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LACTOFERRIN IN CERVICAL MUCUS AS A BIOCHEMICAL MARKER FOR INFLAMMATION

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ABSTRACT

Lactoferrin, an iron-binding protein, has been proposed to act as an anti-infective agent and has been used as a diagnostic marker in several inflammatory disorders. A sensitive enzyme-linked immunosorbent assay developed earlier was used for lactoferrin estimation in cervical mucus. A brief study was undertaken to observe if lactoferrin is detectable in cervical mucus and to correlate its level with reproductive tract infection, if present. One hundred and twelve cervical mucus samples were collected from healthy as well as infected females. Some of these females were using CuT-200 intrauterine copper devices for contraception. Women were at different phases of their menstrual cycle. The presence of detectable amount of lactoferrin in cervical mucus was confirmed. The average level of lactoferrin in cases either with clinical symptoms of cervicitis or with proven infection by PAP smear was significantly ($t=7.6$, $P < 0.01$) higher than the normal controls. CuT users have higher ($P<0.01$) mean level of lactoferrin than corresponding non-users.

KEY WORDS : Lactoferrin, cervical mucus, genital tract infection.

INTRODUCTION

The iron binding protein, Lactoferrin (LF) is a constituent of neutrophil secondary granules and is discharged into the surrounding medium when neutrophils are activated (1,2). It is commonly present in mucus membranes, neutrophil leucocytes, milk, sputum and tears as well as in seminal plasma (3-7). A high content of LF was also observed in cervical mucus of pregnant women (8). The function of LF has been a target of considerable recent interest. It appears to play a role in the transport and utilization of iron in the digestive tract (9). Several studies have focused on its bacteriostatic and bacteriocidal activities, which are based on the removal of a bacterial growth factor, iron. Attempts have been made to use LF as a diagnostic marker in several inflammatory disorders like dental pulp disease, chronic prostatitis, severe spermatogenesis disorders, rheumatoid arthritis and chronic bronchitis (10-14). No report is available from India on the use of LF as a possible marker in any inflammatory disease. Hence, in the present study, we attempted to detect LF in cervical mucus and to correlate its level with presence of reproductive tract infection and intrauterine device (CuT-200).

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MATERIALS AND METHODS

Sample Collection

A total number of 112 cervical mucus samples were consecutively collected from 112 married women attending the family welfare clinics of the Institute over a period of 3 months. The samples (0.5ml) were aspirated by tuberculin syringe and kept in vials containing 1 ml of PBS (phosphate buffer saline, pH 7.2) with sodium azide. When the amount of cervical mucus aspirated was less, the dilution volume of the buffer was accordingly adjusted. There were 21 cases where the volume was less than 0.5ml. These vials were then kept at room temperature for 1 hr., centrifuged and the dissolved LF in the PBS was separated to a different vial. Simultaneously smears were taken from endocervix to detect presence of infection by Papanicolaou-stain (PAP) method. The cases were at different phases of their menstrual cycle (7 to 19 days from last menstrual period, LMP).

Laboratory Procedures

A simple, sensitive Sandwich ELISA (enzyme linked immunosorbent assay) was standardised and used for estimation of LF in these samples. Briefly, anti-LF antibody (rabbit anti-human lactoferrin, Sigma, 1 mg/ml, at 1:500 dil) was coated (0.4 µg/well) on the maxisorp microtitre ELISA modules (Nunc, Denmark) with carbonate/bicarbonate (pH 9.6, 0.1M) binding buffer. The plates were incubated at 4°C overnight, washed with washing buffer (0.15 M NaCl containing 0.05% Tween 20). Three washings each of 3 minutes were performed. The wells were then incubated with 1% bovine serum albumin (Sigma) to block the nonspecific binding for 1 hr. at 37°C followed by 3 washings. Then 200µl of samples at 1:100 dilution were added in duplicate and incubated for 2 hrs. at 37°C. This was followed by three washings and incubation with anti-lactoferrin-HRP (horseradish peroxidase, Sigma) conjugate (1:1000 dil, 200µl/well) at 37°C for 2 hrs. Conjugation of anti-lactoferrin with HRP was carried out using the periodate method. Orthophenylenediamine (OPD) was used as substrate (8mg of OPD dissolved in 20ml of fresh citrate buffer with 50µl of H_2O_2) and incubated for 30 minutes in dark. The reaction was stopped by adding 50µl of 8N H_2SO_4 and read at 492nm. Similarly, standard curve was plotted by running 8 dilution of standard (lactoferrin, Sigma). The results were expressed in µg of LF per ml of cervical mucus. The cervical-endocervical smears were stained by using Papanicolaou method and screened for reproductive tract infections and cervical intraepithelial neoplasia (CIN). After analysis, the status of each case was decoded to reveal the condition of cervix. They were divided into healthy control (C) group, clinically symptomatic (S) and a group with proven infection (P) by PAP cytology. The group S might include some group P subjects. Clinically symptomatic (S) cases had erosion or red inflammed cervix and or with mucopurulent discharge on visual inspection. Each group was sub grouped to two on the basis of use of intra-uterine device (CuT-200) - a family planning device.

Statistical Method

One way analysis of variance, Duncan's Multiple range test was applied to observe the significance of the difference in LF level in

different groups of subjects. Kruskal-Wallis one-way analysis of variance was also carried out to cross-verify the significance of the difference between the groups. Unpaired t-test was done to observe the significance of the difference between two sub-groups.

RESULTS

The sensitivity of LF assay was defined as the smallest amount of the compound which differs from zero at 95% confidence limit and was 2.997 ng/ml. The intra and inter assays coefficients of variation (5.9% and 13.5% respectively) were within the limit.

One hundred and twelve cervical mucus samples were collected for LF analysis. The mean age of this population was 28 years (20-41 years). None was using any oral contraceptive. The results revealed a wide variation in LF content from 0.47 to 40.0 µg/ml in cervical mucus. The mean LF level and its range in each group and sub-group were presented in Table 1. Thirty five subjects did not have any sign or symptoms of cervical erosion or infection clinically as well as in PAP smears. The clinical history as described by these cases also revealed no abnormality and hence were considered as healthy (C). The LF level of group C varied from 0.47 to 21.3 µg/ml.

Of the 35 cases of group C, 18 were not using CuT. The LF levels of these 18 cases were considered for deciding the normal cut off value for LF in cervical mucus. The mean LF value of these 18 cases was 5.01 µg/ml and the standard deviation (S.D.) was $\pm$ 4.51 µg/ml (Table 1). The mean + 2 S.D. of these LF values was 14.03 µg/ml and was taken as normal cut off value. The individual having LF value above this, was considered as having infection or inflammation of the cervix. The level of LF of group S varied from 3.5 to 40 µg/ml (n=52) with a mean level of 18.95 $\pm$ 7.72 µg/ml. Among them 36 were using CuT and 16 were without it.

Fifty nine cases of group P revealed presence of single or multiple infection by PAP analysis. Their lactoferrin level varied from 4.1 to 40 µg/ml (Mean $\pm$ S.D., 18.26$\pm$7.45 µg/ml). Thirty four cases were having clinical symptoms. Different types of organisms were present in different infected individuals. These were *Trichomonas vaginallis*, Bacterial vaginalis, Actimyces like organism, Human papilloma virus and *Chlamydia trachomatis*. In this group 39 cases were using CuT whereas the other 20 cases were without it.

In one group of healthy cases we divided them into sub-groups, according to their LMP (0-6 day, n = 4; 7-12 day, n = 17; 13-16 day, n = 5; 17-22 day, n = 5 and 23-28 day, n = 8) and compared the mean LF level of each group with the other using 't' test. We did not find any significant difference ($P>0.05$) in LF level in these groups (data not shown). When the LF values of individuals were compared with the clinical symptoms considering 14.03 µg/ml of LF in cervical mucus as cut off value, the sensitivity, specificity and accuracy of the test was 71, 62 and 66 percent respectively. When the LF values were compared with the PAP-result, the sensitivity, specificity and accuracy of the test was 71.2, 66 and 69 percent respectively.

The LF levels of individuals belonging to

different groups (C,S and P) and their subgroups (using CuT or not) were compared (Table 1). It was observed that in subgroup of C (healthy control) whether using CuT or not, the mean LF values were significantly less ($P<0.05$) as compared to other two groups (S & P) with or without CuT. Unpaired t-test revealed a significant difference ($P<0.05$) in mean LF value among the subgroups belonging either to group C or group P. The mean LF level of C group was significantly less ($t = 7.6$, $P < 0.01$) as compared to the LF level of S and P. Similarly the CuT users as a group had a significantly higher ($t=2.864$, $P < 0.01$) level of LF (16.67 ± 8.24 μg/ml, $n=70$) than the non-users (11.94 ± 8.82 μg/ml, $n = 42$).

Table 1. Lactoferrin level (μg/ml) in different groups of subjects with or without CuT

Types of Subjects		Groups		
		Normal (C)	Clinically Symptomatic (S)	PAP +ve (P)
With CuT	Mean	9.54 # o *	18.87 *	19.63 @ o
	± S.D.	6.47	7.68	7.57
	Range	1.15 - 21.3	3.5 - 40	4.1 - 40
	n	17	36	39
Without CuT	Mean	5.01 + # ^	19.08 ^	15.39 + @
	± S.D	4.51	8.05	6.87
	Range	0.47 - 15.6	10.42 - 40	4.48 - 29
	n	18	16	20
Total	Mean	7.21 $	18.94 $	18.26 $
	± S.D.	5.93	7.72	7.45
	Range	0.47 - 21.3	3.5 - 40	4.1 - 40
	n	35	52	59

Fote Note : *o Among CuT users mean LF level of Group C was significantly ($p < 0.05$) lower than Group S and P

^ + Among CuT non-users mean LF level of Group C was significantly ($p < 0.05$) lower than Group S and P.

@ The mean LF level of Group C and Group P CuT users is significantly ($p < 0.05$) higher than their corresponding non users.

$ The mean LF level of group C was significantly ($P < 0.01$) lower than group S and P.

DISCUSSION

Lactoferrin is a protein found in the secondary granules of polymorphonuclear cells (PMNs). It has been implicated in the PMN functions of adherence, chemotaxis, anti-microbial killing and oxidative metabolism. Neutrophil LF may function to trap iron from ingested micro-organisms, enabling its removal from sites of inflammation. This may prevent iron from catalysing undesirable oxidative reactions, as well as making it unavailable for growth of microorganisms that survive the killing process (15). The more stable Fe-lactoferrin complex could then transcriptionally regulate genes that are involved in alternative defences against infection or could mediate anti-infective roles by other mechanisms, suggesting the role of LF as a multifunctional immuno regulatory protein (16).

Lactoferrin appears to play a role in placental inflammation, for LF positive cells were significantly enriched in areas of immuno-pathology (17). Measurement of lactoferrin may provide a marker for inflammation (18). In the present study, in normal cases the level remained low with little variation in different phases of the menstrual cycle. But the level of lactoferrin is significantly higher in other two groups of subjects either with clinical symptoms (S) or with cytologically proven infection (P). This observation is similar to several other studies carried out by different investigators. In cases of rheumatoid arthritis (RA), the plasma LF level was significantly higher than in normal subjects (13). Lactoferrin level besides elastase appears to be a valid diagnostic marker of dental pulp diseases (10). The preservation of LF production in severe inflammation or atrophy suggests that LF may be a key component of the inflammatory response within the human prostate (11). Lactoferrin and lysozyme appear to arise in the lower respiratory tract within the airways and their levels are elevated in association with chronic bronchitis (14). The LF level is high even in vaginal mucus just after menstruation (19) and is secreted by the endocervical cells or shed from the endometrium during menses (20). The high concentrations of LF and lysozyme have activities potentially important for the modulation of inflammation. In infected cervix the high level of LF acts as an important factor in the process of inflammation and the elimination of bacteria or associated debris. Similarly presence or insertion of external device (CuT 200) results in secretion of significantly high level of LF from the surrounding epithelium as a host immune response to any external challenge as observed between the users and non-users.

Few cases (n=15) in group S had a low level of LF. Among them in 10 who were with CuT, infection was absent by cytology (PAP). Five of these 10 cases were post-partum cases (4 to 5 months duration) and their symptoms might be due to trauma of parturition and not due to infection. Other five CuT users might have lack of immune response to their external device. Besides, they were clinically symptomatic but cytologically negative for infection and hence might have a low level of LF. Rest 5 cases of the 15 had multiple persistent infection that might have resulted in low LF level as reported earlier, recurrent infection leads to LF deficiency in the patients (18). Hence a case could be diagnosed of having chronic or persistent infection when the level of LF is low despite clinical symptoms.

Degradation of LF is slow (21). Clinical history of a healthy control without CuT having a higher level of LF revealed a reproductive tract infection one year prior to LF estimation. Degradation of LF in this case might take some more time to come down to a normal level. Other 5 control cases using CuT had also a high level of LF, which might be due to host immune response to the external device.

The sensitivity, specificity and accuracy of the assay could have been increased if the condition of each cervix was supported by previous clinical record or using specific diagnostic methods for diagnosis of reproductive tract infection which were not available during that period. No report is available on the effect of infection in any part of body on the LF level of cervical mucus. In the present study, we have only observed the local condition such as clinical symptom and use of a Family Planning device (CuT) on the LF value of the cervical mucus. Further study with a large number of cases may be necessary to evaluate its use in the diagnosis of infection.

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