diversity. The SDSA pathway, the more frequent meiotic pathway, appears to be employed primarily in DNA repair.

Infertility and loss of conceptions appears to be common in animals and plants. Oxidative stress may be an important source of DNA damage during gametogenesis. In humans and mice, mutations in enzymes necessary for recombinational repair cause loss of fertility, suggesting that meiotic HRR is essential for the effective production of functional eggs and sperm. DNA damages caused by a variety of known DNA damaging agents are repaired during mitosis by HRR (between sister homologous chromosomes), and even more effectively during meiosis (by HRR between both sister- and non-sister homologous chromosomes). The HRR processes in meiosis and mitosis seems to be similar in that they employ similar gene products.

During mitosis, double-strand damages can be accurately removed by HRR if they occur after DNA replication when two sister chromosomes are present and adjacent. Because non-sister chromosomes do not ordinarily pair in diploid somatic cells, accurate repair of double-strand damages occurring before replication is not usually possible. However, during meiosis there is systematic pairing of non-sister homologs so that double-strand damages occurring prior to replication may be accurately repaired. Thus a potentially lethal class of DNA damages, that cannot be accurately removed during mitosis, may be accurately removed during meiosis. In multicellular eukaryotes there are typically numerous mitotic cell divisions in the germ line prior to the single meiotic division leading to gamete formation. Double-strand damages occurring subsequent to DNA replication in these mitotic cells can be accurately repaired by HRR between sister-chromosomes. Prior to DNA replication in mitotically dividing cells of the germ line, double-strand damages likely cause programmed cell death (apoptosis). This would avoid the possibility of inaccurate repair of such damages, which would otherwise cause mutations in the germ line that could be passed on to gametes and then to progeny.

The essential characteristic of sex in both bacteria and eukaryotes is recombination associated with mating between different individuals. Mating can be between closely related individuals (inbreeding) or distantly related individuals (outcrossing). Both types of mating provide the benefit of HRR. However, in those eukaryotes whose diploid stage of the life cycle is predominant, as in humans, masking of deleterious recessive mutations also provides a benefit. This benefit, referred to as “genetic complementation” (also “hybrid vigor”), is likely the immediate selective benefit that maintains outcrossing since a switch to inbreeding will cause the expression of the deleterious recessive mutations previously masked (inbreeding depression).

Outcrossing sex also produces genetic variation. This variation may provide long-term benefits to a population, as proposed by numerous investigators. However, in many sexual species, outcrossing (sex between distantly related individuals) is much less frequent than

inbreeding (sex between closely related individuals). Such observations suggest that it is not the possible long-term benefits of outcrossing (increased diversity) that generally maintains sex from one generation to the next, but rather the short-term benefit of DNA repair (due to both CO and NCO HRR).

Parthenogenesis is the development of an individual from an unfertilized egg. Parthenogenesis is not a generally successful strategy. It occurs in less than 0.1% of animal species, and parthenogenetic lineages are ordinarily not long-lasting. It appears that parthenogenetic strategies are favored over the sexual strategy when the costs of mating are unusually high, such as under environmental conditions that only allow low population density, when finding a mate is very difficult. Parthenogenetic strategies appear to suffer the disadvantages of inadequate HRR during meiosis or inadequate masking of deleterious recessive mutations.

In conclusion, we consider that the preponderance of evidence supports the idea that sex arose as an adaptation for repairing DNA damage and that this has remained the primary adaptive benefit of sex throughout the evolution from bacteria to unicellular eukaryotes, and then to complex multicellular eukaryotes, such as mammals.

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PARALYSIS IN ALS PATIENTS: AN OVERVIEW ON ASSESSMENT AND TREATMENT OF MOTOR IMPAIRMENT

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease affecting brain, brainstem and spinal cord motor neurons.

Muscle weakness is a hallmark sign in ALS, occurring in approximately 60% of patients. Early symptoms vary with each individual, but usually include tripping, dropping things, abnormal fatigue of the arms and/or legs, slurred speech, muscle cramps and twitches and/or uncontrollable periods of laughing or crying. There are spinal-onset or bulbar-onset disease forms, according to the first involvement of limbs or speech/swallowing muscles, respectively. With voluntary muscles progressively affected, patients in the later stages of the disease may become totally paralyzed.

In this paper, ALS diagnostic issues, and methods and techniques for assessing severity of motor deficits are discussed. Various treatment options for limb and bulbar palsies are reviewed: in addition to drugs, assistive rehabilitation approaches are presented, from physiotherapy to occupational therapy, from respiratory exercises to swallowing manoeuvres and postures.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS), also called *Lou Gehrig's disease*, is a rapidly progressive, invariably fatal neurodegenerative disease that attacks the neurons responsible for controlling voluntary muscles. It belongs to a group of disorders known as *Motor neuron diseases* (*MND*), including *Primary lateral sclerosis*, *Progressive bulbar palsy*, *Progressive muscular atrophy*, *Flail arm syndrome*, *Flail leg syndrome*, and *ALS with multisystemic involvement* (e.g., *ALS-Dementia*). All these syndromes share a common molecular and cellular pathology comprising degeneration of motor neurons (MNs) in cortex, brainstem and/or spinal cord, and the presence of characteristic ubiquitin and TDP-43-immunoreactive intraneuronal inclusions.

ALS is one of the most common neuromuscular disease worldwide: in Western countries, prevalence ranges from 2.7 to 7.4 per 100,000 [Worms, 2001]. In 90 to 95 percent of all

PALS, the disease occurs apparently at random with no clearly associated risk factors (*sporadic ALS*, *sALS*): patients do not have a family history of the disease, and their family members are not considered to be at increased risk for developing ALS. About 5 to 10 percent of all PALS have a family history of ALS (*fALS*). Often there is a Mendelian inheritance and high penetrance, with most cases having a pattern of inheritance that requires only one parent to carry the gene responsible for the disease (autosomal dominant pattern), although autosomal recessive pedigrees have been reported [Mulder et al., 1986; Gros-Louis et al., 2006].

ALS most commonly strikes people between 40 and 60 years of age, but younger and older people also can develop the disease [Haverkamp et al., 1995]. Men are affected more often than women (about 1.5 to 1.0), although more recent data suggest that the gender ratio may be approaching equality [Abhinav et al., 2007; Logroscino et al., 2008; Zoccolella et al., 2008].

The cause of ALS is not known, and it is not clear why ALS strikes some people and not others, but both genetic [Ticozzi et al., 2011] and environmental factors (including excitotoxicity [Rothstein et al., 1992; Steele and McGeer, 2008] and calcium-mediated intracellular damage [Choi, 1988]; oxidative stress [Ferrante et al., 1997], mitochondrial dysfunction [Curti et al., 1996], lack of neurotrophic factors [Henriques et al., 2010] and apoptosis [Cereda et al., 2006]; immune events [Calvo et al., 2010] and neuroinflammation [Wu et al., 2006]; altered axonal flow (probably due to the cytoskeletal damage [Crippa et al., 2010]) and protein aggregation (related also to defective autophagy [Venkatachalam et al., 2008]); exogenous toxicity [Callaghan et al., 2011]) may play a role.

CLINICAL SIGNS

In patients with ALS (PALS), both the brain upper MNs (UMNs) and the brainstem or spinal cord lower MNs (LMNs) degenerate or die, ceasing to send messages to muscles: unable to function, the muscles gradually weaken, waste away, and twitch, therefore leading to a wide range of disabilities.

Eventually, all muscles under voluntary control are affected, and patients lose their strength and the ability to move their body, but usually maintain control of eye muscles. Only occasionally there are symptoms of bladder dysfunction (such as urgency of micturition), and ALS does not either affect a person's ability to see, smell, taste, hear, or recognize touch; nor impair a person's mind or intelligence (although a small percentage of patients may experience problems with memory or decision-making [Lomen-Hoerth et al., 2003; Ringholz et al., 2005; Raaphorst et al., 2010], and there is growing evidence that some may even develop a form of dementia [Guedj et al., 2007]). Because the disease usually does not affect cognitive abilities, PALS are aware of their progressive loss of function and may become anxious and depressed [Patten et al., 2007].

Approximately 70% of patients with typical ALS have a *spinal form* of the disease: they present with symptoms which may start either distally or proximally in the upper or lower limbs. PALS may have noticed fasciculations (involuntary muscle twitching) or cramps preceding weakness or wasting onset for months, or even years. Rarely, they notice muscle wasting before weakness onset, which may be so subtle that the symptoms are frequently overlooked.

At the beginning weakness is usually asymmetrical (the controlateral limbs being involved sooner or later): body parts affected by early symptoms depend on which muscles in the body are damaged first. Some PALS see the effects of the disease on a hand or arm, as they experience difficulty with simple tasks requiring manual dexterity, such as buttoning a shirt, writing, or turning a key in a lock. In some cases, symptoms initially affect one of the legs, and patients experience awkwardness when walking or running, or they notice that they are tripping or stumbling more often.

Early examination in the course of spinal-onset disease reveals focal muscle atrophy, involving particularly hand, forearm and shoulder muscles in the upper limbs, and proximal thigh or distal foot muscles in the lower limbs. Fasciculations are usually visible in more than one muscle group; spasticity is present in both upper and lower limbs (with clonus and hypertonia). Tendon reflexes are brisk and usually symmetric, including the finger jerks in the upper limbs and positive crossed adductor reflex in the lower limbs, with abnormal spread of tendon reflexes beyond the stimulated muscle group; the Hoffmann's sign may be positive in the upper limbs, and the Babinski's sign in the lower limbs.

Patients with *bulbar-onset* ALS usually present with dysarthria, which arises from either LMN or UMN involvement, leading to slow slurred speech or a nasal quality. They may also develop dysphagia for solid or liquids after noticing speech problems: difficulty in swallowing and chewing impair their ability to eat normally, so that maintaining weight will then become a problem, and the risk of choking increases. Almost all patients with bulbar symptoms develop sialorrhoea with excessive drooling due to difficulty of swallowing saliva and mild UMN-type bilateral facial weakness, which affects the lower part of the face, causing difficulty with lip seal and blowing cheeks. "Pseudobulbar" symptoms, such as emotional lability and excessive yawning, are seen in a significant number of PALS.

The jaw jerk may be brisk, and an UMN type facial weakness affecting the lower half of the face is observed (although often varying degrees of UMN and LMN facial weakness coexist). The gag reflex is preserved and often brisk, whereas the soft palate may be weak. PALS show tongue wasting and fasciculations of the tongue which moves slowly, also due to muscle hypertonia. The other cranial nerves remain intact, although in late stages of the disease patients may very rarely develop a supranuclear gaze palsy or oculomotor palsy [Okuda et al., 1992; Kobayashi et al., 1999].

Bulbar symptoms may develop almost simultaneously with spinal symptoms, and in the vast majority of cases will occur within 1-2 years.

As disease progresses, patients develop the characteristic picture of the combination of UMN (tight and stiff muscles (spasticity) and hyperreflexia, including an overactive gag reflex and Hoffmann's and/or Babinski's signs) and LMN (muscle weakness and atrophy, muscle cramps, and fasciculations) involvement within the same central nervous system (CNS) region, affecting the bulbar, cervical, thoracic and lumbar territories.

About less than 5% of PALS present with respiratory weakness without previous spinal or bulbar signs [Chen et al., 1996; de Carvalho et al., 1996], showing symptoms of respiratory failure or nocturnal hypoventilation, such as dyspnea, orthopnea, disturbed sleep, morning headaches, excessive daytime somnolence, anorexia, decreased concentration, and irritability or mood changes [Polkey et al., 1999]. In later disease stages, when muscles in the diaphragm and chest wall fail, patients eventually lose the ability to breathe on their own and must depend on ventilatory support for survival. Respiratory failure and/or cardiovascular complications are the usual cause of death.

DIAGNOSIS

ALS diagnosis still remains a clinical one supported by neurophysiological evidence: the expert clinician after collection of patient's full medical history and careful neurological examination, in presence of UMN and LMN signs in the same anatomical region(s) with asymmetrical localization, is able to suspect ALS, but he needs a neurophysiological confirmation. On the other hand, suggestive neurophysiological results alone are not adequate for achieving a diagnosis, but must be interpreted in the light of patient's history and clinical findings. Therefore, clinician and neurophysiologist have to rely on each other, since they have the responsibility for the diagnosis of such a dreadful disease. At regular intervals, neurologist usually repeats physical examination to assess whether symptoms (such as muscle weakness and atrophy, hyperreflexia, and spasticity) are getting progressively worse. According to different surveys, the time required to confirm ALS diagnosis from the first signs/symptoms is approximately one year [Chiò and Silani, 2001].

Clinical Criteria

The World Federation of Neurology Research Group on Motor Neuron Diseases have developed the 1994 *El Escorial diagnostic criteria* [Brooks et al., 1994] and the revised 2000 *Airlie House criteria* [Brooks et al., 2000] to aid in diagnosing and classifying patients for research studies and clinical trials. Based on such criteria PALS are classified into "*clinically definite*", "*clinically probable*", "*clinically probable - laboratory supported*" and "*clinically possible*" categories. In the previous 1994 classification, patients with a pure LMN syndrome were classified into the "*clinically suspected*" category, now removed from the revised criteria: however, it is well recognized that several PALS who either have a pure LMN syndrome or early in the disease course do not have obvious UMN signs, will surely develop ALS (or a variant), but they will not fall into the categories of the revised criteria. Such criteria are thus probably more useful for research purposes and treatment trials, rather than daily clinical practice.

A recent rationalization of the El Escorial Criteria during the Awaji Consensus Conference in December 2006 simplifies the criteria [de Carvalho et al., 2008]: the so-called *Awaji-Criteria*, designed for everyday clinical practice, basically recommend that electrophysiological evidence of chronic neurogenic changes should be taken as equivalent to clinical data in the recognition of LMN involvement, and abnormal complex fasciculation potentials should be regarded as equivalent to fibrillation potentials or positive sharp waves.

NEUROPHYSIOLOGICAL METHODS

Conventional Electromyography

Electromyography (EMG) findings can support ALS diagnosis, by documenting LMN dysfunction in clinically involved and uninvolved regions, as it has already shown in the first published criteria for ALS electrodiagnosis [Lambert and Mulder, 1957].

EMG-detected LMN involvement should be found in at least 2 of the 4 regions - brainstem (bulbar MNs); and cervical, thoracic, or lumbar spinal cord (anterior horn MNs). It is enough to demonstrate EMG changes in one muscle (e.g. tongue, jaw, or facial muscles), for the brainstem region; either in the paraspinal muscles at or below the T_6 level, or in the abdominal muscles, for the thoracic spinal cord region; in at least 2 muscles innervated by different roots and peripheral nerves, for the cervical and lumbar spinal cord regions [Brooks et al., 2000]. Such revised El-Escorial criteria require evidence of both *active* or *ongoing denervation* (*fibrillation potentials and positive sharp waves*) and *chronic partial denervation* (*large motor unit potentials* of enhanced duration with an increased proportion of polyphasic potentials, often of enhanced amplitude; *reduced interference pattern* with firing rates > 10 Hz - unless there is a significant UMN component, in which case the firing rate may be < 10 Hz; and *unstable motor unit potentials*), although relative proportions may vary from muscle to muscle [Brooks et al., 2000]. Fasciculation potentials are a remarkable sign in ALS, although they can also be seen in normal muscles (*benign fasciculations*) and are not present in all PALS muscles [de Carvalho et al., 2008].

Nerve Conduction Studies

Because ALS symptoms can be similar to those of a wide variety of other, more treatable diseases, appropriate tests must be conducted to exclude the possibility of other clinical conditions. Nerve conduction (NC) techniques are required for detecting or excluding other disorders of peripheral nerve, neuromuscular junction and muscle that confound the diagnosis of ALS. The findings of NC studies (e.g. motor conduction velocity and distal motor latency) in PALS should generally be normal or near normal [de Carvalho and Swash, 2000], unless the compound muscle potential is small [Brooks et al., 2000].

Motor NC studies are also important in excluding *multifocal motor neuropathy*, by the detection of partial conduction block: a marked reduction of proximal amplitude or negative-peak area as compared with the distal ones (over 50%), in short segments (excluding entrapment sites) [de Carvalho et al., 2001]. F-wave studies are particularly useful in assessing proximal conduction, and abnormalities have been reported in PALS: enhanced F-wave latency with normal frequency and increased amplitude, and reduced F-wave velocity with decreased frequency. Prominent UMN features may be associated with an enhanced F-wave frequency [de Carvalho and Swash, 2000].

Sensory NC studies can show abnormalities in case of entrapment syndromes and coexisting peripheral nerve disease [Brooks et al., 2000]. In 10-20% of PALS, a subclinical sensory system involvement has been reported, suggesting an additional polyneuropathy or sensory ganglionopathy [Pugdahl et al., 2007].

Transcranial Magnetic Stimulation

Transcranial magnetic stimulation (TMS) allows a noninvasive evaluation of the corticospinal motor pathways, and detection of UMN lesions in patients without UMN clinical signs. Motor amplitude, central motor conduction time (CMCT), cortical threshold and silent periods can be easily measured using this method [Eisen and Shtybel, 1990].

Electrophysiological features compatible with UMN involvement include [Brooks et al., 2000]: up to a 30% increase in CMCT; and low firing rates of motor unit potentials on maximal effort. Marked CMCT prolongation is seen in fALS patients with D90A SOD1 mutations [Osei-Lah et al., 2004].

Neuroimaging Techniques

The most important use of neuroimaging in ALS diagnosis is to exclude treatable structural lesions (such as spinal cord tumor, a herniated disk in the neck, syringomyelia, or cervical spondylosis) mimicking ALS by producing varying degrees of UMN and LMN signs [Agosta et al., 2010], especially in patients with clinically probable or possible ALS [Brooks et al., 2000].

Magnetic resonance imaging (*MRI*) can be used in detecting corticospinal tract lesions: the most characteristic finding in ALS is corticospinal tract hyperintensity on T_2-weighted, proton density weighted and FLAIR-weighted MRI scans, and it is best visualised in brain, brainstem and, to a less extent, in spinal cord [Goodin et al., 1988; Waragai, 1997]. T_2-weighted MRI may also show hypointensity of the primary motor cortex, usually along the posterior bank of the precentral gyrus, although this is an inconsistent and non-specific finding [Oba et al., 1993].

More advanced structural neuroimaging modalities (such as *magnetic resonance spectroscopy*, *diffusion-weighted imaging*, *diffusion tensor imaging* and *magnetic resonance voxel-based morphometry*) and functional imaging techniques (*single photon emission computerized tomography*, *positron emission tomography* and *functional MRI*) have a limited role in routine clinical practice, but can be useful in understanding ALS pathophysiology *in vivo*, by identifying potential biomarkers of disease progression and detecting changes earlier in the disease course [Lombardo et al., 2009; Turner et al., 2009].

Laboratory Exams

Muscle Biopsy

Biopsy of skeletal muscle (or other tissues) is not required for diagnosis, unless to rule out a mimic syndrome (e.g. affecting muscle). In addition, muscle biopsy may be used to demonstrate LMN dysfunction in a body region when clinical or electrophysiological findings do not support this evidence. Histological findings that are compatible with ALS diagnosis include: scattered hypertrophied muscle fibers; no more than a moderate number of target fibers; fiber-type grouping of no more than mild-to-moderate extent; the presence of few necrotic muscle fibers [Brooks et al., 2000].

Biochemical Assays

Clinician may order tests on blood, urine and/or cerebrospinal fluid (CSF) samples to eliminate the possibility of other diseases, as well as routine laboratory assays. Clinical laboratory tests that may be abnormal in otherwise typical case of ALS include: a) serum muscle enzymes (creatine kinase, transaminases and lactic dehydrogenase); b) serum creatinine; c) chloremia and serum bicarbonate; d) CSF proteins [Brooks et al., 2000].

Moreover, in our experience, oxidative markers and cytokines/growth factors should be assayed overtime, in order to diachronically assess not only the body redox status, but also the dynamic balance among toxic and trophic factors [Bongioanni et al., 2001; 2002_a; 2002_b; 2008].

FUNCTIONAL ASSESSMENT

Global Functional Scales

Severity of PALS functional impairment has to be assessed at the first visit and monitored over time.

Evaluation of motor deficit implies an assessment of the resulting deficiency or incapacity and final disability. Many assessments have been proposed for patient's follow-up in order to analyze the state of motor function and their consequences on activities of everyday life [Couratier et al., 2006]. Clinimetric scales must be validated and relatively simple to use, and generate ordinate results allowing statistical analysis. The clinician should be aware of the different scales and their relative utility: knowledge of these scales, their validity, their sensitivity to modification, and their specificity and interpretation pitfalls is a prerequisite to good evaluation in daily practice and in clinical research.

The choice of which scale to use depends on the clinical objective: global scales (*Norris Scale* [Norris et al., 1974], *Appel ALS Rating Scale* [Appel et al., 1987], *ALS Severity Scale* (*ALSSS*) [Hillel et al., 1989], *ALS Functional Rating Scale* (*ALSFRS*) [Cedarbaum and Stambler, 1997]) can be employed to evaluate progression of the disability; other scales (*ALS Health State Scale*, *Global Clinical Impression of Change*) are used to classify patients by homogeneous stage of severity.

The Appel ALS Rating Scale

The total Appel score consists of 5 subscores: bulbar, respiratory, muscle strength, and lower extremity and upper extremity function. Each group is composed of individual tests: a group score of 6 is assigned, if there is no dysfunction and group scores of 30-36 points are assigned for maximal dysfunction. The total Appel ALS score is 30 for healthy subjects and 164 for those with maximal impairment [Appel et al., 1987] The rate of change in the Appel ALS Rating Scale is a significant predictor of survival for PALS [Haverkamp et al., 1995].

The ALS Severity Scale (ALSSS)

The ALS Severity Scale (ALSSS) measures 4 functional domains: a) speech; b) swallowing; c) lower extremity and walking; d) upper extremity, dressing and hygiene. Bulbar score results from speech subscore added to swallowing subscore; spinal score from lower extremity subscore plus the upper extremity subscore. Total score ranges from 4 to 40: the lower the score the more impairment is present. The average estimated reliability coefficient interraters is 0.95. The correlation between the speech rating and objective speech

measures is > 0.80. Total score, when combined with measurement of vital capacity, can provide a rapid and accurate assessment of the patient's disease status [Hillel et al., 1989].

ALS Functional Rating Scale

The *ALSFRS* is an instrument, easily administered by a clinician, an allied health professional, or a trained evaluator, for assessing PALS functional status. It is a validated questionnaire-based ordinal score system for measuring physical functions while performing normal daily tasks: it can be used for monitoring diachronically patient's functional changes. The ALSFRS components are grouped into 4 domains encompassing: a) bulbar functions; b) gross motor tasks; c) fine motor tasks, and d) breathing. It consists of 10 items, related to functional activities which are relevant for PALS (speech, salivation, swallowing, handwriting, cutting food and handling utensils, dressing and hygiene, turning in bed and adjusting bed clothes, walking, climbing stairs, and breathing). Each item is rated on a 4 (normal function) to 0 (maximal impairment) scale by the patients and/or caregiver, yielding a maximal score of 40 points [Cedarbaum and Stambler, 1997]. With appropriate training the ALSFRS can be administered with high interrater reliability and test-retest reliability (0.88 for all test items).

ALSFRS results are in close agreement with objective measures of muscle strength and pulmonary function. The ALSFRS may be used as a screening measure for entry into clinical trials, as a surrogate measure of function in situations in which muscle strength cannot be measured directly, or as an adjunct to myometry.

A limit of the ALSFRS as originally designed was that it granted disproportionate weighting to limb and bulbar, as compared to respiratory, dysfunction: authors have then validated a revised version of the ALSFRS, which incorporates additional assessments of dyspnea, orthopnea, and the need for ventilatory support, thus leading to 12 item numerosity (maximal total score: 48/48) [Cedarbaum et al., 1999]. Such a Revised ALSFRS ($ALSFRS_R$) retains the properties of the original scale and shows strong internal consistency and construct validity.

Somatic Muscle Strength Assessment

Besides the abovementioned clinimetric functional global scales, the quantitative assessment of muscle strength (which is a clinically relevant measure of ALS progression) - including the *Manual Muscle Testing* (*MMT*) and the *Maximal Voluntary Isometric Contraction* (*MVIC*) - complete the clinical motor PALS evaluation. Furthermore, the *quantitative EMG* can be regarded as a suitable complement to muscle strength assessment.

Manual Muscle Testing

Muscle strength is measured by a trained evaluator using the British *Medical Research Council* (*MRC*) grading scale in a standardized patient positioning. It was recently demonstrated that if enough muscles are tested, a decline in average grade can be determined early in the disease, and the variability of measurement approximates that of MVIC [Great Lakes ALS Study Group, 2003]. MMT advantages are speed, expense, and the lack of needed equipment; its disadvantages are low sensitivity to change in muscle strength, the fact that

data are ordinal, qualitative and subjective; furthermore, a large number of muscles must be evaluated to reduce variability and improve sensitivity.

Maximal Voluntary Isometric Contraction

The MVIC [Andres et al., 1987] has proven useful as an outcome measure in natural history studies and clinical trials with PALS, and is a valid and reliable measure of disease progression. MVIC can be measured using a hand-held dynamometer or a fixed device with strain gauges. The strength of individual muscle groups is determined quantitatively, and then the scores are normalized and combined into composite scores (called *megascores*). Intrarater and inter-rater reliability have been assessed in a number of clinical trials in ALS: with rigorous training of clinical evaluators, intra- and inter-rater reliability are less than 15% [Hoagland et al., 1997]. The MVIC is a good quantitative measure of the rate of decline of muscle strength, an outcome measure that is highly relevant to the disease. MVIC advantages include good intrarater and inter-rater reliability, sensitivity to small, clinically relevant changes and generation of numerically continuous data which are suitable for parametric statistical analysis. Problems limiting the use of MVIC are: it takes approximately 45 min to perform testing; requires expensive equipment; is not applicable for home visits; and data can not be obtained from very weak muscles.

A faster and portable method uses hand-held dynamometry (HHD) to test isometric strength of multiple muscles, again with standard patient positioning and rigorous training. HHD equipment is inexpensive and takes less than 5 min to complete a test of both upper and lower extremities. It has been directly validated against MVIC in PALS, and shown to change at a similar rate with variability that is only slightly greater than MVIC [Beck et al., 1999]: for both upper and lower extremity muscles, correlations between MVIC and HHD measurements ranged between 0.84 and 0.92, with test-retest variability that was extremely similar as well.

Quantitative Electromyography

Motor unit number estimation (*MUNE*) is a special electrophysiological technique that can provide a quantitative estimate of the number of axons innervating a muscle or group of muscles. MUNE consists of a number of different methods (incremental, multiple point stimulation, spike-triggered averaging, F-wave, and statistical methods), with each having specific advantages and limitations. Despite the lack of a perfect single method for performing MUNE, it may have value in the assessment of progressive motor axon loss in ALS, and may have use as an end-point measure in clinical trials [Bromberg and Brownell, 2008].

Respiratory Muscle Function Assessment

Vital capacity (*VC*), *maximal inspiratory pressure* (*MIP*) and *maximal expiratory pressure* (*MEP*) are the methods most commonly used to evaluate respiratory muscle strength. These measures are widely available, non-invasive and portable.

The *forced vital capacity* (*FVC*) measures volume of air forcefully expired in one breath: usually, the FVC is reported as a percentage of a predicted vital capacity based on subject's height, gender and age. The FVC declines with time in PALS and is a sensitive measure of

disease progression. Both the baseline FVC and the rate of decline in FVC are predictive of survival [Varrato et al., 2001].

MIP measures the maximal negative pressure at the mouth after complete exhalation followed by a single sustained maximal inspiratory effort against an occluded airway; whereas MEP is the maximal positive pressure measured at the mouth after inhalation to total lung capacity followed by a maximal expiratory effort against an occluded airway: both MIP and MEP, easy to determine and reproducible, are sensitive early indicators of respiratory muscle weakness.

However, these tests are associated with increased variability in patients with significant bulbar involvement and require maximal respiratory muscle activation [Lyall et al., 2001]: bulbar or facial weakness can prevent the formation of a tight lip-seal around a mouthpiece; vocal cord spasms and excessive saliva and gagging can also interfere with study performance. Other measures of respiratory muscle function that might be more useful in patients with bulbar involvement and as outcome measures include *maximal sniff esophageal*, *transdiaphragmatic*, and *nasal pressures*.

Swallowing Assessment

Proper evaluation of swallowing problems should begin with a detailed history and oropharyngeal examination by the speech therapist. Generic dysphagia scales are reliable indicators of dysphagia in ALS. The bulbar components of the ALS specific scales is sensitive to dysphagia: the bulbar section of the Norris scale [Norris et al., 1974] can be utilised as an independent and reliable indicator of the severity of dysphagia in ALS.

Clinimetric dysphagia assessment has to be supplemented by instrumental evaluation (videofluoroscopy, fiberoptic examination of the swallowing, and oropharyngoesophageal scintigraphy, OPES) [Fattori et al., 2006].

At present, videofluoroscopy is considered the gold standard for assessing dysphagia in PALS; nevertheless, this procedure allows only qualitative visual evaluation of the swallowing act, without providing any quantitative data. On the other hand, OPES not only permits functional evaluation of swallowing, but it is also used to obtain a detailed semiquantitative assessment of the various stages of swallowing, especially data on transit time and retention of the bolus through the mouth, pharynx, and esophagus; moreover, this technique also provides data on the bolus fraction aspirated into the tracheobronchial tract, if present [Fattori et al., 2006]. As for videofluoroscopy, the main limitation of OPES is a low anatomic definition.

In the absence of availability of detailed instrumental swallowing assessment, clinimetric scales (i.e., the Norris and to a lesser degree the ALSFRS bulbar sections) are adequate to follow clinically significant dysphagia in PALS, and can be used as an indicator for dysphagia treatment initiation.

Speech Assessment

Bulbar involvement leads to progressive dysarthria, unintelligibility and anarthria [Tomik and Guiloff, 2010]. It is associated with significant social and psychological problems:

significant dysarthria can lead to frustration on the part of the patient when others are unwilling to spend the time to carefully listen. Friends and healthcare workers may tend to "do all the talking" and not listen to the patient; there is a temptation to anticipate answers and finish sentences for the patient.

Yorkston et al. [1993] initially suggested that PALS speaking rate reduction precedes decreases in intelligibility; Ball et al. [2001, 2002] reported that speaking rate on the *Speech Intelligibility Test* (*Sentence Subtest*) [Yorkston et al., 2007] is a relatively good predictor of PALS intelligibility deterioration. This computerized test supports the efficient measurement of speaking rate in clinical settings; it helps patients and their families monitor changes over time, prepare for an *Augmentative and Alternative Communication* (*AAC*) evaluation, and reinforces their understanding of rate and intelligibility. Using this test, speaking rate can be accurately monitored over the telephone if a patient lives at a distance, or is unable to travel [Ball et al., 2005]: it should be noted, anyway, that speech intelligibility could not be objectively assessed over the telephone, as a clinical measure of understandability.

TREATMENT

Attempts have been undertaken in the past and present and will be undertaken in the future to bring a stop neurodegeneration in PALS: the ideal outcome for the patient would be complete cessation of symptoms and restitution of full muscle strength and motor control.

At the present time, the neurologist's attempts to treat ALS can be organized under the following multiple modalities of treatment: a *pathogenetic therapy* (encompassing drugs and other biological and electrophysiological approaches) – to counteract MN degeneration; and a *symptomatic therapy* (pharmacological and not, including assistive rehabilitation) – to reduce impairments in motor abilities, in particular body movements in activities of daily living, breathing, nutrition, communication, and a variety of related symptoms, such as spasticity, pain, dyspnea, or sialorrhea.

The appropriate implementation of each one of these types of therapy reflects the difficulties that we now have to face in ALS treatment. Differently from an acute, self-limited disease with expected recovery, the choice of appropriate therapeutic options for PALS raises more difficult concerns, since one must take into account many personal and ethical considerations. Several decisions by PALS and their families regarding treatment hinge on their concept of the quality of life that will result from such treatments.

PATHOGENETIC TREATMENT

This type of treatment is addressed to counteract the biochemical mechanisms leading to MN degeneration, by employing drugs, cells or an electrophysiological approach.

Neuropharmacological Strategies

Over the years several studies have been performed with different types of drugs, acting on the mechanisms involved in MN degeneration. Potential therapies for ALS are being

investigated in animal models: some of this work involves experimental treatments with normal SOD1 and other antioxidants; in addition, neurotrophic factors are being studied for their potential to protect MNs from pathological degeneration. Although no effective cure has yet been found for ALS, investigators are optimistic that these and other basic research studies will eventually lead to treatments for ALS.

In nineties the Food and Drug Administration has approved the first drug treatment for the disease, *Riluzole*: it is believed to reduce damage to MNs by decreasing the release of glutamate. Clinical trials with PALS showed that Riluzole prolongs survival by several months; it also extends the time before a patient needs ventilation support. Nevertheless, it does not reverse the damage already done to MNs, and patients taking the drug must be monitored for liver damage and other possible side effects. However, this first disease-specific therapy offers hope that ALS progression may one day be slowed by new medications or combinations of drugs.

Each mechanism involved in ALS pathogenesis may represent a possible therapeutic approach to counteract MN neurodegeneration. We now focus on some therapeutic clinical trials recruiting PALS or recently concluded, ordered according the pathogenetic mechanism involved.

Excitotoxicity

A pilot study with a noncompetitive modulator of AMPA glutamate receptors, *Talampanel*, showed a slower decline in $ALSFRS_R$ score rate during the 9-month treatment; subsequently, a multinational, multicenter, randomized, double blind, placebo-controlled, parallel-group study to assess the efficacy, tolerability and safety of Talampanel in PALS (ALS-TAL-201) was performed enrolling 550 patients, beginning on September 2008: on May 2010 the pharmaceutical company Teva announced that the results were conclusively negative, since the $ALSFRS_R$ score showed no difference in the progression rate of PALS treated with placebo or either with 2 doses of Talampanel; and side effects were more common in drug-treated participants treated, whereas the dropout rates were very similar in all groups (www.asla.org 2/7/2010).

Given the increase in *excitatory amino acid transporter 2* expression and function documented in transgenic mice overexpressing a mutant SOD1 (mtSOD1) treated with *Ceftriaxone* at symptom onset [Rothstein et al., 2005] and case reports with *cephalosporins* indicating a clinical improvement [Harvey and Martz, 2007], an open label clinical trial with bimonthly drug cycles of Ceftriaxone (2 g/day, for 14 days every two months) was performed: after one-year treatment a significant improvement of antioxidant oxidative stress status was observed, but no clinical improvement [Siciliano et al., 2010]. A wide double blind placebo-controlled clinical trial on about 600 PALS is currently recruiting participants in the United States of America (NCT00349622) [www.clinicaltrial.gov] since July 2006: final data collection for the primary outcome measure (survival and rate of change in $ALSFRS_R$) will be available in June 2012.

Memantine, a low-affinity noncompetitive NMDA receptor antagonist, may be useful in protecting MN from death [Chen and Lipton, 2006]. Recently, the results of a phase II/III, 12-month, double-blinded, single-centre, randomized clinical trial performed to evaluate the efficacy and safety of Memantine (20 mg/day) in 63 PALS have been reported [de Carvalho et al., 2010]: primary (12-month ALSFRS decline) and secondary (FVC, MMT, visual analogue scale, quality of life, MUNE and neurophysiological index) outcomes were not

significantly different between the two groups; Memantine is safe and well tolerated. Actually is ongoing and currently recruiting participants in Canada a "Randomized, double blind, dose-ranging study to determine the effect of Memantine on functional outcomes and motor neuron degeneration in patients with ALS" (NCT00409721).

Oxidative Stress and Apoptosis

Based on several findings of increased oxidative damage in PALS spinal cord and cortex MNs [Ferrante et al., 1997] and imbalance of oxidative stress reported also in CSF [Smith et al., 1998; Ihara et al., 2005], a large variety of antioxidants compounds have been tested in PALS.

Although preclinical studies demonstrated a prolonged survival following treatment with *Coenzyme Q10* [Matthews et al., 1998], an antioxidant, essential mitochondrial cofactor facilitating electron transfer in the respiratory chain, the phase II study in PALS (NCT00243932) showed insufficient promise to warrant Phase III testing [Kaufmann et al., 2009].

After positive results obtained in animal models [Crow et al., 2005], a pilot study with *Manganoporphyrin*, *AEOL 10150*, an antioxidant molecule that catalytically neutralizes superoxide, hydrogen peroxide and peroxynitrite, and inhibits lipid peroxidation [Patel and Day, 1999] showed that single doses of drug, ranging from 3 mg to 30 mg, were tolerated without serious side effects [www.rideforlife.com].

In keeping with maintaining mitochondrial function in ALS, a two-part Phase II randomized, double-blind trial (NCT00647296) with *KNS-760704* including 102 PALS, recently concluded: preliminary results showed that KNS-760704 is safe and well tolerated, and that 300 mg daily may slow the rate of motor function loss [Bozik et al., 2010].

Yoshino and Kimura [2006] investigated the efficacy and safety of *Edaravone*, a free-radical scavenger now widely used for the treatment of acute cerebral infarction for its neuroprotective effect against oxidative damage [Uno et al., 2005], in an open trial design on 20 PALS receiving either 30 mg or 60 mg intravenously once per day. Two weeks of administration were followed by a 2-week observation period: such a 4-week cycle was repeated 6 times. During the 6-month treatment period, the decline in the $ALSFRS_R$ score was significantly less than that in the 6 months prior to Edaravone administration; in almost all patients, CSF 3-nitrotyrosine, a marker for oxidative stress, was markedly reduced to almost undetectable levels at the end of the 6-month treatment period. This study suggested that Edaravone is safe and may delay the progression of functional motor disturbances by reducing oxidative stress in PALS.

Premature apoptosis and/or an aberration in apoptosis regulation is implicated in ALS MN degeneration [Pasinelli et al., 2004]. Transcriptional dysfunction has been implicated in the pathogenesis of many neurodegenerative diseases including ALS [Rouaux et al., 2007]. Therefore, histone deacetylase (HDAC) inhibitors may be promising candidates.

Valproic acid (*VPA*) is a HDAC inhibiting drug [Kanai et al., 2004] that promotes gene transcription and inhibits neuronal cell death by counterbalancing apoptosis, oxidative stress and glutamate toxicity [Morland et al., 2004]. Based on the finding that VPA and other HDAC inhibitors increased survival in mtSOD1 mice [Sugai et al., 2004], 163 PALS received 1,500 mg VPA or placebo daily ("A randomized, double blind, placebo-controlled sequential clinical trial of sodium valproate in ALS" - NCT00136110): unfortunately, VPA at a dose used in epilepsy did not show a beneficial effect on survival or disease progression in PALS.

In the "Long term extension study of TCH346 and placebo administered once daily in patients with amyotrophic lateral sclerosis" (NCT00230074) conducted on 591 PALS enrolled from 42 sites in Europe and North America, patients received orally once daily for at least 24 weeks either placebo or one of 4 doses (1.0, 2.5, 7.5, or 15 mg) of *TCH346*, an antiapoptotic compound exerting its effects by binding to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and blocking the apoptotic pathway in which GAPDH is involved. At the end of the study there were no significant differences between placebo and active treatment groups in the mean rate of $ALSFRS_R$ score decline or in the secondary outcome measures (survival, pulmonary function and MMT).

The administration *Minocycline* to PALS in a multicentre, randomized placebo-controlled phase III trial (NCT00047723) produced negative results with $ALSFRS_R$ score deterioration that was faster in the Minocycline group than in the placebo group [Gordon et al., 2007], even if it was reported to prolong survival by 10-22% in transgenic mouse models of ALS [Kriz et al., 2002; Van Den Bosch et al., 2002].

Having shown preclinical studies antioxidant, antiapoptotic and neuroprotective properties of *Tauroursodeoxycholic Acid* (*TUDCA*) in the CNS, in both *in vitro* and *in vivo* models [Castro et al., 2004], a small Italian phase II trial (NCT00877604) involving 20 people with MND is now underway to assess whether the addition of TUDCA to riluzole can slow the disease progression: the trial will run for 12 months and results are expected quite soon.

Neurotrophic Factors

Insulin-like growth factor 1 (*IGF-1*) is a neurotrophic factor that has been tested in a North American and in an European phase III trial, enrolling 266 and 183 PALS, respectively. The two trials showed contrasting results, since IGF-1 either slowed the rate of functional decline by 26% [Lai et al., 1997], or did not produce positive effects [Borasio et al., 1998]. Subsequently, another phase III trial using IGF-1 treatment for 2 years has been performed on 330 PALS, showing that the drug did not produce beneficial effects on MMT, $ALSFRS_R$ or in delaying tracheostomy [Sorenson et al., 2008].

A double blind, randomized, parallel-group study to evaluate safety and tolerability with *VEGF165* administered intracerebroventricularly (NCT00800501) is currently recruiting participants [www.clinicaltrial.gov].

Neuroinflammation

Thalidomide has potent anti-inflammatory properties through the modulation of inflammatory cytokines, such as TNF-α, and appreciably penetrates the CSF [Franks et al., 2004]. Although a pilot study with Thalidomide was interrupted for safety concerns, a non-randomized, open label phase II study was further performed (NCT00140452), showing that the drug can cause side effects without effectively modulating disease progression [Stommel et al., 2009].

Arimoclomol, a molecule able to determine an enhanced production of the heat shock proteins, thus protecting cells from protein aggregation and death [Kieran et al., 2004], determined a prolonged survival of mtSOD1 mice, either if treatment was started in presymptomatic stage [Kieran et al., 2004] or at the onset of symptoms [Kalmar et al., 2008]. After a Phase IIa Arimoclomol trial showed that the drug is safe and well tolerated

[Cudkowicz et al., 2008], a phase II/III adaptive, randomized, placebo-controlled clinical trial is still recruiting fALS patients (NCT00706147) [www.clinicaltrial.gov].

Autophagy

Lithium is a drug regulating autophagy: it induces autophagy by inhibiting the intracellular enzyme inositol monophosphatase [Sarkar et al., 2005], whereas glycogen synthase kinase-3β inhibition (at higher drug concentrations) determines a negative regulation of autophagy via the mammalian target of *rapamycin*, *mTOR* [Yang and Guan, 2007]. Lithium-induced autophagy counteracts neurodegeneration [Pasquali et al., 2009] and appears to be neuroprotective for MNs both in organotypic slice cultures of spinal cord [Caldero et al., 2010] and in mtSOD1 mice, where it improves motor function and slows disease progression [Fornai et al., 2008].

A 15-month pilot clinical trial in randomized PALS showed that Lithium and Riluzole co-treatment markedly reduced mortality when compared with matched control patients treated with Riluzole alone [Fornai et al., 2008]. However, inconsistent results have also been reported: in a sibling-matched, gender-balanced, investigator-blinded trial, using a standard mouse model of fALS chronic Lithium treatment showed no benefit [Gill et al., 2009]; another study also found no therapeutic or neuroprotective effects of Lithium in female ALS mice [Pizzasegola et al., 2009].

A randomized, double blind, placebo-controlled trial was conducted on 84 PALS by the Northeast ALS and Canadian ALS Consortia: Lithium did not produce safety concerns, but treatment was interrupted for futility at the first interim analysis (24 weeks), when a 43% improvement did not occur in patients actively treated [Aggarwal et al., 2010].

Neurobiological Strategies

Gene Therapy

Accumulation of altered, misfolded and aggregated proteins in both fALS and sALS seems to be involved in the pathogenesis of the disease. Decreasing levels of these proteins, by downregulating the genes responsible for such accumulations, may represent a therapeutic strategy aimed to ameliorate the disease state. *Antisense oligonucleotides* to SOD1 administered near onset in mtSOD1 rats reduced SOD1 in brain and spinal cord, and significantly slowed disease progression [Smith et al., 2006]. Based on these findings, a clinical trial using Antisense oligonucleotides to SOD1 will start in fALS patients with SOD1 mutations [www.alsa.org].

Stem Cell Therapy

Stem-cell transplantation is an attractive treatment approach for neurological diseases. Previous studies performed in neurodegenerative disease animal models generated optimism about restoring functions or delaying neurodegeneration in human beings: autologous or allogeneic stem cells, undifferentiated or transdifferentiated, might represent a new therapeutic strategy for PALS [Silani et al., 2004]. Although scientifically based clinical trials using stem cells to treat neurodegenerative diseases have already been carried out, unfortunately none of them has achieved a real benefit: nevertheless, such treatments are somewhere offered around the world, often underestimating the potential risks of the procedure [Lau et al., 2008].

We need strict laboratory guidelines based upon well established preclinical studies, and rigorous clinical protocols in order to successfully develop a safe and effective stem cell therapy for neurodegenerative diseases; furthermore, we have to carefully deal with ethical, social and economic implications.

Neurophysiological Strategies

Repetitive Transcranial Magnetic Stimulation

Another approach to modulate glutamatergic circuits of human motor cortex is the *repetitive transcranial magnetic stimulation* (*rTMS*). Such a technique has potential therapeutic effects in several neurological diseases, including ALS [Lefaucheur, 2008]. Repetitive stimulation of the motor cortex was performed for 5 consecutive days every month for 6 consecutive months in 20 PALS participating to a double blind, placebo-controlled clinical trial [Di Lazzaro et al., 2006]: both active and sham patients deteriorated during treatment, with active patients having a modest, but significant, slowing of the deterioration rate. Based on these findings, a double blind, placebo-controlled trial on 20 PALS randomly allocated to blinded real or placebo stimulation was performed, to test the hypothesis that rTMS given as continuous theta burst stimulation, repeated for 5 consecutive days monthly for one year, would affect disease progression: treatment was well tolerated, but there was no significant difference in the $ALSFRS_R$ score between patients' groups [Di Lazzaro et al, 2010].

SYMPTOMATIC TREATMENT

Other treatments are designed to relieve symptoms and improve the quality of life for patients. This supportive care is best provided by multidisciplinary teams of health care professionals such as physicians; pharmacists; physical, occupational, and speech therapists; nutritionists; social workers; and home care and hospice nurses [Bede et al., 2011]. Working with patients and caregivers, these teams can design an individualized plan of medical and physical therapy and provide special equipment aimed at keeping patients as mobile and comfortable as possible.

Pharmacological Strategies

Physicians can prescribe medications to ameliorate fatigue, ease muscle cramps, control spasticity, and reduce excess saliva and phlegm. Drugs also are available to help patients with pain, depression, sleep disturbances, and constipation [Bede et al., 2011].

For an exhaustive review on this topic s. Gordon [2011]; as well as the American Academy of Neurology Practice Parameters [Miller et al., 1999; $2009_{a,b}$] for a meta-analysis of peer-reviewed studies on symptomatic treatment of PALS.

Non Pharmacological Strategies

The primary goal of an effective assistive rehabilitation for PALS is the management of disabilities, symptoms and complications arising from the progressive weakness of limb, trunk, and bulbar muscles [Norris et al., 1985]. Further goals include keeping the patient functioning as independently as possible and maintaining quality of life even into the terminal stage.

The rehabilitation program varies depending on whether the patient has a long clinical course or rapid progression of the disease: in the former case, PALS become able to compensate remarkably well for the motor unit loss and are able to continue with their daily activity for several years [Chen et al., 2008]. The success of the rehabilitation approach depends on the active participation of the patient who should be a full partner in the therapeutic team even during the advanced stages of the disease. It may be difficult for the physician to discuss such a fatal illness: however, a direct approach allows the patient to deal most effectively with the disease and its physical limitations. This also helps in decisions about the intensity of the therapeutic effort [Bede et al., 2011; Gordon, 2011].

The family and other caregivers should be encouraged to participate in the patient's early rehabilitation program: the family role will then likely increase as weakness progresses, requirements for assistive devices change, and new problems arise in the management of activities of daily living.

Physical Exercise

Patients and their families often have questions concerning physical therapy: several may have been exposed to exercise or fitness programs in the past, and others will have known patients who received rehabilitation followed neurological or orthopaedic dysfunction. They might reason that if weakness of a muscle group exists, exercise can strengthen it. It is important for PALS to understand that the cause of his/her weakness is a chronic and progressive loss of MNs that will not be replaced by exercise. The patient must understand that, although there may be temporary improvement in function, other muscle groups will become increasingly involved. However, healthy muscle groups can be strengthened by exercise and used to compensate for the weakness of other muscle groups [Chen et al., 2008].

The question of whether regular exercise is beneficial or not in PALS is still controversial [Francis et al., 1999]. Moderate regular exercise is helpful in the management of many neuromuscular diseases [Lui and Byl, 2009], allowing weak muscles to increase their mitochondrial content and to enhance muscle blood flow and strength.

Some epidemiological studies suggest that vigorous physical activity in the form of heavy labor or competitive athletics increases ALS risk [Chiò et al., 2005]. On the contrary, other studies report earlier disease onset among individuals with a greater amounts of leisure time and reduced physical activity [Veldink et al., 2005] or that physical activity is not a risk factor for developing ALS [Qureshi et al., 2006]: anyway, frequently PALS do not perform any type of physical activity in order to preserve their muscle strength and minimize overwork muscle damage [Longstreth et al., 1991].

Preclinical studies in the transgenic mouse model of ALS have demonstrated beneficial effects of physical activity on motor function. The effects of high and moderate levels of exercise were investigated in mtSOD1 mice [Carreras et al., 2010]: in mtSOD1 mice

undergoing moderate exercise, a preservation of motor performance (correlating with higher MN density in the ventral horn of the lumbar spinal cord) was showed.

Investigation of some oxidative stress markers during submaximal incremental exercise at a power level corresponding to the anaerobic lactate threshold in patients), showed an increased production of lactate and lipoperoxides [Siciliano et al., 2002], suggesting that regular aerobic physical activity, rather than anaerobic exercise, may be useful in ALS. Gentle, low-impact aerobic exercise such as walking, swimming, and stationary bicycling can strengthen unaffected muscles, improve cardiovascular health, and help patients fight fatigue and depression. Range of motion and stretching exercises can help prevent painful spasticity and shortening (contracture) of muscles. Physical therapists can recommend exercises that provide these benefits without overworking muscles.

Clinical trials on PALS have suggested that regular physical exercise may be neuroprotective, ameliorating symptoms and improving functionality [Drory et al., 2001; Bello-Haas et al., 2007]. Drory and colleagues [2001] conducted an observational study to determine the effect of moderate regular exercise under professional guidance on motor deficit, disability, fatigue, musculoskeletal pain and perceived quality of life. They randomized 25 PALS to perform either a specific moderate daily exercise program or just their daily physical activity. At 3 months, patients who performed regular exercise showed less deterioration on ALSFRS and Ashworth spasticity scales; at 6 months, there was no significant difference between groups, although a trend towards less deterioration in the treated group on most scales was observed. At 9 and 12 months there were too few patients in each group for statistical evaluation. Their results showed that a regular moderate physical exercise program has a shortlived positive effect on disability in PALS.

Bello-Haas and coworkers [2007] performed a study in which they investigated the effects of resistance exercise on function, fatigue, and quality of life in 27 PALS using ALSFRS, the Fatigue Severity Scale, Short Form-36 (SF-36), FVC and MVIC at baseline and monthly for 6 months. Patients in mild stage of disease were randomly assigned to a resistance exercise group (daily stretching and resistance exercises three times weekly) or to a usual care group (only the daily stretching exercises). Eight of the 13 enrolled resistance exercise patients and 10 of the 14 enrolled usual care patients completed the trial. At 6 months, the resistance exercise group had significantly higher ALSFRS and SF-36 physical function subscale scores and less decline in leg strength, as measured by MVIC. Physical exercise exerts positive effects on healthy MNs, by enhancing dendritic restructuring, protein synthesis, axonal transport and neuromuscular communication [Gardiner et al., 2006; McCrate and Kaspar, 2008].

Physical therapy and special equipment can enhance patients' independence and safety throughout the course of ALS. The role of physical therapy may vary greatly from patient to patient, and it is difficult to evaluate the benefit of therapy in patients whose disease continues to progress: however, the benefits gained, both physical and psychological, are usually worthwhile. Patient function can be improved by the use of supportive appliances and orthotic devices. In many istances, weakness in one area may lead to a loss of balance that impairs function, despite adequate strength in other muscle groups. A device as simple and inexpensive as a soft cervical collar may provide weakened neck muscles with enough support to prevent forward head tilt, resulting in gait imbalance. Use of a support, such as a cane, walker, or brace may greatly improve a patient's ability to ambulate. Many patients deny their problem and refuse to use aids such as a walker: unfortunately, falls secondary to

weakness are common, and resulting injuries such as hip fractures can ultimately worsen disability. PALS can be taught to perform various functions using alternative approaches.

Occupational therapists can suggest devices such as ramps, braces, walkers, and wheelchairs that help patients conserve energy and remain mobile. Small investments in the home and work environment can greatly enhance the quality of life. For instance, a patient unable to button clothing can usually use Velcro fasteners. Self-help books, practical manuals from the ALS associations worldwide, and catalogs from vendors dealing in merchandise for the handicapped can all be valuable resources for PALS and their families: simple solutions are often available for difficult problems, and they need only to be considered.

Respiratory Treatment

Most PALS eventually die of complications due to respiratory dysfunction: these problems may arise from weakness of breathing muscles (leading to hypercapnia, hypoxemia and respiratory failure); and from weakness and incoordination of the bulbar muscles (resulting in a weak cough, aspiration and sleep apnea).

The strength of the respiratory muscles should be monitored with pulmonary function studies (s. Functional assessment – Respiratory muscle function assessment): when pulmonary problems are present, they need to be discussed with the patient and his/her family, and alternatives for respiratory management should also be discussed at that time. Once MIP or MEP falls below 25 cmH_2O or the VC is < 1 l, closed attention must be paid to the patient: if the deterioration in respiratory parameters has been relatively slow, and if the patient is asymptomatic, reliable and well supervised, he/she has to be only carefully followed up [Hardiman, 2011].

Respiratory Exercises

Several strategies can be used to improve respiratory muscle strength and decrease respiratory fatigue: the respiratory muscles, while subject to fatigue like other skeletal muscles, are trainable to potentially achieve greater endurance and strength. The opportunity to increase respiratory muscle strength and endurance, and hence to postpone respiratory failure, must be tempered with the possibility that vigorous exercise may itself induce respiratory muscle failure: this might occur in situations where exercise levels require peak inspiratory pressures greater than the critical level for fatigue. Furthermore, the actual stress caused by exercise could theoretically disrupt muscle fibers. The clinician must keep in mind that endurance and strength-building strategies can only work with intact motor units: muscle fibers becoming denervated will be reinnervated by healthy MNs in the early stages of the disease, but eventually the ability of the nervous system to reinnervate can not keep pace with the increasing number of denervated muscle fibers, which are unlikely to respond to any muscle-training exercise [Hardiman, 2011].

Strategies for improving respiratory muscle strength become most important when the normal work of breathing begins to approach a critical threshold; once it is exceeded, the normal act of breathing will lead to fatigue and ventilatory failure. Patients may exceed this work threshold because of other variables, including minor respiratory tract infections which have to be aggressively treated.

Respiratory muscle strength may be maintained or enhanced with inspiratory-resistive training exercises. Home deep-breathing exercises can be repeated frequently and may be more beneficial with an incentive spirometer. Although the exercises may be performed while the patient is in any position, they are best done with the patient in a supine position. While prolonged benefit from training to improve exercise performance should not be expected in PALS, in many cases, anyway, a training program may delay the onset of ventilatory failure, and give an important psychological support from such an active subject's participation in the fight against his/her disease.

Assisted coughing after a deep breath and chest physical therapy for postural drainage are extremely helpful, and the patient's family may be instructed in these maneuvers [Senent et al., 2011].

Mechanical Ventilation

Whereas a normal $PaCO_2$ does not guarantee that rapid deterioration will not occur, an enhanced $PaCO_2$ (> 45 mmHg) suggests the need for mechanical ventilation. Once MIP and MEP drop below 15 cm H_2O, or the $PaCO_2$ rises to even the smallest extent over normal, the initiation of mechanical ventilation is recommended: the decision concerning long-term mechanical ventilation is a complex one and needs to be approached early in the patient's course and re-evaluated at each visit. Respiratory therapists can help caregivers with tasks such as operating and maintaining ventilators.

Intermittent *noninvasive ventilation* (*NIV*) consists in a positive pressure ventilator (delivering two levels of pressure, inspiratory and expiratory: BiPAP) with a tigh-fitting nasal (or orofacial, or facial) mask. At the beginning, BiPAP will be used nightly, to aid breathing during sleep; then, when muscles are no longer able to maintain O_2 and CO_2 levels, this device will has to be used full time. Such a ventilator can help rest fatigued respiratory muscle and, at least in the short term, may be well tolerated by the patient: however, more chronic use often results in skin breakdown and ulcer formation over the bridge of the nose. NIV can postpone the initiation of *invasive ventilation* (*IV*) for several months; prolong life and improve comfort; allow the PALS to experience a limited form of mechanical ventilation while arriving at a decision concerning full IV [Hardiman, 2011].

NIV, however, is less effective than IV in promoting air exchange. Patients must be told that ventilatory devices are not curative and that further deterioration will occur; furthermore, the upper airway will not be protected as it would be with *tracheostomy*.

Swallowing Treatment

Although PALS with bulbar involvement suffer from more severe swallowing problems (such as aspiration), predominantly nonbulbar PALS may also have dysphagia.

Dysphagia in PALS is known to be characterized by impairment of the oral stage of swallowing, with ensuing impact on the next phase of swallowing, the pharyngeal stage. Deficit in the oral stage first results in increased length of the time required to take a meal: with milder abnormalities, the patient may be able to compensate for any swallowing impairment. A predominant deficit in soft palate muscles may result in nasal regurgitation of fluids, although this symptom is often diminished by concomitant deficit in lingual propulsion.

Several problems related to eating can be treated by relatively simple methods.

Dietary content should be changed to foods that are easy for PALS to swallow: in general, soft but firm foods with a smooth texture are easiest to swallow. Patients should be encouraged to experiment with various textures, temperatures, and types of food. Swallowing water and other thin liquids may prove difficult, due to premature spillover into the pharynx; thicker liquids (e.g. fruit nectars, shakes, and commercial food thickeners) may be used. If chewing is a problem, foods requiring less chewing should be replaced and a blender can be used to homogenize food. As dietary content becomes limited, it can be supplemented with commercially available complete liquid diets; if water intake is reduced due to choking, the nutritionist should suggest adding foods that are high in water content.

Swallowing initiation may be improved by chewing ice or thermal stimulation. Neck extensor weakness can often be successfully managed with a cervical collar. Postures and behavioral modifications can correct swallowing difficulties for a variable interval before other therapeutic interventions are necessary. Documentation of aspiration of a particular food warrants initiation of proper swallowing techniques (postural changes and specific exercises) [Logemann, 2008].

As bulbar muscle weakness progresses, symptoms and signs of decompensated swallowing appear. They are dramatic, and include coughing and choking with feeding, aspiration pneumonia, and asphyxia due to a solid bolus obstructing the airway; frequent throat clearing, wet voice, nasal regurgitation, retention of solid food particles in the pharynx, and delayed food passage.

Eventually, the time comes when a decision has to be made concerning the use of a feeding tube. Patients should be clearly informed that modern techniques for feeding tube placement have greatly decreased the discomfort and morbidity associated with the procedure [Spataro et al., 2011].

Sialorrhea Treatment

Patients complain of drooling or difficulty in controlling their saliva: this problem not only is embarrassing, but also can be life-threatening if secretions are aspirated.

Swallowing difficulties may lead to excessive dribbling of saliva in many PALS. Normally, 1-2 ml of saliva are produced each minute: this is usually swallowed without conscious effort. Weakness of neck and facial muscles will cause a patient to tilt the head forward with lips open: instead of saliva running backwards and being swallowed, it runs forward and drips.

Proper positioning, lip-tightening exercises, and a cervical collar may be helpful.

As the problem progresses, pharmacological intervention may be tried with drying agents such as anticholinergics and certain antidepressants (e.g. *amitryptiline*).

Many patients prefer a suction machine to handle oral secretions while avoiding drug side effects. The use of botulinum toxin and radiotherapy of salivary glands have also been proposed [Guidubaldi et al., 2011; Guy et al., 2011].

Speech Treatment

Dysarthric PALS may benefit from working with a *speech therapist*. These health professionals can teach patients adaptive strategies, such as techniques to help them speak louder and more clearly. In early disease stages, patients can be taught to emphasize certain syllables and slow their speech patterns so that others can understand them better: lip and tongue exercises can sometimes help the patient to enunciate words more clearly on a regular basis.

A recent review on ALS communication research [Hanson et al., 2011] concluded that, due to ALS pathophysiology and the intrinsic degenerative nature of disease, speech treatment strategies designed to increase strength or mobility of the oral musculature are not recommended for PALS. Patients or their caregivers, on the contrary, often request oral exercises to improve strength and mobility for speech, as strengthening exercises seem intuitively to them as a way to increase performance: however, such exercise programs should be discouraged, and PALS should be informed that the speaking that they do each day provides a sufficient amount of speech mechanism activity and exercise.

Speech intervention should focus on learning to conserve energy for priority speaking tasks and to rest often to reduce fatigue, instead of increasing effort with speech exercises. PALS speakers should learn to avoid adverse speaking/listening situations by muting the television, inviting people to speak with them in a quiet place rather than in a crowded room, and using voice amplification when speaking in noisy environments to reduce the effort required [Ball et al., 2007; Yorkston et al., 2010].

Writing my be used as a substitute for speech, and devices as simple as paper and pencil, alphabet cards, portable typewriter, and letter boards may be used by patients with adequate hand function. Becoming speech more and more difficult to understand, many PALS supplement their speech by identifying the first letter of each word on an alphabet board (*alphabet supplementation*), or by identifying the topic on a communication board (*topic supplementation*).

As ALS progresses, speech therapists can help patients develop ways for responding to yes-or-no questions with their eyes or by other nonverbal means, and can recommend aids such as speech synthesizers and computer-based communication systems. These methods and devices help patients communicate when they can no longer speak or produce vocal sounds.

It is frequently difficult for PALS speakers, their caregivers and medical personnel to consider AAC strategies when they are still using residual speech to meet daily communication needs. However, their speaking rate should be clinically monitored so that the referral for an AAC intervention is initiated in a timely manner: Ball et al. [2001, 2002] recommend that patients be referred for AAC assessment when their speaking rates reach 125 words/min (normal value: 190 words/min) on the *Speech Intelligibility Test* (*Sentence Subtest*) [Yorkston et al., 2007]. With sufficient education and preparation, PALS and their caregivers are ready to examine their AAC options. Nevertheless, speech deterioration can be so rapid that individuals can be left with limited communication options, if they are not prepared to act in an opportune manner.

Due to the extended use of AAC with deteriorating levels of physical control, it is imperative that recommended technology has adjustable access options to meet the range of motor capability as the disease progresses, that is, PALS should be fitted with AAC technology that supports multiple access methods, such as allowing them to transition from

hand access to scanning and/or head/eye-tracking. Many AAC devices now incorporate a variety of access options so that the technology can continue to meet the needs of the user despite a decline in physical capability. The sensitivity of dynamic touch screens can be adjusted to allow for lighter touch. The improved sensitivity of head-tracking technology has allowed many to use this access method with minimal head/neck movement control.

Perhaps the most significant advancement in access technology has occurred with the widespread availability of *eye-tracking systems* to allow cursor control with eye movement to access high-technology AAC devices. As the disease progresses, many PALS require the use of eye-tracking for several reasons. Firstly, eye-tracking is often the least fatiguing movement for AAC access.; eye gaze is natural, and eye muscles generally do not fatigue with use. Compared to other access methods such as switch-activated scanning, eye-tracking is often reported to be the least fatiguing access method by PALS [Gibbons and Beneteau, 2010]. Others have reported that eye-tracking technology requires relatively little effort [Calvo et al., 2008; Harris and Goren, 2009]. Secondly, eye gaze may be the only volitional movement that the individual continues to exhibit over time, particularly in cases where invasive ventilation has been chosen [Ball et al., 2010].

Research is needed to objectively document AAC acceptance and use; further research is also needed to clarify the level of cognitive impairment that tends to interfere with AAC intervention [Iversen et al., 2008].

CONCLUSION

PALS functional impairments result from a relentlessly progressive muscle weakness, leading ultimately to a widespread body paralysis. Therefore, all movements become more and more difficult up to a complete immobility.

In the late stages of the disease, eventually patients find themselves in a “locked-in” state, totally unable, in the worst cases, to move neck, trunk and limbs; autonomously breath and feed; and speak. Most of them, however, retain their cognitive capabilities, and assist impotent at their functional decay.

Today, much more than in the past, we can give interdisciplinary assistance to those persons and their families who have to face a dreadful condition and cope with many daily problems: motor, respiratory, nutritional and communicational management is indeed getting better now, although it is not enough to fulfil all patients’ demands.

Actively waiting a “world free from ALS”, we keep fighting against the disease together with PALS and their caregivers, by trying to improve our knowledge of pathogenetic mechanisms; develop novel and effective treatments; help suffering persons to maintain their quality of life as high as possible. Besides scientific and clinical achievements, we look forward to building up a more empathic healthcare enriched with humanity and compassion for sick people.

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CORRELATION BETWEEN SERUM CHROMIUM CONCENTRATION AND THE OXIDATIVE STRESS MARKER MALONDIALDEHYDE IN TYPES I AND II DIABETES

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ABSTRACT

Serum concentration of chromium and oxidative stress (malondialdehyde) were measured in Types I and II diabetic patients. The nature of the relationship between serum chromium and oxidative stress was also assessed in Types I and II diabetic patients. Results showed that both Types I and II diabetes were associated with a significantly elevated serum chromium levels. Serum chromium increased by 22.6% in Type I diabetic patients and 25.3% in their Type II counterparts, $p < 0.05$. Oxidative stress level as indicated by serum malondialdehyde (MDA) was also elevated in both Type I and Type II diabetes. Malondialdehyde concentration was 7.44 ± 0.53 μmol and 9.88 ± 0.91 μmol in Types I and II diabetic patients respectively, values that are higher than the control serum malondialdehyde concentration of 5.51 ± 0.52 μmol, $p < 0.05$.

The correlation between serum chromium and oxidative stress in both controls and Types I and II diabetic patients shows a weak relationship between these parameters. This indicates the difference in the pattern of the influence played by this metal in the phenomenon of oxidative stress induced by diabetes mellitus and the differences in the pathogenesis, etiology and severity of types I and II diabetes. The elevated levels of serum chromium can potentiate further production of reactive oxygen species, and hence contribute to an increase in the oxidative stress phenomenon already found to be associated with both Types I and II diabetes.

Keywords: Diabetes, Malondialdehyde, Oxidative Stress, Chromium, Correlation, Serum.

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INTRODUCTION

Chromium is a naturally occurring heavy metal found in the environment, commonly in trivalent Cr (III), and hexavalent, Cr (VI) forms. Major sources of chromium for humans include foods such as vegetables, high bran breakfast cereals, broccoli, green beans and meat [1-2]. Chromium salts such as chromium polynicotinate, chromium chloride and chromium picolinate (CrP) are used widely as micronutrients and nutritional supplements and have been demonstrated to exhibit a significant number of health benefits in animals and humans [3]. In addition to its significance in maintaining the general health of humans, chromium is also an essential nutrient required by the human body to promote the action of insulin for the utilization of sugars, proteins and fats [4]. CrP in particular has been used as a nutritional supplement to control blood sugar in diabetes and help reduce cholesterol and blood pressure levels. Chromium increases insulin binding to cells, insulin receptor number and activates insulin receptor kinase leading to increased insulin sensitivity [3]. Whereas Cr (VI) is known to be cytotoxic, studies on the prolonged use of Cr (III) in dosages up to 350 times above the reference dose has shown no significant harmful effects [5]. However, with increased levels i.e. > 20 micrograms / ml, oxidative stress, reduction of cytochrome C and DNA breakages may arise [6]. This study is therefore aimed at measuring the concentration of chromium and the levels of oxidative stress (malondialdehyde) in the serum of types I and II diabetic patients and assessing the nature of the relationship between this metal and malondialdehyde in this condition.

SUBJECTS AND METHODS

Subjects and Exclusion Criteria

Twenty five adult Types I and II diabetic patients (mean age, 61.8 years) were involved in this study. There were 25 age-matched healthy volunteers who served as controls. Both patients and controls were recruited from a comparable background (same geographical area and socio-sanitary status). None of the patients or control was using hormones, oral contraceptives or trace-metal containing supplements at the time of the study. All were non-smokers. Patients with concomitant diseases such as rheumatoid arthritis, Wilson's disease, cancer or liver diseases were excluded. Other exclusion criteria were as described in Dee et al., 2006 [7].

Blood Samples. Fasting blood samples (5ml) were collected from patients and controls under sterile conditions into metal-free containers by venepuncture. Serum was prepared by centrifuging the blood at 3000g for 10 minutes at room temperature. The collected sera were stored in metal-free containers at 4°C until analysis.

Determination of Malondialdehyde

Serum malondialdehyde (MDA) was assayed according to the flourometric method described in Wojciech, Neve and Peretz, [8]. Briefly, 50µL of serum of serum was introduced

into 10mL test tubes containing 1 mL of distilled water. After addition of the solution of 29mmol/L thiobarbituric acid (TBA) in acetic acid (pH of the reaction mixture, 2.4 – 2.6) and mixing, the samples were placed in a water bath and heated for 1 hour at 95-100°C. After the samples were cooled, 25µL of 5mol/L HCl was added (final pH 1.6 – 1.7), and the reaction mixture was extracted by agitation for 5min with 3.5mL of *n*-butanol. The butanol phase was separated by centrifugation at 1500g for 10min, and the fluorescence of the butanol extract measured with a flourometer at wavelengths of 525nm (excitation) and 547nm (emission) using a SANCO Fluorescent Photometer – Model 930A.

Determination of Serum Chromium Concentration. All the glassware used in the determination were first dipped in 5% nitric acid for 48 hours, then washed with tap water and distilled deionized water, each three times before drying. Serum samples (0.5ml) were diluted 10-fold with 0.5% (v/v) solution of Triton-X-100 prior to analysis. [9]. The concentration of chromium was determined in duplicate using a Buck Atomic Absorption Spectrophotometer (AAS), Buck Scientific Instruments, USA.

Statistical Analysis. Data was analyzed using Stratigraphics 3.0 Software. Comparison of mean values between controls and Types I and II diabetics was done using one way Analysis of Variance (ANOVA). The method of Least Significant Difference (LSD) was used to assess the difference between means. The nature of the relationship between serum chromium and malondialdehyde was done using the Pearson's Product Moment Correlation Coefficient (r). Data were expressed as mean +/- SD. $p < 0.05$ were considered significant.

Ethics. Ethical approval for this work was obtained from the Ethics Committee of Plateau Hospital Jos, Plateau State Nigeria. In addition, the Council of International Organization of Medical Sciences / World Health Organization (CIOMS / WHO) International Guidelines for the Conduct of Research Involving Human Subjects [10] was also adhered to.

RESULTS

The results obtained showed that both types I and II diabetes are associated with significantly elevated levels of serum chromium, table 1. Serum chromium concentration were 22.6% and 25.30% higher in Types I and II diabetics respectively when compared to the chromium level of 1.15 ± 0.19 mg/l in the controls, $p < 0.05$. No significant difference exist between the serum chromium concentration in Types I and II diabetics. Oxidative stress levels, as indicated by serum malondialdehyde concentration was found to be elevated in both types I and II diabetic patients relative to the control value of 5.51 ± 0.52 µmol.

The serum malondialdehyde concentration was highest in type II diabetics, increasing significantly by a magnitude of 79.30% above the control MDA levels, $p < 0.05$. There was a 35% increase in serum MDA in type I diabetes relative to the control, $p < 0.05$. Among the two patient groups, serum MDA was significantly higher in Type II diabetics; 9.88 ± 0.91µmol relative to the concentration of 7.44 ± 0.53 µmol in their type I counterparts, $p<0.05$.

The correlation matrix shown in table 2 indicates a shift in the pattern of relationship between serum chromium and MDA. With a shift of correlation from $r = 0.39$ in the controls to $r = -0.0012$ and $r = 0.18$ in Types I and II diabetic patients respectively, it indicates that the relationship between serum chromium and MDA is not static in these conditions.

Table 1. Serum chromium and malondialdehyde concentration in Type I and II diabetic patients and control

Subjects	Chromium (mg/l) Malondialdehyde (μmol)
Control	1.15 ± 0.19* 5.51 ± 0.52**
Type I Diabetics	1.41 ± 0.22*,[a] 7.44 ± 0.53**
Type II Diabetics	1.44 ± 0.29*,[b] 9.88 ± 0.91**

* ' ** $p < 0.05$; [a], [b] $p > 0.05$.

Table 2. Pattern of correlation between serum chromium and malondialdehyde (MDA) in Type I and II diabetic patients and control

	Chromium (mg/l)
Malondialdehyde (MDA) (μmol)	Control ($r = 0.39$, $p = 0.053$)
	Type I Diabetics ($r = -0.0012$, $p = 0.99$)
	Type II Diabetics ($r = 0.18$, $p = 0.40$)

CONCLUSION

Types I and II diabetes differ in their etiology. While Type I diabetes is mainly an autoimmune process, only the minority of type II diabetes is based on an autoimmune process. The major immunological process is the destruction of pancreatic insulin-producing β-cells, which is caused not only by T-cell mediated cytotoxicity, but also cytokine-induced cell death, followed by the appearance of autoantibodies [11-12]. Diabetes mellitus is characterized by chronic elevation of blood glucose (hyperglycemia) as consequence of decreased blood levels or decreased action of insulin [13], a hormone responsible for the regulation of the plasma glucose concentration and glucose utilization [14-15]. In type I diabetes, the autoimmune destruction of β-cells by the cellular and humoral immune system in the pancreatic islets of Langerhans leads to impaired insulin secretion and subsequently hyperglycemia. This type of diabetes is characterized by the appearance of antigen-specific T-cells and antibodies in peripheral blood which are directed against a variety of β-cell antigens, including glutamic acid decarboxylase, tyrosine phosphatase 1A-2 and insulin. The onset of type I diabetes frequently occurs before the age of 20 years, but disease manifestation is also common in adult patients. Exogenous administration of insulin is necessary to maintain glucose homeostasis and prevent early and late diabetic complications [12, 16]. In type II diabetes comprising approximately 90% of cases of diabetes mellitus, hyperglycemia is the consequence of relative insulin deficiency and insulin resistance of various tissues including muscle and adipose tissue. While in early type II diabetes insulin resistance and the resulting increased metabolic demand may be overcome by increased pancreatic insulin secretion, failure of β-cells to maintain adequate insulin production and decrease in β-cell mass are common in progressive disease, resulting in chronic hyperglycemia and loss of metabolic control [13, 17-18]. Hyper-insulinemia is associated with down-regulation of insulin receptors, thus further contributing to the exhaustion of insulin production in β-cells [19]. Both types I and II diabetes show a genetic predisposition although only type I diabetes is HLA dependent [12-13, 16, 20]. Complications arising from types I and II diabetes include

diabetic neuropathy, retinopathy, angiopathy and nephropathy, which are mainly due to the accumulation of advanced glycosylation end products (AGES). The risk of diabetic complications increases with duration of diabetes and the grade of hyperglycemia [13, 16]. The results from this study showed significantly elevated levels of oxidative stress (malondialdehyde, MDA) in both types I and II diabetic patients. These findings are in agreement with the results of similar studies reporting an association between diabetes and increased oxidative stress [21-23]. Some workers have further postulated that oxidative stress plays a central role in the onset of diabetes mellitus as well as in the development of vascular and neurologic complications of the disease [24]. One potential source of oxidative stress in diabetes mellitus is a cascade of reactive oxygen species (ROS) leaking from the mitochondria and this process has been associated with the onset of type I diabetes via apoptosis of pancreatic β-cells, and the onset of type II diabetes via insulin resistance [25-26]. The elevated levels of oxidative stress can be accounted for by the hyperglycemia, which is a hallmark of diabetes. Hyperglycemia, defining established diabetes can induce oxidative stress by various mechanisms; excessive levels of glucose reaching the mitochondria leads to an overdrive of the electron transport chain, resulting in over-production of superoxide anions normally scavenged by mitochondrial superoxide dismutase (SOD). Failure of the latter leads to oxidative stress and it was recently proposed that this mechanism is responsible for the activation of all major pathways underlying the different components of vascular diabetic complications (glycation, PKC activation, sorbitol pathway)[27]. Another mechanism whereby high glucose can stimulate oxidative stress is the auto-oxidation of glucose in the presence of transition metals as well as generation of reactive oxygen species during the process of glycation. Indeed the development from Schiff's base to Amadori to advanced glycation end products (AGES) is accompanied by ROS-generating reactions at various steps [28]. Additional sources of increased ROS production and increased oxidative stress consequent to hyperglycemia include changes in redox potential of glutathione, although non-hyperglycemic mechanisms have also been reported e.g. increased activity of xanthine oxidase, a ROS-generating enzyme [29-30]. Furthermore decreased antioxidant defenses have also been observed in diabetes mellitus, including reduction in serum paraoxonase and in total anti-oxidant capacity of plasma. Some of these mechanisms may possibly operate simultaneously in a synergistic fashion [31]. The elevated levels of serum chromium in both types I and II diabetics indicate an altered state of chromium homeostasis in this disease. Indeed out results are in agreement with findings in several studies where subjects with diabetes were reported to have an altered chromium metabolism due to impaired chromium utilization. Diabetics absorb more chromium than non-diabetics but also have urinary losses [4, 32-33]. In addition diabetics appear to sense a need for additional chromium which is illustrated by an increased absorption of the metal, but are unable to utilize the absorbed chromium reflected by increased losses. Therefore while chromium concentration in blood and urinary losses are elevated in diabetics, tissue levels of the metal were found in some studies to be lower than those of the controls [4, 34-35]. The implications of this finding point towards caution in introducing chromium supplementation in diabetics. While chromium supplementation is has been shown to improve glucose tolerance, impaired glucose metabolism in children, adults with varying degrees of impaired glucose tolerance, elderly subjects and hyperglycemics, the relative improvement is proportional to the degrees of glucose intolerance [4]. Since diabetes is already known to be associated with high oxidative stress levels, the elevated oxidative stress can alter micronutrient requirements including

chromium. Additional stresses that can further alter chromium metabolism in humans are glucose loading, high simple sugar diets, lactation, acute and chronic exercise and physical trauma [36]. Chromium once mobilized in response to stress, is not reabsorbed by the kidneys but is lost in the urine [32]. While chromium supplementation and oxidative stress appear as potential therapeutic targets in diabetes, the exploitation of these targets in diabetes should be done bearing in mind the following implications: an elevated chromium concentration in serum can convert this metal into a pro-oxidant thereby accelerating the further production of more reactive oxygen species. Targeting oxidative stress with the aim of reducing it in diabetes should also take into cognizance the fact that the phenomenon plays a role in several important cellular metabolic processes [38-40]. The variation in the nature of the correlation between oxidative stress (MDA) and serum chromium concentration in controls and types I and II diabetics indicates the relative differences in the etiology, pathogenesis and severity of types I and II diabetes.

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CURRENT ADVANCES IN PROSTHESES IN HERNIA REPAIR

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The "Biomaterial Consensus Conference" in 1983, reached an agreement on the definition of biomaterial: the substance (or combination of substances), natural or synthetic, that can be used alone or in combination, and for a variable period of time, that can treat, increase or replace a function of the body. It can therefore be defined as all materials reachable as metals and alloys, ceramics, carbon derivatives, polymers and biological tissues, which have been used and are still used in surgical treatments. Only some of these are components of the prostheses used today in surgery for inguinal hernia.

Amid's classification proposed in 1997, provides three sets of synthetic prostheses in response to the diameter of the pores, but basically marking guidelines application of different prosthetic materials has now been relegated due to the emergence of next-generation prosthesis.

They seek to achieve the best possible integration into the tissues, improve their performance on all interfaces and avoid complications.

The following table shows the classification of Amid:

Classification of synthetic prosthesis (Amid)		
Type I	Macroporous prosthesis: Pores>75 microns	Monofilament polypropylene mesh
Type II	Macroporous prosthesis at least one of three directions) Pores < 10 microns	e-PTFE mesh
Type III	Macroporous prosthesis with multifilament component or microporous	Polyester mesh e-PTFE perforated mesh Monofilament polypropylene mesh
Type IV	Prosthesis with submicrons pores	

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Traditional Classification:

- *Biological materials:* aponeurotic tissue, skin (dermis), muscle.
- *Sintetic materials:* metals and alloys (titanium, iron, steel), Polymers (carbon, polyglycolic acid, polyglactin, PTFE, polivinilic acid, polyester, polypropylene).

Types of actual prostheses in surgery:

- Non-absorbables prostheses: polyester, polypropylene, PTFE-e.
- Absorbables prostheses: polyglactin, polyglycolic acid, Acelular collagen matrix of bovine, pig or human tissue.

NONABSORBABLE PROSTHESIS

Polyester (Dacron)

Dacron is a polyester composed entirely biomaterial, derived from glicoethilen and terephthalic acid. It was used the first time in the United States in 1954 as a prosthesis in vascular surgery and subsequently introduced in Europe (France) in 1967 by Rives, for surgical repair of abdominal wall defects.

In general, all them are composed of tiny strands of braided polyester fibers. The mesh is displayed fine, light, soft, flexible, slightly elastic and fitted with high tensile strength.

Forgetfulness makes plastic adapted to different anatomical situations and is ideal for operations that require the placement of the mesh retromuscular or preperitoneal hernioplasty as Rives, Stoppa and Wantz. The macroporous structure encourages a lively fibroblastic reaction and rapid formation of a periprosthetic capsule. (Type III prosthesis according Amid).

Dacron is a non-resorbable biomaterial that provides excellent biological tolerance and a moderate inflammatory response, but in contact with the viscera, the intense fibroblastic reaction can lead to adhesions and it can be complicated by adhesions: sub-occlusive or obstructive syndrome, or even producing intestinal fistulaes.

The resistance to infection is less than a monofilament prosthesis because the interstices of the polyester fibers are perfect nesting niches that allow the entry of the bacteria but not of macrophages. In case of infections it is often necessary to remove the prosthesis.

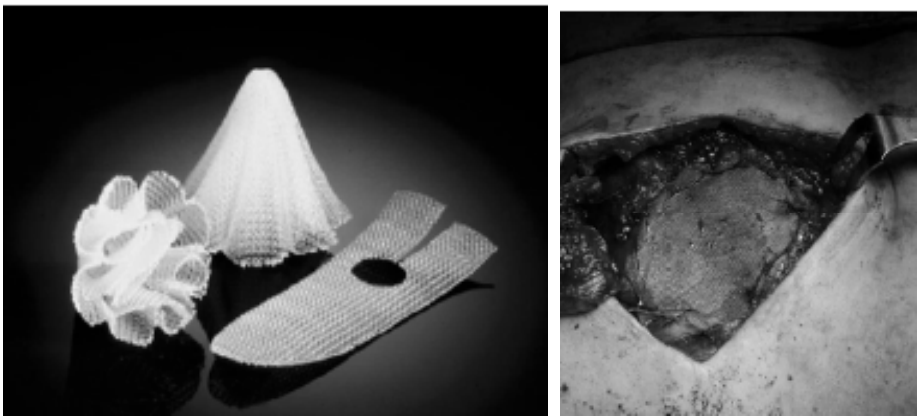
Polypropylene

Usher used the first prosthesis of polypropylene in hernia surgery in 1958. Polypropylene is a synthetic derivative of polyethylene tht has undoubted advantages such as: high tensile strength, the possibility of sterilization, tolerance to infections and many chemicals products, ease of use and comfort for the patient, does not create tension and rarely shows intolerance. Since 1958 the use of polypropylene has become the principal material used for the repair of abdominal wall defects, especially for the treatment of inguinal hernia.

The polypropylene monofilament is available in various forms, which differ mainly by the kind of twisted and the dimensions of the pores.

The type of braided mesh gives its mechanical characteristics: weight, stiffness, memory plastic, two-dimensional elasticity, flexibility, surface roughness (Velcro effect ®), diameter of the pores.

All polypropylene prosthesis share some characteristics such as high tensile strength, encouraging rapid intra-and periprosthetic fibroblastic reaction and unfortunately the susceptibility to infections (prosthetic type I, according to Amid). The polypropylene creates a dense scar tissue, induced by fibroblastic reaction, which in contact with intraperitoneal structures has a high risk of adhesion formation with possibility of evolution to erosions and intestinal fistulas.



Polytetrafluoroethylene (PTFE) and Expanded PTFE (PTFE-e)

The expanded polytetrafluoroethylene (PTFE-e) is a synthetic derivative of Teflon through a procedure discovered in 1963 in Japan, and subsequently redefined in 1970 by Gore in the United States. The e-PTFE has been used initially for the production of prosthetic vascular surgical use. Its use for repair of abdominal wall defects began 1983, Gore-Tex ® Soft Tissue Patch (STP).

The expanded PTFE is one of the most inert and biocompatible biomaterials among those currently available. It is not absorbed, does not cause allergies, triggers a minimal inflammatory response, is not altered by the action of tissular enzymes and it is not modificate by the presence of infections.

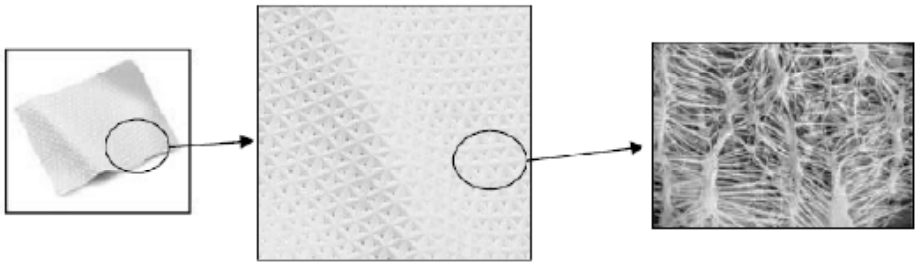
Its microporous structure of trabecular type, consisting of e-PTFE micronet joined together along three dimensions of microfibrils. The micropores have a diameter of 20 microns. The multi-microporous structure allows the penetration of fibroblasts and collagen formation within the mesh. The integration of PTFE by the tissues is quite slow (3-4 weeks). Other prosthesis (Gore-Tex ® Mycromesh) of microporous structure, has macropores of diameter of 0.8 microns, equidistant between them, which ensure rapid and earlier anchor integration. After 7 days of implantation, this mesh appears firmly attached to the tissue with minimal foreign body reaction and extensive vascularization.

Mechanical tests on PTFE shows extremely resistant with high tensile and suture retention, similar to that of polypropylene monofilament. If placed in contact with viscera, also after 2-4 weeks of implantation, are coated with a layer of mesothelial cells, resulting in soft and easily adhesions dissected.

Another feature of PTFE is the high biological inertness. It is a biomaterial which also allows the normal development of the tissue repair processes (there is a fibrous tissue formation rather organized which is placed horizontally along the surface of the prosthesis and perpendicularly deepens the micropores, with little foreign body reaction strange).

The behavior of PTFE-e against bacterial contamination and infection is controversial argument. Due to its hydrophobicity, the e-PTFE has the ability to slow down bacterial penetration, but there are several articles opposed to these theoretical and clinical results, which confirm the low possibility of penetration of neutrophil granulocytes, with subsequent infection and possibility of chronic infection in the mesh. (Gore-Tex ® Dual Mesh: Type II prosthesis according to Amid, Gore-Tex ® Mycromesh: Type III prosthesis according Amid).

The e-PTFE prostheses have added two antimicrobial agents (chlorhexidine diacetate and silver carbonate), (Gore-Tex ® Dual Mesh Plus), which inhibits bacterial colonization of the prosthesis until 10 days after implantation, and makes it the safest in current comparative studies with the rest of the meshes.



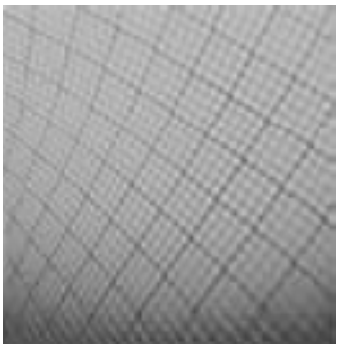
RESORBABLE PROSTHESIS

The absorbable prostheses are emerging and are being widely studied subject to controversy. The principle of the use of absorbable prosthesis for hernia surgery is based on a double presupposition:

They are used as internal reinforcement (offered by the prosthesis itself) and turn cause a stimulus for fibroblast activation with subsequent production of connective tissue to enhance healing.

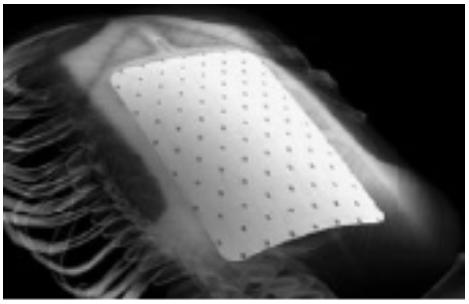
Resorbable synthetic prostheses are a polymer of glycolic acid esters (Dexon ®, Davis and Geck,) or a copolymer derived from the synthesis of the latter with lactic acid (polyglactin 910) (Vicryl ®, Ethicon).

Synthetic absorbable meshes are usually textured multifilament braided. Most prostheses are soft, flexible, extensible, mouldable, and biodegradable. They are gradually resorbed by hydrolysis beginning between 90 days and 6 months, with a gradual reduction in mass and resistance to stress.



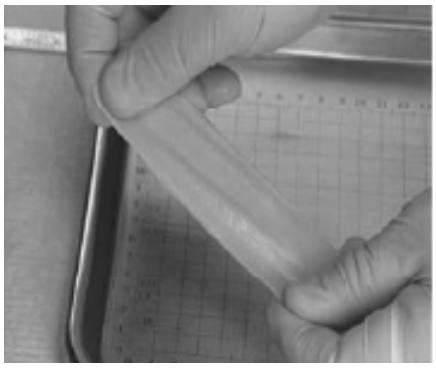
- Other sintetic bioprostheses are made from a new material (PGA-TMC), which is conforming a mesh composed of a microporous structure of synthetic absorbable copolymer fiber (67% polyglycolide and 33% trimethylene carbonate). It is 100% absorbable prosthesis at 6 months of placement, currently marketed as a plug for inguinal hernia (Gore Bioabsorbable Hernia Plug ®). It belongs to the category of microporous mesh, with a particular character, is hydrophilic, which makes it theoretically optimal vector to absorb and release water based products on the site of fixing it.

- Xenograft acellular collagen type I (Surgisis ®, Cook ®) from porcine submucosa, including growth factors and glycosaminoglycans. It produces very little rejection and gradually their fibers are replaced by fibroblastic cells, newly formed vessels, with the creation of extracellular matrix is gradually replacing the mesh.).



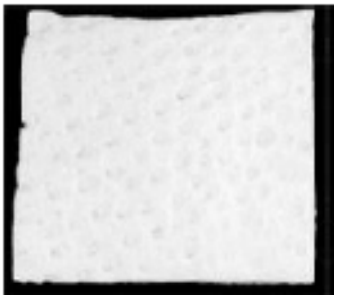
- Alloprostheses human acellular dermal matrix (Alloderm ®, LifeCell ®) is a mesh with excellent characteristics and without risk of intolerance or rejection.

It also provides the advantages of the equivalence with the PTFE with a lower rate of adhesions if you are in contact with viscera.



- Xenograft derived from bovine pericardium (Peri-Guard and Veritas ® Collagen® Matrix, Synovis Surgical Innovations), a material with excellent flexibility and resistance. It is one of the less foreign material remains in the receiver compared with others.

- Xenograft acellular collagen matrix subdermal swine (Permacol ®, TissueScience). The publications choose to use in infected areas and complicated providing an optimal solution to this problem.



- The use of these biological absorbable meshes has been equated with non-absorbable meshes for hernia recurrence purposes. But the advantage offered by these meshes is when preventing or treating infection at the implant site, and is currently the main indication. The absorbable prostheses are therefore presented as a temporary alternative to polypropylene and PTFE-e in the presence of infections. The supplied mesh support reabsorbable until resolution of septic problems may in fact increase the chances of success of the permanent prosthesis.

These types of natural dentures require a very strict controls, given their origin in various animal species (cattle, pigs) for the risk of transmitting diseases, as has happened with lyophilized human dura prosthesis. The characteristics of reabsorbable prosthesis can be exploited positively for the construction of prosthetic joint, particularly useful for ventral hernia surgery.

NEW TRENDS IN PROSTHESES MATERIAL

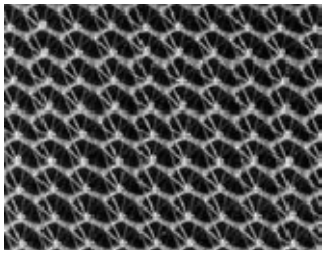
A new classification of the meshes in surgery attempt to: integration and peritoneum formation:

Reticular prostheses are useful for placement in a tissue interface.

The laminates are optimal prosthesis for placement in direct contact with the visceral peritoneum. The composite prosthesis can be placed on all interfaces, but its design is devised in order to be placed in a tissue and a visceral peritoneum interface.

- *Laminar prostheses:* these meshes provoke low formation of new peritoneum, but high integration with the receptor tissues.

Non absorbable: PTFE, Silicone, Poliuretane.
Absorbable: biological material (dermis or submucosa of pig, bovine or human tissue)



- *Reticular prostheses:* these meshes provoke a big formation of new peritoneum, and normal integration.

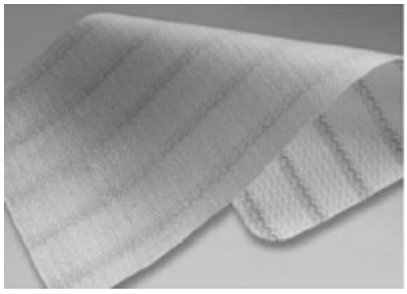
Non absorbable: polyester, polypropylene
Absorbable: polyglactin, polyglycolic acid
Lightweight prostheses: these meshes have a big pore and low density prostheses. This prostheses *lightweight* can be so much of material (one lonely material or mixed materials such non-absorbable and absorbable material).

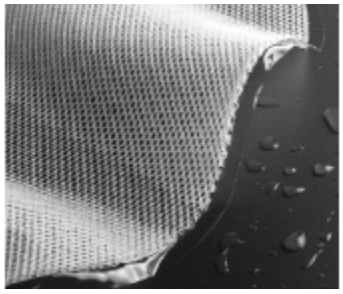
These large pore and low density implants are integrated causes less strange body reaction, because have less biomaterial, with the advantage that the implant maintains the elasticity of the host tissue. Lightweight prostheses can be either non-absorbable material as absorbable material, thereby generating hybrid prostheses. These hybrid prostheses ultimately generate less fibrous reaction favoring the integration of the mesh and obtaining improved clinical outcomes compared with open surgery.

- *Composites prostheses:* This prosthesis provokes high formation of peritoneum and high integration. Two different- materials layers: the first (superior layer) use to be prosthesis of reticular type, destined to increase the fibroblastic reaction, polypropylene or polyester usually. The second component (inferior layer) uses to be a laminar type and it can be absorbable or scarcely reactive material like the PTFE.

In ventral hernia or primary hernia surgery This prosthesis are currently hosting by a series of properties: inducing good tissue integration and prevent the formation of adhesions when placed in contact with the viscera. The composite prosthesis (composites) are generally constituted of two layers of different materials: biomaterials that form the first component (top) are usually reticular prosthesis type, destined to join the fibroblastic reaction, either polypropylene or polyester. The second component (bottom) is usually laminar and can be absorbable or poorly reactive and PTFE-e.

They are especially useful in large hernia defects repair, where is necessary a reconstruction of the abdominal wall, and does not exist resistant tissue where to fix the mesh properly. In laparoscopic surgery of abdominal hernia is useful, where the biomaterial is in contact with the visceral peritoneum.





Classification of prostheses used in the repair of abdominal wall hernial defects (Bellon 2005)	
Reticular prosthesis	
Non absorbables	Polypropylene (high or low density), polyester.
Partially absorbables	Polypropylene/polygalctin 910, polypoprylene/polygrecaprone
Absorbables	Polylactic, polyglactin 910
Laminar prosthesis	
Non absorbables	e-PTFE, silicone, polyurethane
Absorbables	porcine intestinal submucosa,, bovine pericardium, human acellular dermal matrix, others biological meshes.
Composite prosthesis	
Non absorbables components	Polypropylene/e-PTFE, polypropylene/polyurethane
Absorbables components	Polypropylene/polyethylene glycol , polyester/polyethylene glycol, polypropylene/hyaluronic acid, polypropylene/ polidiaxona/ cellulose

BIOPROSTHESES: CURRENT STUDIES IN SURGERY

The bioprostheses are used in hernia processes to cover defects. Although today is still valid herniorrhaphy in some types of hernias, it is increasingly appropriate to use these materials to prevent possible complications, mainly recurrence.

After analyzing several meta-analysis about the current herniorrhaphy (Bassini, MacVay, Shouldice Macintyre) concluded that, that the prosthetic repair is the procedure of choice for inguinal hernia.

Recurrences after repairs materials prosthetic figures have fallen to very low percentages: inguinal hernia recurrence is over 1-2%. The recurrences with no prosthetic techniques were in the 10-12% of cases.

The indication of prosthesis in hernia repair is focused on:

- Big-hernia defects (hernia sac more than 10 cm).
- Multi-recidive Eventrations.
- Eventrations in location-special: close to lumbar bony prominences (Subcostal, in pubis or xiphoid).

Cassar reported recurrence after prosthetic repair around 0-10% in open surgery and laparoscopic surgery on a 0-9%. This accounts for half of recurrences in 4%. The recurrence

without the use of prostheses is between 30 and 50%, so the placement of a bioprosthesis is the technique of choice currently.

Another indication for the use of prosthesis is temporarily closing the abdomen in cases where the closure can generate a compartment syndrome. In these cases you must use a laminar prosthesis.

Therefore the cases of recurrence after repair without prosthetic hernia defects are not acceptable and therefore the repair is imposed by biomaterials even minor defects.

ACTUAL ANALYSIS OF ABSORBABLE-NONABSORBABLE BIOPROSTHETIC

The apparition of new absorbable components in bioprostheses makes the debate to determine which mesh is better and in what situation to use each one. Also between absorbable and nonabsorbable mesh itself there are features that differ widely between them.

Discussed below are the main differences by analysis of current experimental studies and collection of enforcement cases with bioprostheses in vivo.

Most contentious points are:

- Integration of the mesh.
- Postoperative adhesions on contact with the visceral peritoneum.
- Susceptibility to being infected and recover from the infection.
- Biophysical characteristics that avoid hernia recurrence.

Nonabsorbable Bioprosthesis

When analyzing separately nonabsorbable meshes we focus on current articles that have been animal experiments and human studies.

Ott in the study compared resorbable mesh nonabsorbable mesh, concluded with more infection in the nonabsorbable mesh and the postoperative adhesion formation especially.

Martin Cartes and Morales in their experimental study in pigs showed ventral hernia rate of adhesions that occur with nonabsorbable, polypropylene (synthetic prosthesis as the main type lattice) and PTFE-e (as synthetic prosthesis main type lattice). Adhesions to polypropylene were mostly consistent, and the entire surface of the implant, while in PTFE-e showed a clear reduction of adhesions and in addition only the edges of the implant. By using inhibitors of adhesion formation, such as fibrin sealant (Tissucol ®) and hyaluronidase (Thiomucase ®), they found a reduction of adhesions to the prosthesis. This interface has directed many surgeons to use fibrin sealant with the abdominal wall prostheses, especially for laparoscopic surgery to minimize the possible adhesions.

In infection prosthesis, Carbonell described a study which compared the main prosthesis at that time. The prosthesis that resisted better the infection was PTFE-e, above other biomaterials, including the absorbable prostheses. It showed that levels of infection in different biomaterials were higher in synthetic nonabsorbable, except the e-PTFE, where the infection was very low, especially by its antiseptic impregnation.

The Nonabsorbable prostheses are biologically inert and have a structure that ensures unchanged in the formation of a new wall while the prosthesis remains anchored. It is evident by the many existing studies that describe how nonabsorbable meshes are widely used, but with the following problems (in a very low rate): complications in contact with abdominal viscera (adhesions, fistulas, etc.), impaired synthetic products that make the prosthesis and infection of themselves as prime examples.

These are their weaknesses, and ultimately are the source of the study and promotion of the creation of absorbable mesh, which attempt to overcome these shortcomings discussed. We can say further that in minimizing the adverse effects of a nonabsorbable bioprosthesis should be taken into account by their best results, macroporous meshes composed of monofilament that are better integrated, more histocompatibility complex, and are less infection in the mesh.

The Laminar prostheses offer great advantages in the resistance of biomaterial and to the infection, but are more likely to be intolerable and have to be removed if an infection of the mesh.

Burger analyzed 200 rats with various types of mesh: a) nonabsorbable prosthesis: polypropylene (Prolene ®) and PTFE-e (Dualmesh ®) b) Composite Prostheses: polypropylene - polyglecaprone composite (Ultrapro ®), polypropylene + titanium composite (Timesh ®) , polypropylene coated sodium carboxymethylcellulose more hyaluronidase (Sepramesh ®), polyester coated with polyethylene glycol glycerol with collagen (Parietex Composite ®), polypropylene + polydioxanone composite coated activated cellulose (Proceed ®) c) absorbable prostheses: from bovine pericardium (Tutomesh ®). This author and his research group concludes that there is no difference between them in relation to infection of the same concerns and only noteworthy is the significant decrease of adhesion in composites, as well as good integration, so Parietex® and Sepramesh Composite ® are the most appropriate of all the above for use in direct contact with viscera to have the lowest rate of adhesions combined with better integration. Dentures Dualmesh ® and Tutomesh ® also have the characteristics of having a strong retraction.

The new hybrid materials and composites, which combine with nonabsorbable material partially or fully absorbable material, in addition to forming macroporous, low density (ligthweigth) are an excellent alternative in order to minimize the adverse effects of nonabsorbable bioprosthesis.

Nowadays another advance in this type of mesh is the ability to self-adhesion of new prostheses. This is possible by three-dimensional structure or the addition of little hooks of absorbable material, which enables early stabilization of the mesh without using sutures.

Absorbable Bioprosthesis

The creation of these prostheses was born to improve the weak points of the nonabsorbable. The research level in vitro and in animal model demonstrates succeed. It has shown a decrease in the rate of adhesions when placed in contact with viscera and a lower rate of infection of the same, so much so that its current indication is primarily focused in infected or at high risk infection, despite not having antimicrobial properties.

All these advantages are demonstrated by several studies such as the Zheng experimental study: using collagen implants of pig submucosa (Pelvicol ®) in Wistar rats found along the

intervals of 7, 14, 30 90 days the existence of less inflammatory response, fewer adhesions and a correct and orderly formation of collagen.

In 2007, Gaertner made an experimental study of great importance in absorbable prostheses, leaving the biomaterial at 3 and 6 months in rats. In ventral hernia repair used 4 types of reabsobable mesh 2x2 cm, in contact with viscera: two prostheses derived from bovine pericardium (Peri-Guard ® in 15 rats and Veritas ® in 13 rats) human dermal collagen matrix (allograft, Alloderm ® in 10 rats) and collagen subdermal pig (xenograft Permacol ® in 10 rats) and found that all was proper integration with minimal intraperitoneal adhesions, and no difference is in terms of tensile strength dynamometry and pneumoperitoneum applied after the sacrifice of animals. However it is notable that occurred in the prostheses infections derived from bovine pericardium (5 in Peri-Guard ® and 1 Veritas ®) and ulceration the skin also in the same prosthesis (6 in Peri-Guard ®). The prosthesis most favored in resistance, adhesion and reduced integration was Permacol ®; with similar results Peri-Guard ®, but with the disadvantages discussed before.

When searching for information on the results of absorbable prosthesis in patients highlight Franklin, who made a collection of data on infection in 23 hernioplasties potentially or actually contaminated laparoscopically, and using bovine serosa absorbable prosthesis (Surgisis ®) found no complications in the immediate postoperative patients with a normal recovering.

It follows from this study that reabsorbable mesh is a good alternative in these circumstances, although the authors acknowledge that their number is very short and studies are needed long time. However there are conflicting studies also present in humans, where it is not clear superiority of these meshes.

Ueno presented a review of 20 patients requiring hernia surgery but in the context of dirty surgery (strangulated hernia and perforated enterocutaneous fistula, infected prostheses need to remove and correct the hernia defect) using porcine submucosal collagen prosthesis (Surgisis). In this review they wanted to check whether there are harmful effects of the xenograft, and what is their behavior in dirty surgery. They conclude that there is no deterioration due to the porcine origin of the mesh, but nevertheless there is a 50% postoperative complications (8 patients presented with wound infection, including loss of mesh and fasciitis necrotizing), and a recurrence at 15 months of 30%. The bioprosthesis offers great advantages, but the problem is the infected abdominal wall and the pathologies of patients.

Van't Riet made one of the latest revisions of absorbable mesh in patients in late 2007, specifically polyglactin 910 mesh versus nonabsorbable mesh. In this study, the absorbable meshes do not perform better than non-absorbable regard to complications and mortality.

The advantage of crosslinked mesh versus non-crosslinked mesh remains a controversial area. Early investigation showed increased stiffness for two crosslinked biologic mesh products (porcine dermis and bovine pericardium) compared to the non-crosslinked bovine pericardium mesh. Greater cell infiltration was seen in the non-crosslinked mesh. Future investigation is warranted as to whether these characteristics are clinically important or if the crosslinked mesh poses an increased risk for infection by preventing collagen breakdown and macrophage migration. Chemical cross-linking of collagen is performed not only in hernia prosthetics, but also in bone, cartilage, and vascular implants, and in degrees from low to high density.

The cross-linking density is bigger, so does fibroblast encapsulation and implant resistance to enzymatic degradation. However, the rate of cellular infiltration decreases. The optimum cross-linking pattern and density to balance graft strength and durability with cellular in-growth and remodeling remain unclear.

A lack of quality long-term clinical experience and data makes it difficult to decide which product has the optimal balance. In conclusion: the placement of any synthetic material in the presence of intra-abdominal infection has a high risk of complications, regardless of whether the graft absorbable (polyglactin) or non-absorbable (polypropylene or polyester), so that biomaterials should be avoided if possible in the presence of infection.

COMPLICATIONS IN PROSTHESES

The recurrence rates have decreased drastically thanks to prostheses, but other new problems have been presented in hernioplasty: Infection, adherences and intolerance.

We have made surgical experimentations to study these problems, and there are the most frequent solutions:

Recidive: the correct election of the prostheses is essential in the surgery.

- Choose a reticular mesh (lightweight) in open surgery (without contact with viscera) is the best option. If the intraabdominal content could be in contact with thee mesh, best use a laminar or composite mesh is justified.
- A laminar or composite mesh in laparoscopic surgery is the most extended and correct option.

The good election of the mesh, according with the hernia characteristics, reduces the recurrence.

Infection: the antisepsis methods and a correct technique could be completed with antibiotic prophylaxis. In the infection of prostheses several actions could be attempts: remove the laminar mesh, cures in reticular meshes, and finally use the absorbable (biological essentially) meshes to repair the defect in infected tissue or after remove the infected mesh. The use of antimicrobial drugs in the infection place is a new concept that can be useful in this complication.

In the case of reticular mesh:

- If the infection is not in all the mesh, it is possible remove only the infected area of the mesh and washing it several times to decrease the grown rate of the bacteria.
- If the mesh shows a complete infection the best choice is to remove all the mesh and use another surgical procedure (placing a new biological mesh, using a VAC therapy or performing an anatomical reconstruction such a herniorrhaphy or separation of components).

In the case of laminar mesh:

- Although the infection was only a small portion of the mesh, the most often treatment is removes all the mesh and uses the anterior surgical procedures.
- A new generation of mesh has an antibiotic impregnation (Vancomicin), and they can be used in the infection in hernia repair.

Adhesions: The laminar and composites meshes have low rates of adhesion to the intraperitoneal organs. Substances such fibrin glue or hialuronidasa cream decreases the adhesion.

- The adhesion with intraperitoneal organs when using a reticular mesh must be repaired with a redo to remove the mesh and looking for any perforation or erosion of bowels and the others intraabdominal organs.
- The adhesion using a laminar mesh is not frequent. The best option is a composite mesh to avoid the adhesion when the mesh is or could be in contact with viscera.

Intolerance: this problem was frequent in the past, nowadays the prostheses are very good tolerates without this problem.

- If the intolerance appears it could be a chronic infection of the mesh. The existence of infection must be investigating (blood test, cultures, imaging test…).
- If despite of all this, the intolerance is confirmed, the best option is replace it for other kind of mesh (depending of the place of the anterior mesh and the contact with viscera).

The new advances in prostheses go to use one material (or mixed material) with medium or big pore and low molecular weight, which increases a proper integration in the receptor and minimize de foreign inflammatory response. This material must to be elastic and strong enough to use it in laparoscopic approach in the hernia surgery.

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MELANOGENESIS AND NATURAL HYPOPIGMENTATION AGENTS

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ABSTRACT

Human melanin is synthesized in melanosomes located in melanocytes of the skin, hair, eyes, ears, and leptomeninges. Melanin not only determines skin color, but also protects the skin from UV damage by absorbing UV light. Congenital pigmentary disorders that result in skin and hair depigmentation, such as Hermenksky Pudluk Syndrome, Chediak Higashi Syndrome, and Griscelli Syndrome are due to various gene mutations that cause defects in melanin synthesis. Excessive production of melanin, which occurs in response to UV-induced DNA damage, inflammation, or other skin injuries, however, can result in skin hyperpigmentation including freckles, melasma, solar lentigo, age spots, and post-inflammatory hyperpigmentation. In this article we review the synthesis of melanin, the signaling pathways related to the regulation of melanogenesis, the factors influencing melanogenesis and various pigmentation disorders, as well as the effectiveness of various natural products at reducing hyperpigmentation.

ABBREVIATIONS

ACTH, adrenocorticotropin melanocyte stimulating hormone;
AHA, α-hydroxy acids;
ASP, agouti signaling protein;
ATP, adenosine 5'-triphosphate;
BBI, Bowman Birk inhibitor;
bFGF, basic fibroblast growth factor;
BHAs, β-hydroxy acids;
cAMP, cyclic AMP;

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CRE, cAMP response element;
CREB, cAMP-response element binding protein;
CRH, corticotropin-releasing hormone;
DCT, DOPAchrome tautomerase;
DHI, 5,6-dihydroxyindole;
DHICA, 5,6-dihydroxyindole-2-carboxylic acid;
DKK1, dickkopf-related protein 1;
DPPH, 1,1-diphenyl-2-picryl-hydrazyl;
ECE, ET converting enzyme;
ERK2, extracellular signal-regulated kinase 2;
ET-1, endothelin-1;
ETBR, endothelin B receptor;
FOXD3, forkhead-box transcription factor D3;
GM-CSF, granulocyte-macrophage colony-stimulating factor;
GSK3β, glycogen synthase kinase-3β;
HGF, hepatocyte growth factor;
HQ, hydroquinone;
IL, interleukin;
ITF2, immunoglobulin transcription factor-2;
L-DOPA, 3,4-dihydroxyphenylalanine;
LIF, leukemia inhibitory factor;
LT, leukotrienes;
MAP kinase, mitogen-activated protein kinase;
MC1-R, melanocortin 1 receptor;
MITF, microphthalmia-associated transcription factor;
MOPB, methylophiopogonanone B;
NGF, nerve growth factor;
NHKC, normal human keratinocytes;
NHMC, normal human melanocytes;
NO, nitric oxide;
NRG, neuregulin;
PAR-2, protease activated receptor 2;
PAX3, paired box 3;
PGs, Prostaglandins;
PIAS3, protein inhibitor of activated STAT3;
PKA, protein kinase A;
PKC, protein kinase C;
PLA2, phospholipase A2;
POMC, proopiomelanocortin;
ROS, reactive oxygen species;
RSK, ribosomal S6 kinase;
SA, salicylic acid;
SCCE, stratum corneum chemotrypic enzyme;
SCF, stem cell factor;
SEM, skin equivalent model;

SOX, Sry-related HMG box;
STAT3, signal transducer and activator of transcription 3;
STI, soybean trypsin inhibitor;
TGF-β1, transforming growth factor-β1;
TNF-α, tumor necrosis factor α;
TPA, 12-*O*-tetradecanoylphorbol-13-acetate;
TPR1, tyrosinase-related protein 1;
TRP-2, tyrosinase related protein-2;
UV, ultraviolet;
α-MSH, α-melanocyte-stimulating hormone;

INTRODUCTION

Variations in human skin, hair, and eye color are due to the type, amount, stage, and distribution of melanin [1]. Melanin, one of the most widely distributed pigments, is a heterogeneous polyphenol-like biopolymer with a complex structure and color varying from yellow to black [2]. More than 150 genes regulate and contribute to skin pigmentation [3, 4]. In addition to contributing to the color of skin and hair, melanin also protects skin from physical (such as ultraviolet (UV) irradiation damage), chemical (such as environmental pollutants, heavy metals, and oxidative stress), and biochemical (such as bacteria) challenges [5, 6]. Overexposure to solar UV irradiation can result in photoaging, mutagenesis, and photocarcinogenesis in human skin [7, 8]. The incidence of skin cancer is increasing at a rate of 3% to 4% per year, and the mortality rate associated with skin cancer (melanoma) is increasing more rapidly than the mortality rate associated with any other cancer [9]. Melanocytes transfer melanosomes through their dendrites to surrounding kerotinocytes where they form melanin caps. This accumulation of melanin plays a protective role against UV irradiation by absorbing and transforming UV energy into harmless heat. Melanin can also scavenge toxic xenobiotics and reactive oxygen species (ROS) as well as bind to drugs, thereby protecting human skin against chemical and biochemical challenges [5, 6, 10-12]. However, excessive production of melanin and its accumulation in the skin can cause pigmentation disorders, including melasma, solar lentigo, and post-inflammatory hyperpigmentation [13]. Overproduction of melanin is not only a dermatological issue but also poses esthetic problems, especially among patients in Asian cultures. In this article we review the synthesis of melanin, the signaling pathways related to the regulation of melanogenesis, the factors influencing melanogenesis and various pigmentation disorders, as well as the effectiveness of various natural products at reducing hyperpigmentation.

MELANOSYNTHESIS

Variations in dermal pigmentation depend on the number, size, composition, and distribution of melanocytes as well as the activity of melanogenic enzymes. Melanin synthesis by melanocytes within membrane-bound organelles (melanosomes) and their transfer to keratinocytes within the epidermal melanin unit determines cutaneous pigmentation. Melanin synthesis is characterized by an increased number of melanocytes in

the basal layer of the epidermis, the size, maturation, and number of melanosomes, the production of melanin, the dendricity of melanocytes, the transfer of melanosomes from melanocytes to keratinocytes, the proliferation of keratinocytes, and the thickening of the epidermis and stratum corneum.

MELANOCYTES AND MELANOSOMES

Melanin is synthesized in melanocytes, which are localized at the basal layer of the epidermis. Each melanocyte is functionally related to underlying fibroblasts in the dermis and to keratinocytes in the epidermis. Each melanocyte transfers pigment-containing melanosomes via dendritic melanocytes to approximately 36 basal and suprabasal keratinocytes – the so-called epidermal melanin unit [3, 14, 15] (Figure 1). This inter-cell cross-talk regulates the function and phenotype of human skin [16]. Protease-activated receptor 2 (PAR-2) plays an important role in melanosomal transfer [17, 18]. PAR-2, a G protein-coupled receptor, mediates the phagocytosis of melanosomes in a Rho-dependent manner [19]. The amount and type of melanin produced and transferred to the keratinocytes with subsequent incorporation, aggregation, and degradation influences skin complexion coloration [20].

Melanoblasts, melanocyte precursor cells, are derived from the neural crest and migrate to target sites such as dermis and eyes [21]. Melanoblasts differentiate into melanocytes when they reach their destination and start to produce melanosomes, the organized elliptic membrane-bound organelles where melanin is synthesized. Melanin synthesis starts with the exportation of structural proteins from the endoplasmic reticulum to the cytosol, where they fuse with melanosome-specific regulatory glycoproteins that have been released in coated vesicles from the Golgi apparatus.

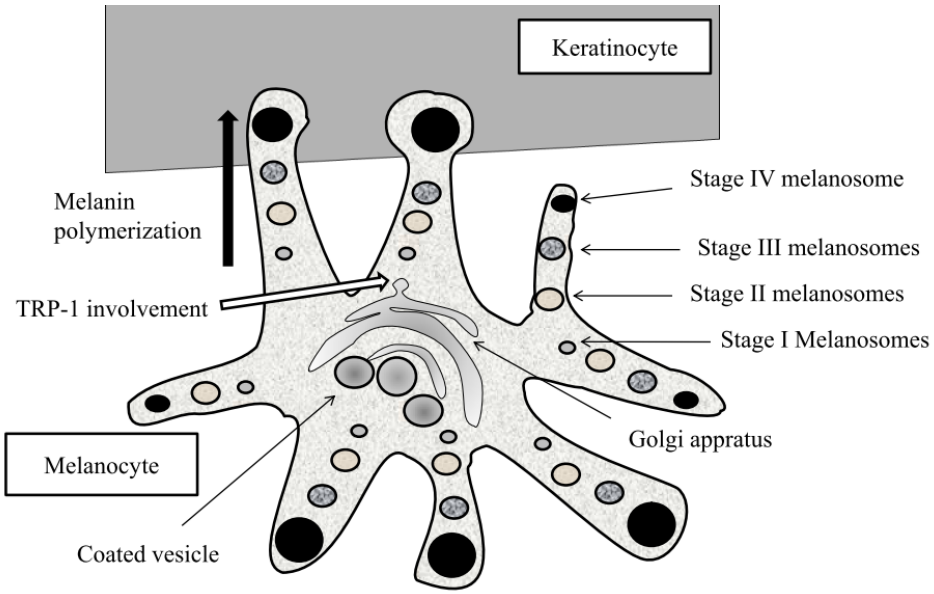

Figure 1. Melanosome transfer.

Melanin synthesis ensues subsequent to the sorting and trafficking of these proteins to melanosomes [22, 23]. Melanosomes are divided into four maturation stages according to their structure and to the type and amount of melanin produced [24, 25]. 'Early' melanosomes (stages I and II) present with little or no pigment, while 'late' melanosomes (stages III and IV) present with some to complete pigment. Stage I melanosomes are spherical vacuoles lacking tyrosinase activity and internal structural components. Stage II melanosomes are elongated, fibrillar organelles containing tyrosinase and little melanin [26, 27]. After stage II, melanin synthesis starts. Stage III melanosomes have uniformly deposited pigment on the internal fibrils. Mature melanosomes (stage IV) are either elliptical or ellipsoidal in shape, are electron-opaque due to complete melanization, and have minimal tyrosinase activity. Highly pigmented melanocytes are rich in Stage IV melanosomes which are transferred by melanocyte dentrites to keratinocytes [16].

The trafficking of sorting vesicles to their target organelles is controlled by two classes of microtubule-associated motor proteins – kinesins and cytoplasmic dyneins [28]. Kinesins power plus-end-directed microtubule-based motility, while cytoplasmic dyneins drive minus-end-directed motility [29, 30]. Dyneins and kinesins also play roles in retrograde and in anterograde transport of melanosomes [31-34], whereas dyneins and spectrin dominate the movement of early melanosomes [35].

The methods of melanosome transfer from melanocytes to keratinocytes include cytophagocytosis of melanocyte dendrite tips [36, 37] and exocytosis of melanosomes into the extracellular space and their subsequent uptake by phagocytosis into keratinocytes [38, 39], either by filopodia-mediated melanosome transfer [40-42] or the filopodial-phagocytosis model [43]. Rab, melanophilin, and myosin Va have been shown to be involved in the movement of melanosomes [37, 44].

MELANIN BIOSYNTHESIS

Melanins are polymorphous, multifunctional biopolymers. The major types of melanins include eumelanin, pheomelanin, a combination of eumelanin and pheomelanin (mixed melanin), and neuromelanin (Figure 2). Eumelanin is a blackish-brown heterogeneors polymer consisting of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA). Pheomelanin is yellowish-red in color and consists of sulfur-containing benzothiazine derivatives [2, 45]. Neuromelanin is produced in dopaminergic neurons of the human substantia nigra, the dorsal motor nucleus of the vagus nerve, and the median raphe nucleus of the pons. Neuromelanin has the capacity to chelate redox-active metals such as Cu, Mn, and Cr as well as toxic metals such as Cd, Hg, and Pb to avoid neuron degeneration [46]. If the level of neuromelanin decreases, dopamine synthesis may be diminished, resulting in diseases associated with neuronal degradation. Massive loss of dopamine-producing pigmented neurons in the substantia nigra has been found in patients with Parkinson's disease [47].

The biosynthetic pathway governing melanin formation is well established [48-52] (Figure 2). Synthesis of melanin starts with the conversion of the amino acid L-tyrosine to dopaquinone by tyrosinase, a copper-containing glycosylated type I membrane-bound glycoprotein that catalyzes the rate-limiting step of melanin biosynthesis [53, 54]. Tyrosinase is synthesized by melanosomal ribosomes on the rough endoplasmic reticulum [55].

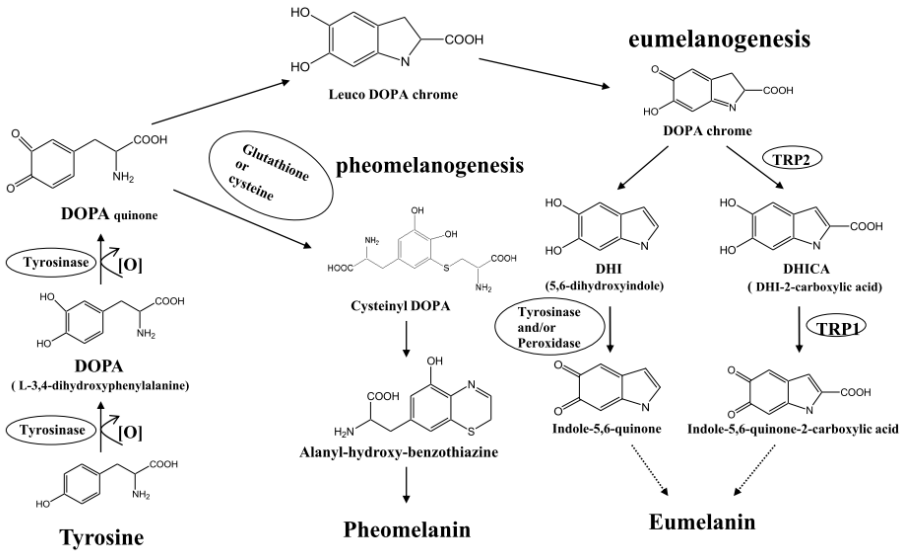

Figure 2. Pathway of melanin biosynthesis.

The enzyme is glycosylated *en route* to and within the Golgi apparatus, and subsequently delivered to melanosomes via coated vesicles [55, 56]. Tyrosinase is the most common target for therapeutic agents intended to alleviate hyperpigmentation [57-59]. Tyrosinase catalyzes two distinct oxidation reactions. First, tyrosinase catalyzes the oxidation of monophenol (L-tyrosine) to *o*-diphenol (3,4-dihydroxyphenylalanine, L-DOPA (monophenolase activity). Second, L-DOPA is oxidized to *o*-quinone (dopaquinone) (diphenolase activity). Tyrosinase gene transcription has been shown to correlate with the differentiation of lysosomes and/or peroxisomes into melanosomes [60, 61]. Tyrosinase-related protein 1 (TPR-1) and DOPAchrome tautomerase (DCT, also known as TRP-2) subsequently metabolize dopaquinone into eumelanin through a process referred to as eumelanogenesis. Dopaquinone is transferred to DHI via multiple processes including decarbxylation, oxidation, and polymerization and DOPAchrome is converted to DHICA. Pheomelanogenesis refers to the process through which dopaquinones conjugate with thiol-containing cysteines or glutathiones to form pheomelanin. As mentioned above, dopaquinone plays pivotal roles both in eumelanogenesis and pheomelanogenesis [16]. Eumelanogenesis involves the activation of tyrosinase, TRP-1, and TRP-2 whereas the synthesis of pheomelanin only requires the activation of tyrosinase [16, 62]. Following the synthesis of those pigments, melanin-containing melanosomes are transferred to neighboring keratinocytes. However, without successful transfer of melanosomes to keratinocytes, the skin can appear essentially unpigmented [63].

FACTORS REGULATING MELANIN BIOSYNTHESIS

UV radiation from the sun stimulates melanin synthesis in skin. After UV exposure, melanocytes increase their expression of pro-opiomelanocortin (POMC, the precursor of α-MSH) and its receptor melanocortin 1 receptor (MC1-R), tyrosinase, TRP-1, protein kinase C (PKC), and other signaling factors [64-66] (Figure 3).

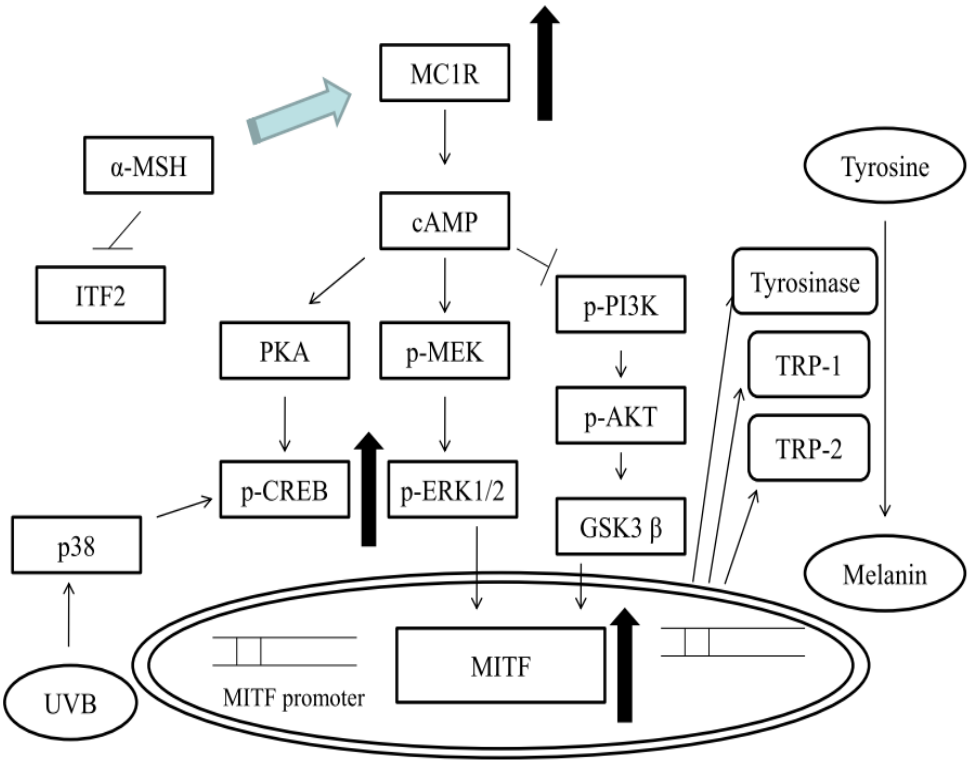

Figure 3. Factors regulated melanin biosynthesis.

Upon exposure to UV irradiation, fibroblasts release the above-mentioned cytokines, growth factors, and inflammatory factors, which then stimulate melanin production and/or stimulate melanin transfer. UV also stimulates the production of endothelin-1 (ET-1) and POMC in keratinocytes, factors that then act in a paracrine manner to stimulate melanocyte function [67, 68]. Other keratinocyte-derived factors that regulate the proliferation and/or differentiation of melanocytes include α-MSH, adrenocorticotropin melanocyte stimulating hormone (ACTH), basic fibroblast growth factor (bFGF), nerve growth factor (NGF), endothelins, granulocyte-macrophage colony-stimulating factor (GM-CSF), steel factor, leukemia inhibitory factor (LIF), and hepatocyte growth factor (HGF) [69]. Melanocytes have been shown to increase the production of intracellular nitric oxide (NO), which in turn triggers signal transduction cascades to initiate melanogenesis [70, 71] through the enzyme tyrosinase. In addition, human melanocyte proliferation requires cross-talk between several signaling pathways including the cAMP/PKA, PKC, and tyrosine kinase pathways; therefore, the mechanisms by which various factors increase skin pigmentation are closely inter-related [52, 72-75].

UV radiation has been shown to influence melanogenesis through a paracrine regulation process involving keratinocytes [52, 76] (Figure 3). Both autocrine and paracrine cytokine networks are involved in UV-induced upregulation of melanogenesis [77]. α-MSH is a major mediator of the response of melanocytes to UV [78]. The POMC gene is activated in the pituitary gland but POMC-derived peptides are also generated in keratinocytes and melanocytes [79, 80].

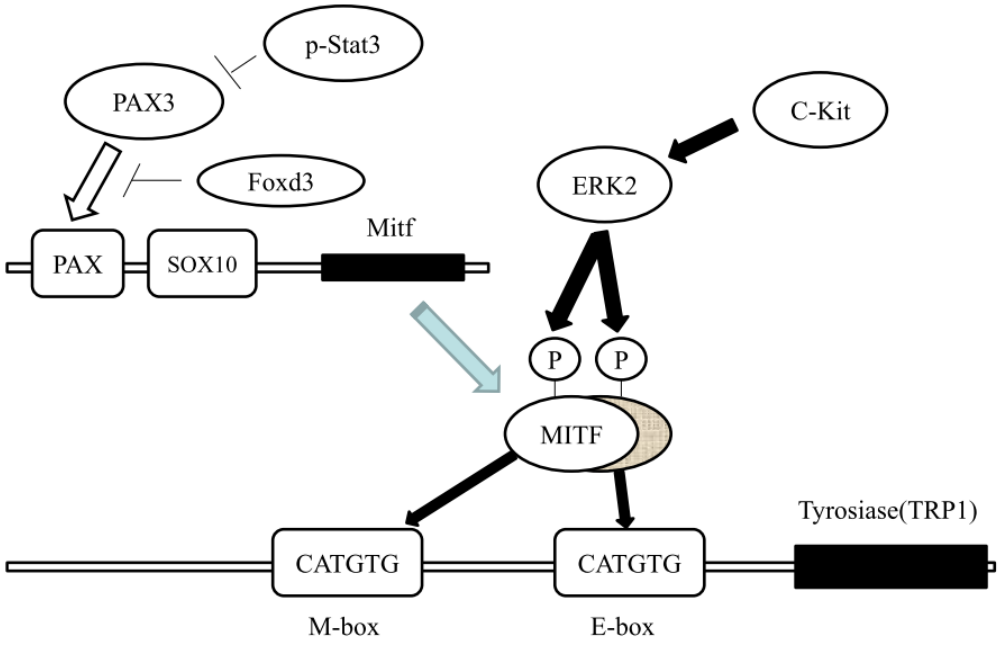

Figure 4. Gene expression of tyrosinase.

The POMC gene encodes a large precursor protein, which is then enzymatically cleaved to form several different peptides including α-MSH, ACTH, melanocortin, and β-endorphin [80]. The binding of α-MSH and ACTH to MC1-R on the melanocyte membrane [81] activates intracellular adenylate cyclase through G proteins, which then catalyze the conversion of adenosine triphosphate to cyclic AMP (cAMP) [82]. cAMP exerts its function through protein kinase A (PKA) [83]. The intracellular elevation of cAMP increases the protein expression of microphthalmia-associated transcription factor (MITF), tyrosinase, and TRP-2, but not tyrosinase or TRP-2 mRNAs [84]. PKA promotes the activation of the cAMP-response element binding protein (CREB) that binds to the cAMP response element (CRE) that is present in the M promoter of the MITF gene [85, 86]. MITF is a transcription factor with a basic helix-loop-helix-leucine zipper motif. MITF regulates melanocyte cellular differentiation and the transcription of melanogenic enzymes such as tyrosinase, TRP-1, and TRP-2 and the transcription of melanosome structural proteins including MART-1 and Pmel17 [87-90]. Pmel17 is a structural matrix protein and an amyloid protein required for the generation of the internal fibril [91]. The promoter sequences of tyrosinase, TRP-1, and TRP-2 share a highly conserved motif known as the M-box, which contributes to their melanocyte-specific expression [92, 93] (Figure 4).

TRP-1 promoter activity is up regulated by paired box 3 (PAX3) [94]. The M-box (AGTCATGTGCT) is an extended E-box (ACATGTGA) and is necessary for promoter up-regulation by MITF [16, 95]. The E-box is more important than the M-box in promoting the transcription factor MITF [96] (Figure 4). MITF is exclusively expressed in melanocytes. It binds to the M-box promoter elements of tyrosinase and modulates TRP-1 and TRP-2, resulting in hyperpigmentation [97-99]. In addition to the process of melanization, MITF also regulates melanocyte proliferation, differentiation, development, apoptosis, and survival [100-102].

A transient increase in MITF leads to the up-regulation of tyrosinase, TRP-1, and TRP-2[103] as well as to increased dendricity [88]. Many transcription factors including Sry-related HMG box (SOX) 9 and 10, PAX3, signal transducer and activator of transcription 3 (STAT3), protein inhibitor of activated STAT3 (PIAS3), lymphoid-enhancing factor-1 (LEF-1), immunoglobulin transcription factor-2 (ITF2), and forkhead-box transcription factor D3 (FOXD3) are able to modulate the expression and/or transcriptional activity of MITF *in vivo* [104] (Figure 4). The transcription factor SOX9 may play an important role in UVB-induced melanocyte differentiation and pigmentation through MITF regulation [105]. SOX10 regulates the expression of MITF and TRP-2. SOX10 has been demonstrated to activate the TRP-2 promoter-reporter construct and to work in synergy with MITF [106, 107]. PAX3 binds to the MITF gene promoter to regulate MITF expression [108]. In addition, PAX3 has been shown to act in synergy with SOX10 to up regulate the expression of MITF [109]. The transcriptional activity of MITF is regulated through the interaction between STAT3 and PIAS3. LEF-1, a transcription factor involved in the Wnt signal transduction pathway, initiates and facilitates MITF expression, while ITF2 and FOXD3 down regulate MITF expression [104, 110-112].

The transcriptional activity of MITF is regulated by phosphorylation of tyrosinase residues on extracellular signal-regulated kinase 2 (ERK2) following signals from c-kit (tyrosinase-type receptor) and then by phosphorylation of the 73rd serine residue in the N terminal domain of MITF [113] (Figure 4). The tyrosinase gene and TRP-1 promoter zones share a CATGTG motif. When MITF is activated, binding to the formed dimmers serves to regulate the expression of the tyrosinase gene TRP-1. MITF is also regulated at the transcriptional level by interleukin-6 (IL-6) and the Wnt signaling pathway and it is post-transcriptionally regulated by phosphorylation via ribosomal S6 kinase (RSK), glycogen synthase kinase-3β (GSK3β), p38 stress signaling, and the mitogen-activated protein kinase (MAP kinase) pathways [89, 90, 98, 114-116] (Figure3). α-MSH also stimulates p38 MAP kinase, which in turn phosphorylates upstream transcription factors that bind to the tyrosine promoter [52]. Human placental lipid upregulates p38 activation and subsequent tyrosinase expression, thereby promoting melanogenesis [117]. Down-regulation of p38 expression leads to an increase in expression of biomarkers associated with differentiation such as tyrosinase and tyrosinase-related proteins. The mechanism involved in the p38-mediated regulation of melanogenesis is the ubiquitin-proteasome pathway, through which melanogenic enzymes are degraded [118]. In addition, inhibition of ERK and AKT signaling via MITF up-regulation plays a key role in inducing hyperpigmentation [119]. ERK activation results in phosphorylation of MITF and its subsequent ubiquitination and degradation [120]. Sphingosine-1-phosphate, C2-ceramide, and sphingosylphosphorylcholine activate ERK and may play important roles in the inhibition of melanogenesis [120-122].

Transforming growth factor-β1 (TGF-β1) inhibits melanogenesis by mediating the down-regulation of MITF promoter activity as well as by reducing the production of tyrosinase, TRP-1, TRP-2, and MITF protein levels. In addition, TGF-β1 inhibits the expression of PAX 3, which in turn inhibits melanogenesis [123]. It has been reported that TGF-β1 influences the ERK pathway and down regulates MITF and the production of melanogenic enzymes [115, 124, 125].

The agouti signaling protein (ASP) can down regulate MITF gene expression and compete with α-MSH in binding to MC1R, causing inhibition of α-MSH signaling on the MC1R receptor. ASP modulates the frequency, rate, and extent of eumelanin and

pheomelanin generation [4]. Studies have demonstrated that high levels of ASP are associated with yellow-pigmented bands in mouse hair because ASP inhibits α-MSH binding to MC1R [16]. Thus, MC1R and its ligands, α-MSH and ASIP, regulate the switch between eumelanin and pheomelanin synthesis in melanocytes [79, 126].

UVB exposure activates the transcription factor p53, which in turn induces the expression of POMC. Expression of that α-MSH precursor leads to the secretion of α-MSH and the up-regulation of melanogenesis via MC1R in keratinocytes [127, 128]. In addition, p53 directly stimulates the expression of the genes encoding tyrosinase and TRP1 in melanocytes [129]. UVB also induces the expression of corticotropin-releasing hormone (CRH) in melanocytes, which is mediated by the CREB–PKA signaling pathway with consequent stimulation of POMC expression through the CRH-R1 receptor. The POMC gene has been shown to be p53-responsive following UV irradiation [130]. Kichina et al. demonstrated that stable transfection of wild-type p53 into pigmented melanoma cells leads to overexpression of wild-type p53 and a decrease in tyrosinase mRNA levels and tyrosinase activity [131]. Khlgatian et al. have shown that UV irradiation results in increased p53-dependent tyrosinase mRNA levels in melanoma cells and that p53 is required for the thymidine dinucleotide-induced increase in tyrosinase function in mouse epidermis [132]. They also reported that tanning is part of a p53-mediated adaptive response of mammalian skin to UV-induced DNA damage [132].

Other hormones, such as steroids and sex hormones, can influence pigmentation [79, 127, 133], and it has been reported that cholesterol is capable of increasing the expression of MITF and its target genes in melanocytes through the up-regulation of the CREB protein [134]. Two fibroblast-derived paracrine factors, namely dickkopf-related protein 1 (DKK1) and neuregulin-1 (NRG1), regulate melanogenesis. DKK1 is a factor secreted by fibroblasts. DKK1 has been shown to suppress growth of melanocytes, strongly inhibit melanin production, and inhibit binding of Wnt proteins to their receptors, which results in down-regulation of melanogenesis [102, 135]. In addition, DKK1 suppresses melanocyte growth and function by inhibiting the Wnt/b-catenin signaling pathway [136, 137]. DKK1 has also been shown to regulate the expression of PAR-2 [137].

PIGMENTARY DISORDERS

Hyperpigmentation disorders are characterized by the overproduction of melanin and include melasma, postinflammatory hyperpigmentation, freckles, moles, chloasma, age spots, and lentigines [138-140]. Hypopigmentation disorders are characterized by the underproduction of melanin and include disorders such as oculocutaneous albinism, Hermansky-Pudlak syndrome, Griscelli syndrome, Chediak-Higashi syndrome, and Waardenburg syndrome.

Oculocutaneous albinism is an inherited autosomal recessive disorder characterized by deficiency or complete absence of melanin [61]. At least 10 types of oculocutaneous albinism exist. Patients with the disorder present with hypopigmention of the skin, hair, and eyes as well as reduced visual acuity with nystagmus and photophobia. Furthermore, in these patients there is often complete lack of tyrosinase activity [141, 142]. Oculocutaneous albinism type 2, which is characterized by a congenital reduction or absence of melanin pigment in the skin, hair, and eyes, is the most common type and the incidence is highest in black Africans [16].

Hermansky-Pudlak syndrome (HPS) is a genetically heterogeneous group of related autosomal recessive conditions. It is divided into eight types according to the HPS genes that carry mutations [143]. Defects in proteins encoded by these genes can affect the biogenesis or function of intracellular organelles such as melanocytes and retinal pigment epithelial cells. Hermansky-Pudlak Syndrome is also associated with lung disease, inflammatory bowel disease, renal disease, and bleeding problems due to platelet dysfunction [16].

Griscelli syndrome is an autosomal recessive disorder characterized by pigmentary dilution of the skin and the accumulation of large and abnormal end-stage melanosomes in the center of melanocytes [144]. It may be caused by defects in the formation of the Rab27a-Mlph-MyoVa protein complex in melanocytes, an important protein that connects melanosomes to the actin network [144].

Chediak-Higashi syndrome is an autosomal recessive disorder similar to oculocutaneous albinism [145]. Patients with this syndrome are susceptible to infection because they lack natural killer cell function and are at risk for developing lymphofollicular malignancy and peripheral neuropathies [146, 147].

Mutations in the human homolog of the MITF gene are associated with auditory and pigmentary abnormalities in patients with Waardenburg syndrome type IIA [109, 148, 149]. Mutations in the PAX3 gene are associated with Waardenburg syndrome type I, while SOX 10 mutations are characteristic of Waardenburg syndrome type IV [4, 51].

MECHANISMS OF DEPIGMENTATION

Studies on the processes of cellular melanogenesis and the response of pigment-producing cells to UV radiation have been instrumental in promoting the development of depigmenting agents [57, 58, 114, 150, 151]. The mechanisms of action by which biological and chemical agents cause hypopigmentation include (i) tyrosinase inhibition, maturation, and enhancement of its degradation; (ii) inhibition of tyrosinase mRNA transcription; (iii) inhibition of MAP kinases, TRP-1, TRP-2, and MITF; (iv) downregulation of MC1R activity; (v) interference with melanosome maturation and transfer; and (vi) melanocyte loss and desquamation [57, 114, 150-153]. Tyrosinase inhibition is the most common approach to achieve skin hypopigmentation as this enzyme catalyses the rate-limiting step of pigmentation [114, 152]. Tyrosinase inhibitors can be classified as competitive, uncompetitive, mixed type, and non-competitive inhibitors [57, 154]. Tyrosinase can be inhibited at the transcriptional and post-transcriptional levels by inhibiting tyrosinase mRNA transcription and disrupting tyrosinase glycosylation by using competitive or non-competitive inhibitors to attenuate the catalytic activity of tyrosinase, by accelerating tyrosinase degradation, and by modulating tyrosinase stability [155, 156].

NATURAL HYPOPIGMENTATION AGENTS

Hydroquinone, ascorbic acid, and retinoic acid have been shown to be effective skin-whitening agents; however, they are associated with harmful side effects, thereby limiting their clinical use [155]. Compounds derived from natural products, on the other hand, have been shown to be as effective as chemical-based products at reducing hyperpigmentation.

Some natural skin-depigmenting products have been reported to directly effect melanogenesis; enhance tyrosinase degradation; interfere with melanosome maturation and its transfer; inhibit inflammation-induced melanogenesis; and accelerate skin desquamation [51, 154, 157-159].

1. Effect on Melanogenesis

As shown in Table 1, whitening agents derived from natural products can be divided into three groups: phenols, polyphenols, and others [58]. Table 1 also presents the plants from which the compounds are derived, the mode of action including tyrosinase inhibition, other enzyme inhibition (OEI; TRP1 and TRP2), melanin inhibition (MI), and other mechanisms of action, as well as the IC_{50} values of said compounds. The mechanisms of tyrosinase inhibition can be evaluated by measuring enzyme inhibition kinetics using Lineweaver-Burk plots with varying concentrations of L-DOPA as the substrate. Moraceae, Anacardiaceae, Chloranthaceae, Ericaceae, Lamiaceae, Sapindaceae, and Fabaceae are rich in phenols and polyphenols that have anti-melanogenesis activity. Most studies used B16 melanoma cells as a model to investigate the mechanism of action governing melanin inhibition. Some of the studies used mouse melan-a melanocyte cultures or normal human melanocytes (NHMC) as experimental models. Data from studies that involved the use of NHMC cells are probably more reliable because those cells mimic the response to stimuli seen *in vivo*. Human melanocyte proliferation and enhancement of melanin synthesis require cross-talk between several cytokines and hormones that are released from keratinocytes. Co-cultures of melanocytes and keratinocytes from mouse [160, 161] or human skin [162] also more closely mimic the response seen *in vivo*.

The brownish guinea pig (GP) model is commonly used to study the effects of skin-whitening agents on reducing hyperpigmentation induced by UV or exposure to exogenous α-MSH (Table 1). In human studies, the activities of skin-whitening agents are normally investigated by evaluating skin color changes using a Chromameter or a Mexameter or by histochemical investigations of DOPA positive cells [163, 164]. Beginning in September 2009, the Commission of the European Communities established a prohibition to test finished cosmetic products and cosmetic ingredients on animals (European Commission - Consumer Affairs). Commercially available skin equivalent models (SEMs), a keratinocyte and melanocyte co-culture system [165], and MatTek's MelanoDerm™ (MatTek Corporation), a human three dimensional skin-like tissue structure, are useful *in vitro* models for evaluating the ability of cosmetic and pharmaceutical agents to modulate skin pigmentation. A common vertebrate model organism that is used for whitening studies is the zebrafish, which has been [165, 166] proved to be a useful model for demonstrating the *in vivo* toxicity of whitening agents.

2. Enhancing Tyrosinase Degradation

Fatty acids are ubiquitous components of cell membranes and serve as a biological energy source. They also play important roles in intracellular signaling and as precursors for ligands that bind to nuclear receptors [152, 167-169].

Table 1. Whitening ingredients from natural sources effect on melanogenesis

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
Phenols						
Anacardic acid, 6-[8(Z),11(Z),14-pentadecatrienyl]-salicylic acid, 5-[8(Z),11(Z),14-pentadecatrienyl] resorcinol	*Anacardium occidentale* cashew fruit (Anacardiaceae)	Yes (c)				[249]
10'(Z)-heptadecenylhydroquinone	*Rhus succedanea* (Anacardiaceae)	Yes IC_{50} = 37 μM			Yes IC_{50} = 40 μM	[250]
2-Hydroxy-4-methoxybenzaldehyde	*Rhus vulgaris* Meikle *Sclerocarya caffra* Sond (Anacardiaceae) *Mondia whitei* (Hook) Skeels (Asclepiadaceae)	Yes (m) ID_{50} =0.03 mM				[251]
3,4-Dihydroxyacetophenone	*Ilex pubescens* (Aquifoliaceae)	Yes IC_{50} = 10 μM		Reduction of TYR and MITF protein level	Yes	[252]
p-Coumaric acid	*Panax ginseng* (Araliaceae)	Yes (m) IC_{50} = 3.65 mM				[253]
p-Coumaric acid	*Sasa quelpaertensis* (Gramineae)	Yes (c)		Reduction of TYR protein level	Yes	[254]
2',4',6'-trihydroxydihydrochalcone	*Greyia flanaganii* (Greyiaceae)	Yes IC_{50} = 69.15 μM				[255]
protocatechuic aldehyde	*Salvia miltiorrhiza* (Lamiaceae)	Yes (c) IC_{50} = 19.92 μM				[256]

Table 1. (Continued)

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
protocatechualdehyde	*Phellinus linteus* (Hymenochaetaceae)	Yes (c)				[257]
protocatechuic acid methyl ester	Black Rice Bran	Yes IC_{50} = 0.28 μM				[258]
phloroglucinol (1), eckstolonol (2), eckol (3), hlorofucofuroeckol A (4), ieckol (5)	*Ecklonia stolonifera* brown alga extracts (Laminariaceae)	Yes [(1) and (2), -- (c)] (1) IC_{50} = 92.8 μg/mL (2) IC_{50} = 126 μg/mL Yes [(3) , (4) and (5) -- (n)] (3) IC_{50} = 33.2 μg/mL (4) IC_{50} = 177 μg/mL (5) IC_{50} = 2.16 μg/mL			Yes	[259]
Phloroglucinol dieckol, eckol	*Ecklonia cava* (Lessoniaceae)	Yes Dieckol (88.9% of TYR at 50 μM)		Reduction of UV-B induced cell damages	Yes	[260]
7-phloroeckol	*Ecklonia cava* (Lessoniaceae)	Yes (nc) IC_{50} = 0.85 μM			Yes	[261]
Cinnamaldehyde (1), 2-methoxy cinnamaldehyde (2), cinnamic acid (3), O-coumaric acid (4), icariside DC (5), dihydromelilotoside (6), dihydromelilotoside (7)	*Cinnamomum cassia* (Lauraceae)	Yes (1) IC_{50} = 0.52 ± 0.03 mM, (2) IC_{50} = 0.42 ± 0.02 mM, (3) IC_{50} = 0.41 ± 0.01 mM, (4) IC_{50} = 0.67 ± 0.03 mM, (5) IC_{50} = 0.71 ± 0.03 mM, (6) IC_{50} = 0.57 ± 0.01 mM, (7) IC_{50} = 0.63 ± 0.02 mM				[262]
Mulberroside F	*Morus alba* leaves (Moraceae)	Yes TYR (mushroom) IC_{50} = 0.29 μg/mL ; TYR (mammalian) IC_{50} = 68.3 μg/mL		Superoxide scavenging activity	Yes (30.6% of MI at 1 mg/mL)	[263]

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
4-Substituted resorcinols	*Artocarpus incises* (Moraceae)	Yes (c)				[264]
Macelignan	*Myristica fragrans* (Myristicaceae)	Yes IC_{50} = 30 μM	TRP-1 TRP-2	Reduction of TYR, TRP-1 and TRP-2 protein level	Yes IC_{50} = 13 μM	[265]
Americanin A (1), 3,3'-Bisdemethylpinoresinol (2)	*Morinda citrifolia* seeds (Rubiaceae)	Yes (1) IC_{50} = 2.7 mM (2) IC_{50} = 0.3 mM		SOD-like activity		[266]
3-Caffeoylquinic acid, 4-Caffeolyquinic acid, 5-Caffeoylquinic acid, 5-Feruloylquinic acid, 3,4-Dicaffeoylquinic acid, 3,5-Dicaffeoylquinic acid, 4,5-Dicaffoylquinic acid	green coffee beans (Rubiaceae)	Yes				[267]

Table 1. (Continued)

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
3,4-Hihydroxycinnamic acid (1), 4-Hydroxy-3-methoxycinnamic acid (2)	*Pulsatilla cernua* (Ranunculaceae)	Yes (nc) (1) IC_{50} = 0.97 mM (2) IC_{50} = 0.33 mM				[268]
4-Acetonyl-3,5-dimethoxy-p-quinol (1), cis-p-Coumaric acid (2), trans-p-Coumaric acid (3), p-Hydroxybenzoic acid (4), Vanillic acid (5)	*Synsepalum dulcificum* (Sapotaceae)	Yes (1) IC_{50} = 208.1 μM, (2) IC_{50} = 197.9 μM, (3) IC_{50} = 168.7 μM, (4) IC_{50} = 358.6 μM, (5) IC_{50} = 174.4 μM				[269]
Cardamonin	*Alpinia katsumadai* Hayata (Zingiberaceae)	Yes		MITF	Yes	[270]
Isopanduratin A (1), 4-Hydroxypanduratin A (2)	*Kaempferia pandurata* (Zingiberaceae)	Yes (1) IC_{50} = 10.5 μM (2) IC_{50} > 30 μM		Reduction of TYR protein level	Yes (1) IC_{50} = 10.64 μM (2) IC_{50} = 23.25 μM	[271]
Curcumin Yakuchinone A, Yakuchinone B, Eugenol, Ferulic acid	*Syzygium aromaticum* (Myrtaceae) and *Alpinia oxyphylla* (Zingiberaceae)	Yes (c) (Curcumin and yakuchinone B)				[272]
polyphenols						
1,2,3,4,6-penta-O-galloyl-â-D-glucose	*Galla rhois* (Anacardiaceae)	Yes (nc)				[273]

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
2,3,4,6-tetra-O-galloyl-D-glucopyranose (1), 1,2,3,6-tetra-O-galloyl-beta-D-glucopyranose (2), 1,2,3,4,6-penta-O-galloyl-beta-D-glucopyranose (3)	*Rhus chinensis* (Anacardiaceae)	Yes (nc) (1) IC_{50} = 54 μM, (2) IC_{50} = 30 μM, (3) IC_{50} = 15 μM			Yes	[274]
Tannic acid	*Rhus javanica* leaves (Anacardiaceae)	Yes (c) IC_{50} = 22 μM				[275]
Icariside I (1), Icariside II (2), Icaritin (3)	*Epimedium grandiflorum* (Berberidaceae)				Yes (1) IC_{50} = 49.04 μM, (2) IC_{50} = 10.53 μM, (3) IC_{50} = 11.13 μM	[276]
Xanthohumol	*Humulus lupulus* L. (Cannabaceae)	Yes	Reduction of TRP-1 and TRP-2 mRNA level	Reduction of cAMP, MITF protein and its mRNA expression and TYR protein expression	Yes	[277]
GB-2 (biflavanones)	*Garcinia kola* Seed (Clusiaceae)	Yes IC_{50} = 582 μM				[278]
GS contained 2 biflavonoids; 2R,3S-5,7,4',5",7",3'",4'"-heptahydroxy-flavanone[3-8"] flavone (1), 5,7,4',5",7",3'",4'"-heptahydroxy[3-8"] biflavanone (2)	*Garcinia subelliptica* (Clusiaceae)	Yes (1) IC_{50} = 2.5 μM (2) IC_{50} = 26 μM				[279]

Table 1. (Continued)

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
3 flavonols: Quercetin (1), Kaempferol (2), Morin (3), 2 flavones: Luteolin (4), Luteolin 7-*O*-glucoside (5)	*Heterotheca inuloides* (Asteraceae)	3 favonols-(c) (1) ID_{50} = 0.07 mM (2) ID_{50} = 0.23 mM (3) ID_{50} = 2.32 mM 2 flavones-(n) (4) ID_{50} = 0.19 mM (5) ID_{50} = 0.50 mM				[280]
Luteolin		Yes		Inhibition of adenyl cyclase activity	Yes	[281]
N-feruloylserotonin (1), N-(*p*-coumaroyl)serotonin (2), acacetin (3)	*Carthamus tinctorius* L. (Asteraceae)	Yes (1) IC_{50} = 0.023 mM (2) IC_{50} = 0.074 mM (3) IC_{50} = 0.779 mM			Yes IC_{50} = 0.191 mM IC_{50} = 0.245 mM IC_{50} > 20 mM	[282]
Inulavosin	*Inula nervosa* (Asteraceae)			Mistargeting of tyrosinase to lysosomes	Yes	[283]
Anastatin A (1), Isosilybin A (2), Isosilybin B (3), Luteolin (4), Quercetin (5), (+)-Dehydrodiconiferyl alcohol (6), (+)-Balanophonin (7), 3,4-Dihydroxybenzaldehyde (8)	*Anastatica hierochuntica* (Cruciferae)			Isosilybin A (2) and Isosilybin B (3) inhibit the mRNA expression of TRP-2	Yes (1) IC_{50} = 16 μM, (2) IC_{50} = 10 μM, (3) IC_{50} = 6.1 μM, (4) IC_{50} = 14 μM, (5) IC_{50} = 15 μM, (6) IC_{50} = 16 μM (7) IC_{50} = 15 μM (8) IC_{50} = 17 μM	[284]

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
Silymarin	*Silybum marianum* (milk thistle) (Asteraceae)	Yes		Reduction of TYR protein levels	Yes IC_{50} = 28.2 μg/mL	[285]
5,2',4'-trihydroxy-2'',2''-dimethylchromene-(6,7:5'',6'')-flavanone	*Dalea elegans* (Fabaceae)	Yes [(m) L-tyrosine IC_{50} = 0.26 μM] [(nc) L-DOPA IC_{50} = 18.61 μM]				[286]
Kuraridin (1), Kurarinone (2), Norkurarinol (3)	*Sophora flavescens* (Fabaceae)	Yes (1) IC_{50} = 1.1 μM (2) IC_{50} = 1.3 μM (3) IC_{50} = 2.1 μM				[287]
Sophoraflavanone G (1), Kurarinone (2), Kurarinol (3)	*Sophora flavescens* (Fabaceae)	Yes (1) nc- IC_{50} = 4.7 μM, (2) nc- IC_{50} = 2.2 μM (3) c- IC_{50} = 0.1 μM				[288]
Sophoraflavanone G (1), Kuraridin (2), Kurarinone (3)	*Sophora flavescens* (Fabaceae)	Yes IC_{50} = 6.6 μM IC_{50} = 0.6 μM IC_{50} = 6.2 μM				[289]
Kurarinone (1), Kushnol F (2)	*Sophora flavescens* (Fabaceae)	Yes (1)-nc IC_{50} = 4.6 μ g/mL IC_{50} = 9.0 μg/mL				[290]
Kurarinol (1), Kuraridinol (2)	*Sophora flavescens* (Fabaceae)	Yes (1,2)-nc (1) IC_{50} = 8.60±0.51 μM (2) IC_{50} = 0.88±0.06 μM			Yes (1) IC_{50} = 29 μM, (2) IC_{50} = 17 μM	[291]
5,2',4'-Trihydroxy-2'',2''-dimethylchromene-(6,7:5'',6'')-flavanone	*Dalea elegans* (Fabaceae)	Yes [(m) L-tyrosine IC_{50} = 0.26 μM] [(nc) L-DOPA IC_{50} = 18.61 μM]				[*286]*

Table 1. (Continued)

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
Kuraridin (1), Kurarinone (2), Norkurarinol (3)	*Sophora flavescens* (Fabaceae)	Yes (1) IC_{50} = 1.1 μM (2) IC_{50} = 1.3 μM (3) IC_{50} = 2.1 μM				[287]
N-Feruloyl-N′-cis-feruloyl-putrescine	*Sophora japonica* (Fabaceae)	Yes (m) IC_{50} = 85.0 μM				[292]
Dalbergioidin	*Lespedeza cyrtobotrya* (Fabaceae)	Yes (nc) IC_{50} = 20 μM			Yes IC_{50} = 27 μM	[293]
Haginin A	*Lespedeza cyrtobotrya* (Fabaceae)	Yes (nc) IC_{50} = 5.0 μM	TRP-1 protein level	Reduction of TYR, and MITF protein level, Induction of ERK and Akt/PKB protein level	Yes Melan-a cells IC_{50} = 3.3 μM ; HEMn cells IC_{50} = 2.7 μM	[294]
Glycyrrhisoflavone (1), Glyasperin C (2)	*Glycyrrhiza uralensis* (Fabaceae)	Yes (2) IC_{50} = 0.13 μg/mL			Yes 63.73 ± 6.8 % inhibition at 5 μg/mL 17.65 ± 8.8 % at 5 μg/mL	[295]
Licuraside (1), Isoliquiritin (2), Licochalcone A (3)	*Glycyrrhiza uralensis* (contains compond 1 and 2) *Glycyrrhiza inflate* (contains compond 3) (Fabaceae)	Yes 1, 2 and 3 (c) (1) IC_{50} = 0.072 mM (2) IC_{50} = 0.038 mM (3) IC_{50} = 0.0258 mM				[296]

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
Calycosin	*Astragalus membranaceus* (Fabaceae)	Yes IC_{50} = 38.4 μM			Yes IC_{50} = 40 μM	[297]
Butin	*Spatholobus suberectus* (Fabaceae)	Yes IC_{50} = 35.9 μM	Reduction of TRP-1 and TRP-2 protein and mRNA level	Reduction of TYR protein and mRNA level	Yes 29.26% at 100 μM	[298]
Gallocatechin (1), Epi-gallocatechin gallate (2), Quercitrin (3)	*Distylium racemosum* (Hamamelidaceae)	Yes (1) IC_{50} = 4.8 μg/ mL, (2) IC_{50} = 30.2 μg/ mL, (3) IC_{50} = 37.7 μg/ mL				[299]
Quercetin (1), Tiliroside (2)	*Marrubium velutinum* and *Marrubium cylleneum* (Lamiaceae)	yes 100% inhibition (1) 49.67 ± 1.16 mM (2) 30.19 ± 9.60 mM				[300]
Kaempferol	*Crocus sativus* L. (Iridaceae)	Yes (c) ID_{50} = 0.23 mM				[301]
Bibenzyl xyloside-1 (1), Bibenzyl xyloside-2 (2), Bibenzyl xyloside-3 (3)	*Chlorophytum arundinaceum* (Liliaceae)	Yes (1) IC_{50} = 1.6 μM (2) IC_{50} = 0.43 μM (3) IC_{50} = 0.73 μM				[302]
Resveratrol (1), Oxyresveratrol (2)	*Veratrum patulum* (Liliaceae)	Yes (1) IC_{50} = 43.5 μM (2) IC_{50} = 1.2 μM				[303]
2"- O-Feruloylaloesin, aloesin	*Aole vera* extracts (Liliaceae)	Yes (n)				[304]
Aloesin		Yes		in vitro pigmented skin equivalent model	Yes	[305]

Table 1. (Continued)

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
Artocarpfuranol(1), Dihydromorin (2), Steppogenin (3), Norartocarpetin (4), Artocarpanone (5), Artocarpesin (6), Isoartocarpesin (7)	*Artocarpus heterophyllus* (Moraceae)	Yes (1) IC_{50} = 47.93 μM (2) IC_{50} = 10.34 μM (3) IC_{50} = 0.57 μM (4) IC_{50} = 0.46 μM (5) IC_{50} = 1.54 μM (6) IC_{50} = 0.52 μM (7) IC_{50} = 0.66 μM				[306]
Norartocarpetin (1), Resveratrol (2)	*Artocarpus gomezianus* (Moraceae)	Yes				[307]
3-Prenyl luteolin	*Artocarpus heterophyllus* (Moraceae)	Yes IC_{50} = 76.3 μM			Yes IC_{50} = 57.6 μM	[308]
1,3-Diphenylpropanes: Kazinol C (1), Kazinol F (2), Broussonin C (3), Kazinol S (4)	*Broussonetia kazinoki.* (Moraceae)	Yes (c) (1) IC_{50} = 15.5 μM (2) IC_{50} = 0.96 μM (3) IC_{50} = 0.43 μM (4) IC_{50} = 17.9 μM				[309]
Chlorophorin	*Chlorophora excelsa* (Moraceae)	Yes (c) IC_{50} = 1.3 μM				[310]
4-[(2''*E*)-7"-hydroxy-3",7" -dimethyloct-2" -enyl]-2' ,3,4',5-tetrahydroxy-*trans*-stilbene	*Chlorophora excelsa* (Moraceae)	Yes (c) IC_{50} = 96 μM				[310]
(±)2,3-cis-Dihydromorin (1), 2,3-trans-Dihydromorin (2), Oxyresveratrol (3)	*Cudrania cochinchinensis* (Moraceae)	Yes (1) IC_{50} = 31.1 μM (2) IC_{50} = 21.1 μM (3) IC_{50} = 2.33 μM				[311]
2,4,2',4'-Tetrahydroxy-3-(3-methyl-2-butenyl)-chalcone	*Morus nigra* (Moraceae)	Yes (c) IC_{50} = 0.95 μM			Yes	[312]
Oxyresveratrol	*Morus alba* L. (Moraceae)	Yes (nc) IC_{50} = 1 μM				[313]

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
Polyphenols: Compound 1,5,9	*Morus lhou* (Moraceae)	Yes (c) IC_{50} = 1.3 μM IC_{50} = 1.2 μM IC_{50} = 7.4 μM				[314]
Betulinic acid	*Morus alba* L. *and Morus rotundiloba* K. (Moraceae)	Yes				[315]
Crude extract (C-AP) Anthocyanins: Cyanidin-3-alpha-O-rhamnoside (1), pelargonidin-3-alpha-O-rhamnoside (2)	*Malpighia emarginata*. acerola fruit (Malpighiaceae)	Yes (C-AP) IC_{50} = 15 μg/mL, (1) (2) – (nc) (1) IC_{50} = 40 μM, (2) IC_0 = 19.1 μM			Yes (data no shown)	[316]
2R,3S-5,7,4',5",7",3'",4'"-heptahydroxy-flavanone[3-8"] flavone, and 5,7,4',5",7",3'",4'"-heptahydroxy[3-8"] biflavanone	*Hibiscus tiliaceus* (Malvaceae)	Yes				[279]
Globulusin A (1), Eucaglobulin (2)	*Eucalyptus globules* (Myrtaceae)				Yes	[194]
Kaempferol (1), Quercetin (2), Mudanpioside B (3), Benzoyl-oxypaeoniflorin (4), Mudanpioside H (5), Pentagalloyl-β-D-glucose (6)	*Paeonia suffruticosa.* (Paeoniaceae)	Yes (1) to (5) --(c) (1) IC_{50} = 0.12 μM (2) IC_{50} = 0.11 μM (3) IC_{50} = 0.37 μM (4) IC_{50} = 0.45 μM (5) IC_{50} = 0.32 μM (6) -- (nc) (6) IC_{50} = 0.06 μM				[317]

Table 1. (Continued)

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
2,3-dihydro-4',4'''-di-O-methylamentoflavone	*Podocarpus macrophyllus var. macrophyllus* (Podocarpaceae)	Yes IC_{50} = 0.10 mM	Reduction of TRP-2 mRNA		Yes	[318]
Anthraquinones	*Polygonum cuspidatum* (Polygonaceae)	Yes				[319]
(2R,3R)-(+)-taxifolin	*Polygonum hydropiper* L. (Benitade) (Polygonaceae)	Yes IC_{50} = 0.24 mM				[320]
3,4-Dihydroxycinnamic acid (1), 4-hydroxy-3-methoxycinnamic acid (2)	*Pulsatilla cernua* (Ranunculaceae)	Yes (nc) (1) IC_{50} = 0.97 mM (2) IC_{50} = 0.33 mM				[268]
Quercetin	*Rosa canina* L. (Rosaceae)	Yes			Yes Reducing melanin content to 64% at 10 μM, 34.5% at 20 μM, 17.5% at 17.7% at 40 μM	[321]
3,3'-Bisdemethylpinoresinol (1), Quercetin (2)	*Morinda citrifolia* (Rubiaceae)	Yes (1) IC_{50} = 0.3 mM, (2) IC_{50} = 0.1 mM				[266]
Nobiletin	Peel of *Citrus* fruit (Rutaceae)	Yes IC_{50}=46.2 μM				[322]
Betulin, Lupeol, Soyacerebroside I	*Guioa villosa* (Sapindaceae)	Yes				[323]
(+)-*epi*-Syringaresinol (1), *N*-cis-Feruloyltyramine (2)	*Synsepalum dulcificum* (Sapotaceae)	Yes (1) IC_{50} = 200 μM, (2) IC_{50} = 215.5 μM				[269]

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
Acetone extract, epigallocatechin gallate (1), Procyanidin B_1(2)	*Sideroxylon inerme* (Sapotaceae)	Yes Acetone extract IC_{50}=63 μg/mL, (1) IC_{50} = 30 μg/mL (2) IC_{50}＞200 μg/mL			yes	[324]
Negundin A (1), Negundin B (2), 6-hydroxy-4-(4-hydroxy-3-methoxy)-3-hydroxymethyl-7-methoxy-3,4-dihydro-2-naphthaledehyde (3), Vitrofolal E (4), (+)-lyoniresinol (5), (+)-lyoniresinol-3α-O-β-D-glucoside (6), (+)-(－)-pinoresinol (7),? (+)-diasyringaresinol (8)	*Vitex negundo Linn.* (Verbenaceae)	Yes (1) IC_{50} = 10.06 μM (2) IC_{50} = 6.72 μM (3) IC_{50} = 7.81 μM (4) IC_{50} = 9.76 μM (5) IC_{50} = 3.21 μM (6) IC_{50} = NA (7) IC_{50} = 15.13 μM (8) IC_{50} = 5.61 μM				[325]
Isopanduratin A (1), 4-Hydroxypanduratin A (2)	*Kaempferia pandurata* (Zingiberaceae)	Yes (1) IC_{50} = 10.5 μM (2) IC_{50} > 30 μM			Yes IC_{50} = 10.64 μM, IC_{50} = 23.25 μM	[271]
Gentol	*Gnetum* genus (Zingiberaceae)	Yes IC_{50}=4.5 μM			Yes	[326]
(-)-epigallocatechin gallate (EGCG) (1), (-)-gallocatechin 3-0-gallate (GCG) (2), (-)-epicatechin gallate (ECG) (3)	Green tea	Yes (c) (1) IC_{50} = 34.10 μM (2) IC_{50} = 17.34 μM (3) IC_{50} = 34.58 μM				[327]
1,2,3,6-Tetra-Ogalloyl-b-D-glucose (1), 1,2,3,4,6-Penta-O-galloyl-b-D-glucose, (2), 2,3,4,6- Tetra-O-galloyl-D-glucose (3)	10 Chinese Galls	Yes (nc) (1) IC_{50} = 30 μM (2) IC_{50} = 15 μM (3) IC_{50} = 54 μM			Yes	[274]

Table 1. (Continued)

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
N,N′-dicoumaroyl-putrescine (DCP), N,N′-diferuloyl-putrescine (DFP)	Corn bran	yes (DCP) IC_{50} = 181.73 μM (DFP) IC_{50} = 291.3 μM			Yes (DCP) IC_{50} = 3169.5 μM (DFP) IC_{50} = 733.64 μM	[328]
7,8,4'-Trihydroxyisoflavone (1), 7,3',4'-Trihydroxyisoflavone (2), Genistein (3)	Korean fermented soybean paste (Doenjang)	Yes (1) IC_{50} = 11.21 ± 0.8 μM (2) IC_{50} = 5.23 ±0.6 μM			Yes (1) IC_{50} = 12.23 ±0.7 μM (2) IC_{50} = 7.83 ± 0.7 μM (3) IC_{50} = 57.83 ±0.5 μM	[329]
5,7-Dihydroxyflavone (chrysin)	propolis			Blocking adenylyl cyclase activity	Yes (51.6% at 10 μM, 40.90% at 100μM)	[330]
others						
Eextract	*Salicornia herbacea* (Amaranthaceae)	Yes			Yes	[331]
70% Acetone extract	*Rhus chinensis* (Anacardiaceae)	Yes IC_{50} = 22 μg/mL			Yes	[274]
Isoimperatorin Imperatorin	*Angelica dahurica* (Apiaceae)	Yes		Reduction of TYR mRNA levels	Yes	[332]
Anisic acid	*Pimpinella anisum* (Apiaceae)	Yes (u) IC_{50} = 0.68 mM				[268]
Anisaldehyde	*Pimpinella anisum* (Apiaceae)	Yes (nc) IC_{50} = 0.38 mM				[268]

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
Cumic acid (1), Cuminaldehyde (2)	*Cuminum cyminum* (Apiaceae)	Yes (nc) (1) IC_{50} = 0.26 mM (2) IC_{50} = 0.05 mM				[333]
Ethanolic extract	*Areca catechu* (Arecaceae)	Yes IC_{50} = 0.48 mg/mL			Yes	[334]
	Lichen species: *Graphina glaucorufa, Graphina multistriata, Graphina salacinilabiata, Graphis assamensis, Graphis nakanishiana, Phaeographopsis indica*	Yes				[335]
(2Z,8Z)-Matricaria acid methyl ester	*Erigeron breviscapus* (Asteraceae)	Yes			Yes IC_{50} = 25.4 μM	[336]
Selina-4(14),7(1)-dien-8-one	*Atractylodis Rhizoma Alba.* (Asteraceae)	Yes	TRP-1, TRP-2		yes	[337]
Extract	*Lepidium apetalum* (Brassicaceae)	Yes		Reduction of TYR mRNA and MITF protein level	yes	[338]
2 Germacrane-type Sesquiterpenes	*Chloranthus henryi* (Chloranthaceae)	Yes IC_{50} = 325 μM and 269 μM				[339]
Tianmushanol (1), 8-O-methyltianmushanol (2)	*Chloranthus tianmushanensis* (Chloranthaceae)	Yes (1) IC_{50} = 358 ±3 μM (2) IC_{50}=312 ±3 μM				[340]
3β,21,22,23-Tetrahydroxycycloart-24(31),25(26)-diene	*Amberboa ramosa* (Asteraceae)	Yes IC_{50} = 1.32 μM				[341]

Table 1. (Continued)

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
1β-Hydroxy arbusculin A (1), Costunolide (2), Reynosin (3)	*Saussurea lappa* Clarke (Asteraceae)				Yes (1) IC_{50} = 11 μg/mL, (2) IC_{50} = 3.0 μg/mL (3) IC_{50} = 2.5 μg/mL	[342]
methanolic extract fraction	*Arbutus andrachne L.* (Ericaceae)	Yes IC_{50} = 1000 mg/mL				[343]
Esculetin	*Euphorbia lathyris* L. (Euphorbiaceae)	Yes(c) IC_{50} = 43 μM				[344]
three steroids: Stigmast-5-ene-3 beta,26-diol (1), Stigmast-5-ene-3-ol (2), Campesterol (3)	*Trifolium balansae* (Fabaceae)	Yes (1) stronger than (2) and (3) (1) IC_{50} = 2.39 μM				[345]
	Stryphnodendron barbatimao, *Entada africana* *Prosopis africana* (Fabaceae) *Cariniana brasiliensis* (Lecythidaceae) *Portulaca pilosa* (Portulacaceae)	Yes				[346]
Trifolirhizin	*Sophora flavescens* (Fabaceae)	Yes IC_{50} = 506.77±4.49 μM			Yes IC_{50} = 36 μM	[291]
Methyl gallate	*Distylium racemosum* branches (Hamamelidaceae)	Yes IC_{50} = 40.5 μg/ mL				[299]

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
5-Hydroxymethyl-2-furaldehyde	*Phellinus linteus* (Hymenochaetaceae)	Yes (nc) IC_{50} = 90.8 μg/mL				[257]
Crocusatin-K	*Crocus sativus* (Iridaceae)	Yes IC_{50} = 260 μM				[347]
Trans-cinnamaldehyde	*Cinnamomum cassia* (Lauraceae)	Yes (c)				[348]
Linderanolide B and Subamolide A	*Cinnamomum subavenium* (Lauraceae)	Yes			Yes	[349]
Extract	*Portulaca pilosa* (Lecythidaceae)	Yes				[346]
(-)-N-formylanonaine	*Michelia alba* D.C. (Magnolianceae)	Yes IC_{50} = 74.3 μM		DPPH, reducing power, and chelating metal ions.	yes	[350]
1',3'-dilinolenoyl-2'-linoleoylglycerol	*Flammulina velutipes* (Marasmiaceae)	Yes IC_{50} = 16.1 ± 0.5μg/mL				[351]
Ethanolic extract of mulberry twigs (EEMT), Ethanolic extract of mulberry root bark (EEMR)	*Morus alba* (Moraceae)	Yes				[352]
A series of α,β-unsaturated aldehydes	*Olea europaea L.* (Oleaceae)	Yes (n)		Their ability to form a Schiff base with a primary amino group in the enzyme		[353]
(2E)-alkenal (C 7)	*Oliva olea L.* (Oleaceae)	Yes (nc)				[353]
Acetonic extract	*Osmanthus fragrans* (Oleaceae)	Yes (u) IC_{50} = 2.314 mg/mL			Yes	[354]
Methanol extract	Lichen species: *Usnea ghattensis* (Parmeliaceae)	Yes IC_{50} = 8.5 μg/mL				[355]

Table 1. (Continued)

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
Methanol extract	Lichen species: *Arthothelium awasthii* (Parmeliaceae)	Yes IC_{50} = 17.8 µg/mL				[35])
Sesamol (3,4-methylenedioxyphenol)	*Sesamum indicum* L. (Pedaliaceae)	Yes(c) IC_{50} = 1.9 µM			Yes 63% decreased in 100 mg/mL	[356]
5-(Hydroxymethyl)-2-furfural	*Dictyophora indusiata* (Phallaceae)	Yes (nc) ID_{50} = 0.98 mM				[357]
Piperlonguminine	*Piper longum* (Piperaceae)	Yes		Reduction of TYR mRNA, and MITF protein level, phosphorylates CREB	Yes	[358]
Geranic acid	*Cymbopogon citrates* (Poaceae)	Yes IC_{50} = 0.14 mM (trans) IC_{50} = 2.3 mM (cis)				[359]
Extract	*Coccoloba uvifera* (Polygonaceae)	Yes IC_{50} = 68.84 µg/ml				[360]
Ethanol extract and distilled water extract	*Ganoderma lucidum* (Polyporaceace)	Yes IC_{50} = 0.32 mg/mL				[361]
Dichloromethane fraction	*Cimicifuga heracleifolia* (Ranunculaceae)	Yes	TRP1	Phosphorylates MEK, ERK1/2 and Akt, MITF	yes	[362]
50% Ethanolic extract	*Citrus hassaku* (Rutaceae)	Yes IC_{50} = 4.7 mg/mL			Yes	[363]
Extract	*Dimocarpus longan* (Sapindaceae)	Yes IC_{50} = 2.9–3.2 mg/mL				[364]

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
1-O-Methyl-fructofuranose	*Schisandra chinensis* (Turcz.) Baill (Schisandraceae)	Yes		Activation of MEK/ERK and PI3K/Akt signaling pathway and subsequent MITF downregulation.	Yes	[365]
Ethanol extract, water extract, adenosine (1), Ethyl-*α*-D-glucopyranoside (2)	*Stichopus japonicas* (Stichopodidae)	Yes (m) ethanol extract 0.49–0.61 mg/mL, water extract 1.80–1.99 mg/mL, (1) IC_{50} = 0.13 mg /mL, (2) IC_{50} = 0.19 mg /mL				[366]
Hirsein A, hirsein B	*Thymelaea hirsuta* (Thymelaeaceae)	Yes	TRP1, TRP2	Decrease PKC activity, MITF, TRP1, TRP2,	Yes	[367]
Metallothionein (protein)	*Aspergillus niger* (Trichocomaceae)	Yes (m)				[368]
9-Hydroxy-4-methoxypsoraln	*Angelica dahurica* (Umbelliferae)	Yes (nc) IC_{50} = 2.0 μg/mL				[369]
Alpinia galanga extract	*Alpinia galanga* Rhizome (Zingiberaceae)	Yes IC_{30} = 18.5 μg/mL			Yes	[370]
Extract	*Curcuma aromatica* Rhizome (Zingiberaceae)	Yes IC_{30} = 8.9 μg/mL			Yes	[370]
Partial purification	*Curcuma longa* (Zingiberaceae)	Yes	TRP	Phosphorylates MEK, ERK1/2 and Akt, MITF, and TRP-2 protein level	Yes	[371]
Triacylglycerols; triolein (1), Trilinolein (2)	Sake lees	Yes (nc) TI 2 > 1 (1) IC_{50} = 30 μM (2) IC_{50} = 8.4 μM				[372]

Table 1. (Continued)

Compounds (phenol, polyphenols, others)	Source	Mode of action				Refs.
		TI	OEI (TRP-1, TRP-2)	other	MI	
Aqueous extracts	green asparagus	Yes (m) IC_{50} = 1.21 mg/mL		radical scavenging, chelating activities and protected liposome against oxidative damage.		[373]
Ursolic acid		Yes		Tyrosinase mRNA and protein	Yes	[374]
San-bai-tang	San-bai-tang	Yes IC_{50} = 215.6 ± 10.3 μg/mL	TRP1, TRP2	MITF	Yes IC_{50} = 254.8 ± 14.5 μg/mL	[375]

TI: tyrosinase inhibiton, (c) competitive (u) uncompetitive (nc) noncompetitive and (m) mixed mode, OEI: other enzyme inhibition, MI: melanin inhibition, TRP-1: tyrosinase related protein-1, TRP-2: tyrosinase related protein-2, PKC: protein kinase C, MITF: microphthalmia-associated transcription factor.

Fatty acids act as intrinsic factors that modulate the proteasomal degradation of membrane glycoproteins such as tyrosinase. In addition, they regulate the selective degradation of melanogenic enzymes through the ubiquitin-proteasome pathway [170]. Ando et al. found that fatty acids regulate the ubiquitination of tyrosinase and are responsible for modulating the proteasomal degradation of the enzyme [170] and that they had remarkable regulatory effects on melanogenesis in cultured B16F10 murine melanoma cells by modulating proteolytic degradation of tyrosinase [171]. Physiological doses of oleic acid and linoleic acid have been shown to increase the proteolytic activity of 20S proteasomes in rat skeletal muscle [172].

3. Interference with Melanosome Maturation and Transfer

Table 2 presents the natural products that have been shown to interfere with melanosome maturation and transfer.

(1) Soybean Extract

Protease-activated receptors (PARs) are a subfamily of related G protein-coupled trans-membrane receptors that are proteolytically activated by serine proteases (including trypsin or mast cell tryptase). PAR-2 is expressed in keratinocytes but not in melanocytes. Stimulation of this receptor enhances the rate of phagocytosis of keratinocytes, which in turn leads to increased melanin transfer [173]. Soybean contains small serine proteases, such as Bowman Birk inhibitor (BBI) and soybean trypsin inhibitor (STI, Kunitz-type trypsin inhibitor), that have been shown to inhibit the PAR-2 pathway in keratinocytes. Interference with the PAR-2 pathway was shown to induce depigmentation by reducing the phagocytosis of melanosomes by keratinocytes, thereby diminishing melanin transfer [17, 174-176]. Interestingly, only unpasteurised soybean milk exhibits this activity.

(2) Centaureidin

Centaureidin (5,7,3'-trihydroxy-3,6,4'-trimethoxyflavone), a flavone from yarrow, has been shown to reduce melanosome transfer and melanocyte dentrite outgrowth [177]. Centaureidin either directly or indirectly activates Rho, a small GTP-binding protein that acts as a master regulator of dendrite formation. Ito et al. reported that activation of Rho in cells exposed to centaureidin resulted in dendrite retraction and reduced melanocyte trafficking of melanin to keratinocytes [178]. In addition, Saeki et al. found that centaureidin inhibited melanogenesis and reduced the total amount of tyrosinase, but not TRP-1 [177].

(3) Methylophiopogonanone B (5,7-Dihydroxy-6,8-Dimethyl-3-(4-Methoxybenzyl) Chroman-4-One, MOPB)

Studies have shown that MOPB-induced activation of Rho causes reversible dendrite retraction, microtubule disorganization, and tubule depolymerization, which in turn leads to reduced melanosome transfer. The effect MOPB has on melanogenesis, however, is not the same as the effect centaureidin has on melanin synthesis. Ito et al. showed that MOPB did not influence melanin synthesis or the expression of melanogenic enzymes [179].

(4) Niacinamide

Niacinamide (nicotinamide; 3-pyridinecarboxamide), the amide form of vitamin B_3, is a biologically active form of niacin found in many root vegetables as well as in yeast. Studies have shown that niacinamide down regulates melanogenesis via inhibiting the transfer of melanosomes from melanocytes to keratinocytes [162, 180]. Other studies have reported that niacinamide is a tyrosinase inhibitor [181, 182].

(5) Lectins and Neoglycoproteins

Cellular recognition between melanocytes and keratinocytes is an important process in melanosome transfer. Lectins and neoglycoproteins are glycosylated residues on melanocyte and keratinocyte membranes that play inhibitory roles in the process of receptor-mediated endocytosis, a process that facilitates melanosome transfer [63]. Specifically, plasma membrane lectins and their glycoconjugates are thought to interrupt melanocyte and keratinocyte contact and interaction by binding to their specific plasma membrane receptors, resulting in inhibition of melanosome transfer [183]. This inhibition is reversible and has been shown to be enhanced in the presence of niacinamide [162].

3. Inhibition of Inflammation-Induced Melanogenesis

Some mediators produced by keratinocytes after exposure to primary inflammatory stimuli or UV exposure, such as interleukin-1α (IL-1α), tumor necrosis factor α (TNF-α), ET-1, and Stem cell factor (SCF) are able to promote melanogenesis. ET-1 shows a unique behavior in exerting stimulatory effects both on DNA synthesis and melanization in human melanocytes [65, 184-186]. Activation of epidermal ETs is determined by the enzymatic cleavage of inactive prepolypeptides by an endopeptidase termed ET converting enzyme (ECE), which is regulated by the primary inflammatory cytokine IL-1α [187]. The SCF expressed in keratinocytes is involved in melanocyte growth and the synthesis, migration, and maintenance of melanin. UV exposure stimulates the overexpression of SCF, which binds to its receptor, c-kit, resulting in enhanced melanogenesis [188]. Arachidonate-derived chemical mediators, namely the cysteinyl leukotrienes (LTC) LTC4 and LTD4, and thromboxanes, such as TXB2, are released from membrane phospholipids by phospholipase A2 (PLA2). Leukotrienes not only significantly up-regulate tyrosinase, but also enhance the transfer of melanosomes to keratinocytes. These results suggest that PLA2 itself triggers melanin synthesis following UV irradiation or inflammation, thereby resulting in hyperpigmentation [52, 189]. Prostaglandins (PGs) synthesized from arachidonic acid by cyclooxygenase are responsible for regulating cellular growth, differentiation, and apoptosis. In the skin, PGs (especially PGE2, PGF2α) are produced and rapidly released by keratinocytes after exposure to UV irradiation, resulting in hyperpigmentation [190]. Therefore, anti-inflammatory compounds could be useful for the prevention or treatment of post-inflammatory hyperpigmentation.

Table 3 lists some natural products that have been shown to be effective treatments for inflammation-induced hyperpigmentation. Topical application of *Matricaria chamomilla* extract has been shown to inhibit UVB-induced pigmentation by supprerssing ET-1-induced DNA synthesis. The extract, however, did not affect IL-α-induced ET-1 production or tyrosinase activation [184].

Table 2. Whitening agents from natural sources interference with melanosome maturation and transfer

Source	Compounds	Mode of action			Refs.
		maturation	transfer	others	
Soybean extract	Bowman Birk inhibitor (BBI), soybean trypsin inhibitor (STI)		Yes		[175]
Achillea millefolium, Yarrow	*Centaureidin*	Yes	Yes	Inhibition of melanogenesis and reduction the amount of tyrosinase.	[177, 179]
Ophiopogon japonicus	*Methylophiopogonanone B*	Yes	Yes		[179]
Root of vegetable and yeast	Niacinamide		Yes		[162, 180-182]
	Lectins and Neoglycoproteins		Yes		[162, 376, 377]

Table 3. Whitening agents from natural sources inhibiting on inflammation-induced melanogenesis

Source	Compounds	Mode of action	Refs.
Matricaria chamomilla	*Matricaria chamomilla* extract	Antagonist for ET-receptor (*in vitro* and *in vivo*)	[184]
Sanguisorba officinalis L.		Suppression of endothelin-converting enzyme-1α (*in vitro* and *in vivo*)	[191]
Althaea officinalis L.	roots extract	Inhibits both the secretion and action of ET-1 (*in vitro*)	[192]
Sea algae	fucoxanthin	Suppression of prostaglandin (PGE2) synthesis and melanogenic stimulant receptors (neurotrophin, PGE2 and α-MSH). (*p.o*.)	[193]
Fenugreek seed (*Trigonella foenum-graecum* L.)	steroidal saponins	Inhibition of TNF-*α* and melanogenesis (*in vitro*)	[195]
Eucalyptus globulus	Globulusin A and eucaglobulin	Anti-inflammatory and anti-melanogenesis activity (*in vitro*)	[194]
Azadirachta indica seed	nimolicinol	Inhibition of melanogenesis (*in vitro*) and TPA-induced inflammation (*in vivo*)	[378]
Guava (Psidium guajava L.)	leaves extract	Suppression of skin inflammation and melanogenesis (*p.o*.)	[196]
Anti-melanogenesis may due to antioxidant activity			
Glabridin	superoxide anion productions and cyclooxygenase activities	Inhibition of UVB-induced pigmentation and erythema (*in vivo*), inhibition of superoxide anion productions and cyclooxygenase activities (*in vitro*)	[379]
	luteolin	Inhibiting adenyl cyclase induced by MSH, anti-oxidant activity in DPPH, NBT/XO and intracellular ROS and xanthine oxidase (*in vitro*)	[281]
Pine bark	Pycnogenol (catechin, epicatechin and epicatechin-4-(2-hydroxyethyl)thio ether)	Inhibition of tyrosinase and melanin biosynthesis, suppressing $^{\cdot}O_2$, $NO^{\cdot}$, $ONOO^{-}$, and $\cdot OH$ in (*in vitro)*	[380]
Ecklonia cava (brown alga)	Phlorotannins (dieckol)	Inhibition of tyrosinase activity and reduction of intracellular ROS induced by UV-B radiation (*in vitro*)	[381]
Ishige okamurae (marine algae)	diphlorethohydroxycarmalol	Inhibition of tyrosinase activity and reduction of intracellular ROS induced by UV-B radiation (*in vitro*)	[382]

Hachiya et al. reported that a 50% ethanol extract of *Sanguisorba officinalis* root inhibited UVB-induced pigmentation of brownish guinea pig skin. The results of their study suggest that the mechanism governing the inhibition of ET-1 production in human keratinocytes is via the suppression of endothelin-converting enzyme-1α [191]. Kobayashi et al. reported that a 45% 1,3-butylene glycol extract of *Althaea officinalis* roots inhibited both the secretion of ET-1 from normal human keratinocytes (NHKC) and the action of ET-1 on normal human melanocytes (NHMCs), mainly by suppressing ET-1-induced calcium mobilization. They found that binding of ET-1 to the endothelin B receptor (ETBR) on the cell surface of NHMCs induced the mobilization of intracellular calcium [192]. Fucoxanthin, a carotenoid derived from edible sea algae, exhibited anti-pigmentary activity when applied either topically or orally in an animal model of UVB-induced melanogenesis. This effect of fucoxanthin may be due to suppression of PGE2 synthesis and melanogenic stimulant receptors (neurotrophin, PGE2 and MC1R) [193, 194].

Globulusin A and eucaglobulin, monoterpene glycosides isolated from *Eucalyptus globules,* not only have DPPH free radical scavenging activity, thereby inhibiting phorbol myristate acetate-induced expression of tumor-necrosis factor-α and interleukin-1β, but also inhibit melanogenesis *in vitro* [194]. In addition, a methanolic extract and its steroidal saponins, 26-*O*-*β*-*D*-glucopyranosyl-(25*R*)-furost-5(6)-en-3*β*,22*β*,26-triol-3-*O*-*α*-*L*-rhamno-pyranosyl-(1″→ 2′)-*O*-[*β*-*D*-glucopyranosyl-(1‴ → 6′)-*O*]-*β*-*D*-glucopyranoside, minutoside B, and pseudoprotodioscin isolated from Fenugreek seed (*Trigonella foenum-graecum* L. Fabaceae) inhibited the production of phorbol-12-myristate-13-acetate-induced inflammatory cytokines, namely TNF-*α* and melanogenesis *in vitro* [195]. Guava leaf extracts have been shown to suppress UVB-induced skin inflammation. Takashi et al. found that the skin color of guinea pigs that had been exposed to UVB irradiation followed by treatment with guava extract (p.o.) became lighter as a result of the tyrosinase inhibitory activity of guava leaf extract [196]. Nimolicinol, a limonoid isolated from *Azadirachta indica* seeds, shows inhibitory effects both on melanogenesis in B16 melanoma cells and on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice.

Many studies have found that compounds with potent free radical scavenging activities inhibit tyrosinase expression. Some of the most potent compounds with free radical scavenging ability and tyrosinase inhibiting activity include glabridin, diarylheptanoids and phenolic compounds from *Acer nikoense*; luteolin and pycnogenol from pine bark; phlorotannins from *Ecklonia cava*; diphlorethohydroxycarmalol from *Ishige okamurae*; and [6]-gingerol from ginger (Table 3).

4. Accelerating Skin Desquamation

Desmosomes, which are classified as a molecular complex of cell adhesion proteins consisting of desmoglein and desmocollin, are mainly responsible for the adhesion between epidermal cells. As the cells move upward from the basal layers to the stratum corneum, the desmosome attachments become weaker. This weakening action is accelerated by enzymes, namely the stratum corneum chemotrypic enzyme (SCCE) and Cathepsin D, by breaking the bonds of the desmosomes, resulting in the sloughing off of cells. Keratinization refers to the turnover of the stratum corneum and begins at the basal layer and gradually moves upward to the stratum corneum corneocytes. This desquamation process normally takes about four

weeks and is normally more efficient in younger skin. The process stimulates the growth of newer cells at a deeper level; however, in skin of advanced age, the intercellular desmosomes become glue-like in their ability to cement cells together. As a result, cell sloughing becomes more difficult, which leads to a thicker skin with a dull appearance. The stratum corneum has a pH of 7 at the bottom layer and a pH ranging from 4.5-5.4 at the surface [197]. The optimal pH for SCCE and Cathepsin D activity in the final desquamation stage ranges from 4 to 6, which explains why those enzymes are most active at the surface of the stratum corneum [198-200].

The capability of a compound to accelerate the turnover of epidermal layers and/or disperse melanin pigment can result in skin lightening. Depigmenting agents lighten the skin by stimulating the removal of pigmented keratinocytes [155, 201]. Pigmented spots, such as freckles or actinic lentigines, melasma spots, and post-inflammatory hypermelanosis macules may be removed by the peeling of corneocytes and epidermal keratinocytes.

CHEMICAL EXFOLIANTS AND THEIR MODE OF ACTION

(1). α-Hydroxyacids

α-Hydroxyacids (AHA, i.e. lactic acid, glycolic acid, and malic acid) are weak organic acids found in fruits, plants, and milk [202]. Studies on cell cohesion and skin pH changes indicate that keratin bonds may became weaker at low pH values. AHA solution is activated under low pH conditions and may dissolve the desmosome protein linkages causing a burst in skin exfoliation. AHAs have also been used to successfully treat pigmentary lesions such as solar lentigenes, lesions caused by melasma, and post-inflammatory hyperpigmentation macules. AHAs promote exfoliation by decreasing corneocyte cohesion and by stimulating dermal cell growth in the basal layer at low concentrations, while at higher concentrations AHAs promote epidermolysis and dispersal of basal layer melanin. The accelerated desquamation of the stratum corneum by AHAs is complemented by a direct inhibition of tyrosinase, without influencing mRNA or protein expression [201-203]. Lactic acid can be isolated from sour milk [201]. Glycolic acid can be isolated from natural sources, such as sugarcane, sugar beets, pineapple, cantaloupe, and unripe grapes. Both glycolic acid and lactic acid affect the skin layers in the same manner as described above. Furthermore, additional beneficial effects unique to lactic acid include an increase in dermal glycosaminoglycans (GAGs-natural moisturizers) and ceramides (epidermal barrier lipids), and improved water barrier properties. Glycolic acid stimulates collagen synthesis in a manner similar to that of lactic acid [204]. Yamamoto et al. studied the histological differences between patients who received a six-week treatment of topical AHA, glycolic acid, lactic acid, or citric acid as treatment for photo-aged skin and found that patients who had received AHA showed increased epidermal thickness, decreased melanin deposition, and up-regulated collegen levels relative to patients who received topical glycolic acid, lactic acid, or citric acid [205]. In addition, the authors found that AHA treatment not only decreased melanin deposition, but also resulted in the remodeling of the epidermis and the acceleration of desquamation [205]. The Cosmetic Ingredient Review, a panel endorsed by the Esthetics Manufacturers and Distributors Alliance of the American Beauty Association suggests that

consumers should not use glycolic acid or lactic acid products with concentrations exceeding 10% or at a pH of 3.5; for professional use, the limits are extended to 30% and the lowest advisable pH value is 3.0.

(2). β-hydroxyacids (BHAs)

Salicylic acid (SA) is a β-hydroxyacid (BHA) found in willow bark and sweet birch. It is also a phytohormone that acts similar to hormones that regulate cell growth and differentiation. SA functions as a desquamating agent by penetrating and dissolving the intercellular matrix of the stratum corneum [114, 202]. Unlike lactic acid, salicylic acid does not hydrate the skin and does not help to normalize epidermal anatomy or physiology. Salicylic acid, which is primarily a keratolytic agent, dissolves the stratum corneum layer by layer from the outside in, resulting in a thinning of the stratum corneum. The effect of salicylic acid on hyperpigmentation inhibition has been demonstrated in a number of studies, but only at very high concentrations (50%). SA is more lipophilic than AHAs, enabling it to penetrate sebaceous substances in the hair follicles and exfoliate the pores. The water solubility of AHAs is lower than that of SA. Since SA has a much stronger comedolytic effect than AHAs, it can be used in acne therapy.

(3). Retinol

Retinol (Vitamin A) is a potent skin exfoliant and antiaging agent. Retinol has been shown to improve the visible signs of photoaging as well as normal chronological aging when used on a daily basis. Studies have shown that retinol slows down collagen degradation in skin that has been chronically exposed to sunlight. In addition, retinol has been demonstrated to inhibit enzymes that are responsible for the degradation of collagen, such as collagenase [206].

(4). Liquiritin

Liquiritin, a flavonoid glycoside derived from liquorice, significantly reduces hyperpigmentation in patients with bilateral and symmetrical idiopathic epidermal melasma [207].

Zhu et al. found that a 20% liquiritin cream was effective at inducing skin lightening by dispersing melanin in a clinical trial involving patients with melasma [208]. The proposed mechanisms involve melanin dispersion by means of the pyran ring of its flavonoidal nucleus and acceleration of epidermal renewal.

4. Whitening Agents Verified By Clinical Trials

Whitening agents derived from natural products that have been tested in clinical trials are listed in Table 4 and described below:

Table 4. The effect of whitening agents have been verified by clinical trials

Compounds	Indication of clinical trials	Refs.
3% Arbutin	Treating hyperpigmentary disorders, such as melasma	[212]
3% Deoxyarbutin	Acceleration of the fading of UV-induced tan	[384]
1% Kojic acid	Treating hyperpigmentary disorders, such as melasma, post-inflammatory hyperpigmentation, age spots, and freckles	[216]
10% Magnesium L-ascorbic acid 2-phosphate	Effective for reducing melasma and age spots	[220]
SLM (skin lightening moisturizer containing 3% magnesium ascorbyl phosphate	Reduction of hyperpigmented spots on the face	[385]
0.3% Rucinol	Treating hyperpigmentary disorders, such as melasma	[222]
0.5% Ellagic acid	Effective for treating UVB-induced hyperpigmentation of the skin	[224]
Ellagic Acid (200 mg, 100 mg/oral administration)	Inhibitory effect on a slight pigmentation in the human skin caused by UV irradiation	[386]
Ellagic acid	Melasma	[387]
0.5% Chamomilla extract	Effective for treating UVB-induced hyperpigmentation of the skin	[225, 226]
0.5% 5,5'-Dipropyl-biphenyl-2,2'-diol	Effective for treating UVB-induced hyperpigmentation of the skin	[228]
0.5% 5,5'-Dipropyl-biphenyl-2,2'-diol	Effective in treating hyperpigmentary disorders, such as melasma and senile lentigo	[229]
2 % Rhododendrol	Effective for treating UVB-induced hyperpigmentation of the skin	[153]
20% Azelaic acid	Melasma	[115]
Tranexamic acid	Treating melasma	[388]
3% adenosine Monophosphate Disodium Salt	Effective for treating hyperpigmentary disorders, such as melasma	[239]
4% N-acetyl-4-S-cysteaminylphenol (4-S-CAP)	Melasma	[240]
0.1% Linoleic acid	Effective for treating melasma and to lighten UVB-induced hyperpigmentation of the skin	[241-243]
5 % Glycolic acid	Whitening	[244]
10% Glycolic acid	Melasma	[245]
Lactic acid, full strength (92%; pH 3.5)	Peeling agent in the treatment of melasma	[246]
8% Glycolic acid and 8% lactic acid	Hypopigmentation	[247]
30% Salicylic acid peels	Skin whitening	[248]

a) Arbutin and Its Derivatives

The compound 4-hydroxyanisole has been shown to act as an alternative substrate for tyrosinase both *in vivo* and *in vitro* [209]. However, 4-hydroxyanisole and other phenolic compounds have the potential to generate toxic quinone products and have, therefore, been used in various studies to evaluate the toxic effects mediated by tyrosinase in melanoma cells [210, 211]. Hydroquinone (HQ) was widely used as an effective skin-whitening agent before

it was banned by the US Food and Drug Administration in 2006 because animal studies in South Africa, the United Kingdom, and the USA revealed that HQ was a potential carcinogen and was associated with an increased incidence of ochronosis. HQ is defined as a drug since its cancer-causing properties have not yet been proved in humans. Other phenolic compounds that have been used to evaluate the toxic effects mediated by tyrosinase include arbutin, kojic acid, and ascorbic acid derivatives (Table 4). Arbutin is a glycosylated form of HQ that is present in bearberry extracts but it can also be synthesized from HQ by glucosidation. Its principal modes of action are competitive inhibition of tyrosinase and TRP-1 activity, inhibition of UV-induced formation and elongation of melanocyte dendritric processes, and inhibition of production of O_2^- and OH. It has been shown that a 3% arbutin-containing formulation is effective for treating hyperpigmentary disorders, such as melasma [212]. A combination therapy comprising a YAG laser and 7% α-arbutin solution has been shown to be an effective and well-tolerated treatment for refractory melasma [213]. Deoxyarbutin inhibits tyrosine hydroxylase and DOPA oxidase activities of tyrosinase. *In vitro* studies have demonstrated that the inhibition constant (Ki) of mushroom-derived tyrosinase is 350-fold lower than the Ki of arbutin. In a human clinical trial, topical treatment with deoxyarbutin for 12 weeks resulted in a significant reduction in overall skin lightness in a population of light-skinned individuals and a slight reduction in overall skin lightness and improvement in solar lentigines in a population of darkskinned individuals [214, 215].

b) Kojic Acid

Kojic acid is a γ-pyrone compound produced during the fermentation of aspergillus species, penicillium species, and filiform bacteria. Kojic acid exerts a slow-binding inhibition of tyrosinase activity, mainly by chelating copper, and inhibits the polymerization of DHI and DHICA. In a clinical trial, Mishima et al. showed that a 1% kojic acid-containing formulation was effective at treating melasma, post-inflammatory hyperpigmentation, age spots, and freckles [216]. In 2003, however, the Japanese Ministry of Health, Labor, and Welfare notified suppliers of kojic acid to delay manufacture or import of the product because of concerns about possible carcinogenic effects in animals [217]. However, in 2005, kojic acid was deemed to be a safe cosmetic ingredient, and continues to be used as a skin-lightening quasi-drug [218].

c) Ascorbic Acid and Its Derivatives

Ascorbic acid is highly unstable when exposed to heat or highly acidic conditions; derivatives of ascorbic acid, however, are much more stable. Some of the more commonly administered ascorbic acid derivatives include magnesium ascorbyl phosphate, ascorbyl glucoside, sodium ascorbyl phosphate, and 3-*O*-ethyl ascorbic acid. Ascorbic acid is a potent reducer of DOPA quinone and melanin. It has been reported that ascorbyl glucoside releases ascorbic acid gradually through hydrolysis due to the action of α-glucosidase in living organisms [219]. In a clinical trial, Kameyama et al. found that a 10% magnesium ascorbyl phosphate-containing formulation was shown to be effective at reducing the number of melasma patches and age spots [220]. In another clinical trial, Miyai et al. found that a 2% ascorbyl glucoside-containing cream was effective at accelerating the disappearance of UVB-induced hyperpigmentation [221]

d) Rucinol

Rucinol (4-n-butylresorcinol) has been reported to be an inhibitor of tyrosinase and TRP1 activity. Katagiri et al. found that a 0.3% Rucinol®-containing lotion was effective at alleviating UV-induced pigmentation and melasma patches [222].

e) Potassium Methoxysalicylate

Hideya et al. found that potassium methoxysalicylate inhibits melanin synthesis via a mechanism involving competitive inhibition of tyrosinase activity. This mechanism is similar to the mechanisms governing the modes of action of arbutin and rucinol [153].

d) Ellagic Acid

Ellagic acid, a polyphenolic compound, is found in strawberries, apples, and a variety of plants. Shimogaki et al. demonstrated that ellagic acid is a potent antioxidant and that it inhibits tyrosinase activity through copper chelation [223]. Kamide et al. showed that application of 0.5% ellagic acid-containing cream was effective for treating UVB-induced hyperpigmentation and melasma patches [224].

f) Chamomilla Extract

Chamomilla extract is a crude plant extract. It inhibits melanin synthesis by binding to endothelin receptors and by inducing the synthesis of inositol triphosphate. Ichihashi et al. demonstrated that a 0.5% chamomilla extract-containing cream was effective at treating UVB-induced hyperpigmentation in humans [225, 226].

g) 5,5'-Dipropyl-biphenyl-2,2'-diol (Magnolignan®)

5,5'-Dipropyl-biphenyl-2,2'-diol is a biphenyl compound isolated from *Magnolia heptapeta.* It has been shown to inhibit melanin synthesis by interfering with the process of tyrosinase maturation [227]. Takeda et al. found that a 0.5% Magnolignan®-containing formulation was effective at treating melasma, senile lentigo, and UVB-induced hyperpigmentation in humans [228, 229].

h) Rhododendrol (4-(4-hydroxyphenyl)-2-butanol)

Rhododendrol is a phenolic compound derived from White Birch and Nikko Maple. Rhododendrol inhibits melanin synthesis through competitive inhibition of tyrosinase activity. In 2010, Kanebo Cosmetics Inc. obtained approval from the Japanese Ministry of Health, Labor, and Welfare to use Rhododendrol as a whitening agent [153].

i) Azelaic Acid

Azelaic acid is a naturally occurring saturated nine-carbon dicarboxylic acid. Its use originated from the finding that Pityrosporum species can oxidize unsaturated fatty acids to dicarboxylic acids, which competitively inhibit tyrosinase. Azelaic acid was initially developed as a topical drug for the treatment of acne. However, because of its effect on tyrosinase, it has also been used to treat melasma, lentigo maligna, and other hyperpigmention disorders [230, 231]. In addition, azelaic acid has been shown to be effective at treating postinflammatory hyperpigmentation due to acne by inhibiting the production of free radicals [232, 233]. In the USA, 20% azelaic acid is only indicated for treatment of acne, although it has off-label use for hyperpigmentation. However, studies have found that 20% azelaic acid is equivalent to or better than 2% hydroquinone for the treatment of melasma [233, 234].

5. Tranexamic Acid and Tranexamic Acid Cetyl Ester Hydrochloride

Plasmin, a protease found in blood serum, not only enhances the intracellular release of arachidonic acid, a precursor of prostaglandins [235], but also elevates the levels of α-MSH [236]. Tranexamic acid has been shown to inhibit UV-induced plasmin activity in keratinocytes by preventing the binding of plasminogen to keratinocytes, which ultimately results in less free arachidonic acid and a diminished ability to produce PGs, thereby decreasing the activity of tyrosinase in melanocytes [189, 237]. Both arachidonic acid and α-MSH can activate melanin synthesis in melanocytes. Therefore, the anti-plasmin activity of tranexamic acid is thought to play a role in its topical effectiveness at treating melasma. The effect of tranexamic acid cetyl ester hydrochloride in treating hyperpigmentary disorders is due to its ability to inhibit UVB-induced inflammation, leading to the quiescence of active melanocytes. This mechanism is similar to the mechanisms of action of chamomilla extract and tranexamic acid.

6. Adenosine Monophosphate Disodium Salt

Adenosine is the building block of adenosine 5'-triphosphate (ATP), the main intracellular source of energy. Since energy is essential for cell proliferation and maturation, supporting ATP levels with topical adenosine safely accelerates epidermal turnover [238]. Adenosine monophosphate has the potency to increase the amount of intracellular glucose uptake, which is necessary for the biosynthesis of adenosine triphosphate. Therefore, adenosine monophosphate disodium salt accelerates epidermal turnover by elevating intracellular energy metabolism, which leads to the excretion of melanin from the skin. A clinical trial found that topical administration of a 3% adenosine monophosphate disodium salt-containing formulation was effective at treating hyperpigmentary disorders, such as melasma [239].

7. N-acetyl-4-S-cysteaminylphenol

N-acetyl-4-S-cysteaminylphenol is a tyrosinase substrate, and, on exposure to tyrosinase, it forms a melanin-like pigment. The depigmentation effect of *N*-acetyl-4-S-cysteaminylphenol is associated with a decrease in the number of functioning melanocytes and in the number of melanosomes transferred to keratinocytes. A 4% N-acetyl-4-S-cysteaminylphenol emulsion (O/W) was shown to be effective for treating melasma [240].

8. Linoleic Acid

Linoleic acid accelerates tyrosinase degradation, resulting in the down-regulation of melanin synthesis. In clinical trials, topical application of a 0.1% linoleic acid-containing liposomal formulation alleviated melasma symptoms [241] and UVB-induced hyperpigmentation of the skin [242, 243].

9. AHAs and BHAs

Many clinical studies on the effectiveness of AHAs such as glycolic acid and lactic acid as peeling agents for accelerating skin desquamation have been conducted in patients with pigmentation disorders. For example, a 5% glycolic acid topical cream was shown to improve skin texture and photoaging-induced discoloration [244]. In addition, a 10% glycolic acid lotion has been reported to be effective at improving symptoms of melasma [245]. Furthermore, a 92% lactic acid (pH 3.5) formulation has been shown to be effective at treating melasma [246]. A combination of 8% glycolic acid and 8% lactic acid creams has been shown to be modestly useful in ameliorating mottled hyperpigmentation, sallowness, and roughness due to chronic cutaneous photodamage [247].

A clinical trial showed that 30% salicylic acid in absolute ethanol was effective at treating acne and postinflammatory hyperpigmentation [248].

CONCLUSION

In this article we have reviewed the synthesis of melanin, the signaling pathways related to the regulation of melanogenesis, the factors influencing melanogenesis and various pigmentation disorders, as well as the effectiveness of various natural products at reducing hyperpigmentation. Plant origin, plant cultivation, and extraction methods can influence the content of active components in crude extracts or fractions. Therefore, the cultivation, harvesting, collecting, and storage of medicinal plants as well as the methods of extraction of active components from said plants need to be standardized. In addition, multi-functional formulations may increase the efficacy of skin-whitening products.

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