EVALUATION OF OXIDATIVE STRESS, ANTIOXIDANTS AND PROLACTIN IN INFERTILE WOMEN

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ABSTRACT

The objective of the study was to estimate the serum concentrations of oxidative stress markers- lipid hydroperoxide (MDA-Malondialdehyde), lactate dehydrogenase (LDH), nitrite; and ferric reducing antioxidant power (FRAP) - a measure of total antioxidant capacity in serum. Serum nitrite levels were high in fertile women than in infertile women ($p < 0.001$), whereas MDA was high in infertile women than in fertile group ($p < 0.018$). On classifying the entire group of women with infertility based on the etiology, serum MDA and LDH levels were higher in women with infertility due to unexplained factor than in women with other causes contributing to infertility compared to controls ($p < 0.001$, $p < 0.02$). A positive correlation among serum prolactin and nitrite suggested that hyperprolactinemia could contribute to infertility by inducing oxidative damage. Serum FRAP levels showed no significant difference in the groups studied. Lipid peroxidation was high in women with unexplained infertility which may be due to hyperprolactinemia.

KEY WORDS

Infertility, FRAP, Nitrite, LDH, MDA.

INTRODUCTION

It is proposed that oxidative stress (OS) precipitates the range of pathologies that currently are thought to afflict the reproductive function (1). The possibility that reactive oxygen species (ROS) and O_2^- radicals might be involved in human reproduction was suggested nearly 60 years ago (2). Since then the results of numerous studies have implicated O_2^- radicals and ROS in both oligo and astheno-zoospermia (3). The effects of ROS result from damage to lipids, nucleic acids, proteins and other cellular materials (4). In vivo the damaging effects of oxygen radicals are usually prevented or limited by endogenous antioxidants/scavengers of free radicals (5).

In contrast, the role of antioxidants and ROS in relation to female reproduction has received relatively little attention,

despite the evidence of both physiological and pathological effects (6). As reduced O_2 supply is reflected in the modification of numerous metabolic pathways, intensified peroxidation may be one of the involved processes. Therefore it was of interest to measure the concentrations of three different oxidative stress markers- lipid hydroperoxides (MDA), lactate dehydrogenase (LDH) and nitrites along with a marker of total antioxidant capacity- FRAP in serum of women attending the infertility clinic. Since hyperprolactinemia is thought to be a frequent cause of chronic anovulation and infertility (7) serum prolactin (PRL) levels were also studied as a marker of infertility and its relation to OS and antioxidants were determined.

MATERIALS AND METHODS

A total of 76 women aged 20-40 years, with primary infertility attending the infertility clinic of Manipal Assisted Reproductive Centre (MARC) - a wing of Department of Obstetrics and Gynecology, Kasturba Medical College, Manipal, India were included in the study. This entire group was further classified into four groups based on the etiology of infertility as- infertility due to female factor (group-2), infertility due to male factor (group-3), that due to both the partners (group-4) and unexplained infertility (group-5) and the results were compared

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and correlated with controls (group-1). 25 age matched female with history of atleast one childbirth were included as controls. The biochemical investigations were carried out in the Department of Biochemistry, Kasturba Medical College, Manipal following approval by the institutional ethics committee of Kasturba hospital, Manipal.

Collection of serum samples : Under aseptic precautions, 4ml of blood was drawn in plain vacutainer tubes from antecubital veins of both patients and controls in the fasting state on day-2 of their menstrual cycle. The collected blood was allowed to clot and then centrifuged at 3000rpm for 10minutes for clear separation of serum. The serum was assayed immediately following its separation.

Reagents and materials : Chemicals like 2, 4, 6 Tri (2-Pyridyl) 1, 3, 5-triazine (TPTZ), tetramethoxypropane, Griess reagent and reduced nicotinamide adenine dinucleotide (NADH) were obtained from Sigma-Aldrich chemicals, USA. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, phosphate buffer saline (PBS), sodium pyruvate, thiobarbituric acid, sodium acetate buffer, phosphotungstic acid, H_2SO_4 , glacial acetic acid and n-butanol were of analytical grade and obtained from Merck India limited and Ranbaxy laboratories limited, India. Serum prolactin kit was obtained from Roche diagnostics, USA.

Estimation of FRAP (Ferric Reducing Antioxidant Power)

The FRAP assay uses antioxidants as reductants in a redox – linked colorimetric method with absorbance measured using a spectrophotometer [Genesys 10UV]. A 300mmol/L acetate buffer of pH 3.6[3.1gm of sodium acetate. $3\text{H}_2\text{O}$ + 16ml of glacial acetic acid made upto 1 liter with distilled water], 10mmol/L TPTZ [3.1mg/ml in 40mmol/L HCl] and 20mmol/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ [5.4mg/ml in distilled water] were mixed together in the ratio of 10:1:1 respectively, to give the working FRAP reagent. 50 μl of serum was added to 1ml of freshly prepared FRAP reagent and mixed uniformly. Absorbance measurement was taken at 593nm exactly 10minutes after mixing using 50 μl of water as the reference. The results obtained were interpolated using a standard graph obtained by dissolving 50 μl of standard $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (1mmol/L) in 1ml of FRAP reagent in serial dilutions ranging from 100-1000nmol/L (8).

Estimation of serum nitrite : 400 μl of serum was treated with 100 μl of 0.9%NaCl and 500 μl of Griess reagent. 400 μl of PBS was taken as blank and treated similarly, incubated at 37°C for 5minutes. The absorbance obtained at 543nm was interpolated against the standard graph obtained by using serial dilutions of standard sodium nitrite solutions of 5-25 $\mu\text{mol/L}$ concentrations and treated as for samples. The

nitrite concentration was expressed as $\mu\text{mol/L}$ of serum (9).

Estimation of LDH : 2.4ml of phosphate buffer (100mmol/L, pH 7.4 prepared by dissolving 13.97gm of K_2HPO_4 and 2.69gm of KH_2PO_4 in 1 liter of distilled water); 0.1ml of serum and 0.1ml of NADH (2.5mg in 1ml phosphate buffer) were taken in a test tube and allowed to stand for 20minutes at 25°C. 0.1ml of sodium pyruvate solution freshly prepared (2.5mg/ml of phosphate buffer) was added to the above solution, mixed and absorbance noted at 340nm using spectrophotometer (Genesys 10UV). The rate of change of extinction at 340nm at 25°C was monitored at 60seconds interval for 3minutes. The LDH activity obtained was expressed as U/L (10).

Estimation of MDA : MDA in serum was determined fluorimetrically by thiobarbituric acid reaction (TBA). 0.2 ml of serum was taken in a stoppered centrifuge tube, made upto 1ml with 0.9% NaCl aqueous solution, treated with 4ml of N/12 H_2SO_4 and shook gently. 0.5ml of 10% phosphotungstic acid was added and mixed well followed by centrifugation at 3000rpm for 10minutes. Supernatant was discarded and the sediment was treated with 2ml of N/12 H_2SO_4 and 0.3 ml of 10% phosphotungstic acid followed by centrifugation again for 10minutes. The supernatant was discarded and to the sediment 4ml of distilled water and 1ml of TBA reagent were added. 4ml of distilled water and 4ml of 0.5nmol/L of tetramethoxypropane were taken as blank and standards respectively and 1ml of TBA reagent was added to both the tubes. All the tubes were then incubated at 95°C in a boiling water bath for 60minutes. At the end of this, the tubes were removed, cooled and treated with 5 ml of n-Butanol. Tubes were mixed vigorously and centrifuged at 3000rpm for 15minutes. The upper n-Butanol layer was then taken for fluorimetric measurement at 553nm emission and 515nm excitation. TBA reacting substance was expressed in terms of MDA as nmol/ml of serum (11).

Estimation of serum prolactin : Prolactin estimation kit was obtained from Roche diagnostics and estimated in Elecsys 2010, automated analyser (12).

Statistical analysis : Results were expressed as Mean $\pm$ SEM. Statistical analysis was performed by applying the statistical package for social sciences (SPSS/PC: SPSS-9, Chicago, USA). One way analysis of variance (ANOVA) was applied to compare the mean values of different groups of infertility followed by multiple comparison post-Hoc Tukey HSD test. Independent't' test was applied to compare the values of different groups. Correlation analysis was done by Pearson

correlation. A "p" value of <0.05 was considered statistically significant.

RESULTS

The oxidative markers and antioxidants in serum of controls and patients with infertility are indicated in Table 1. Patients with primary infertility were further sub grouped into 4 groups based on the etiology and the results obtained in individual groups are depicted in Table 2.

Table 1 : Oxidative stress markers and antioxidants in serum of fertile and infertile women (Mean $\pm$ SEM)

Parameters	Control (n=25)	Primary infertility (n=76)
FRAP ($\mu\text{mol/L}$)	859.60 $\pm$ 31.13	921.19 $\pm$ 24.52
Nitrite ($\mu\text{mol/L}$)	5.00 $\pm$ 0.52	2.88 $\pm$ 0.31**
LDH (U/L)	67.90 $\pm$ 3.53	73.23 $\pm$ 1.97
MDA (nmol/L)	2.82 $\pm$ 0.15	3.28 $\pm$ 0.10*
Serum Prolactin (ng/ml)	18.1 $\pm$ 3.1	23.07 $\pm$ 2.11

**p=0.001, *p=0.02

On statistical analysis, serum nitrite was comparatively higher in controls, whereas the serum MDA levels were higher in infertile women than in fertile women (Table 1). On classifying the infertile patients into four groups based on their etiology, nitrite was significantly high in fertile women than in any other group. LDH and MDA levels were comparatively high and statistically significant in infertile women with unexplained etiology than that in control group. The serum LDH levels were not significantly different in any of the groups (Table 2).

By multiple comparison post-hoc tests, serum MDA levels were significantly high in women with infertility due to female as well as male factor than that in controls (p=0.023 and p=0.003 respectively). In infertility due to unexplained etiology also the

MDA levels were higher than in controls (p=0.001).

A statistically significant positive correlation was observed between nitrite and MDA (r=0.399, p=0.0001) as well as between nitrite and PRL (r=0.447, p=0.001) in women with infertility, etiology not being considered. A significant positive correlation was observed between nitrite and PRL in women with both partners contributing to infertility (r=0.673, p=0.023). FRAP and serum PRL correlated positively in serum of women with unexplained infertility (r=0.638, p=0.047).

DISCUSSION

Successful pregnancy results from an interaction between myriad physiological processes in both men and women. Any disruption to this interactive system, whether in a man or woman can result in an inability to have a biological child-called infertility (13). ROS exert their cytotoxic effects by causing peroxidation of membrane phospholipids, which results in an increase in membrane permeability, loss of membrane integrity, enzyme inactivation, structural damage to DNA and cell death (14).

In the present study, the various causes of female infertility and the role of ROS and OS on various etiologies of female infertility was examined. It has also been emphasized that free radicals have important physiological functions in the female reproductive tract and excessive free radicals may precipitate female reproductive tract pathologies (13). High circulating levels of PRL may inhibit ovarian function and ovulation by both central and peripheral mechanisms (15-19).

Elevated MDA levels in serum of infertile women, than in fertile women signifies that the oxidative damage in infertile women. This is also true when the entire group of infertile women were divided into various groups based on the etiology of infertility,

Table 2 : Oxidative stress markers and antioxidants (Mean $\pm$ SEM) in serum of women with infertility due to various etiologies

Parameters	Group1 (n=25)	Group2 (n= 29)	Group3 (n= 21)	Group4 (n= 13)	Group5 (n= 13)
FRAP ($\mu\text{mol/L}$)	859.60 $\pm$ 31.13	937.50 $\pm$ 36.25	892.85 $\pm$ 42.10	926.42 $\pm$ 56.68	876.15 $\pm$ 26.54
Nitrite ($\mu\text{mol/L}$)	5.00 $\pm$ 0.52	3.18 $\pm$ 0.59 *	2.50 $\pm$ 0.32 *	2.80 $\pm$ 0.50 **	3.0 $\pm$ 0.43 **
LDH (U/L)	67.90 $\pm$ 3.53	74.90 $\pm$ 3.27	71.69 $\pm$ 3.03	71.73 $\pm$ 3.82	83.40 $\pm$ 4.81 ***
MDA (nmol/L)	2.82 $\pm$ 0.15	3.36 $\pm$ 0.16 ****	3.24 $\pm$ 0.16 *****	3.14 $\pm$ 0.17	3.92 $\pm$ 0.23 *****
Serum Prolactin(ng/ml)	18.1 $\pm$ 3.1	21.91 $\pm$ 2.82	22.13 $\pm$ 3.83	27.17 $\pm$ 5.39	14.97 $\pm$ 1.92

*p<0.042, **p<0.0001, ***p<0.005, ****p<0.02, *****p<0.003, *****p<0.001 compared to controls

Group 1-Control, Group 2-Female factor contributing to infertility, Group 3-Male factor contributing to infertility, Group 4-Infertility due to both partners, Group 5-Unexplained infertility

wherein serum MDA was high in all groups than that in control group except for the group with infertility contributed by both partners where no significant increase was observed. This emphasizes on the possibility of increased peroxidative damage in infertile women than in fertile women thus decreasing the possibility of conception.

An increase in serum MDA in women with infertility due to male partner, suggests the possibility of other factors contributing to oxidative damage thereby decreasing the possibility of conception. This could include psychological (20) or drug induced oxidative damage as a result of repeated ovarian stimulation (21). This requires further evaluation, as women appearing to be normal during routine infertility workup may fail to conceive if other factors causing oxidative stress are not ruled out.

Serum LDH is also high in the infertile group with unexplained infertility than that in the control group signifying the apoptotic damage in this group. Idiopathic/unexplained infertility accounts for 3-18% of all female infertility (22). Yongjin wang et al reported an increase in ROS in peritoneal fluid of patients with idiopathic infertility than in controls thus suggesting the importance of ROS in contributing to idiopathic infertility (23).

The present study also emphasizes on an increase in ROS, as indicated by elevated MDA and LDH in serum of infertile women than in fertile women especially the unexplained infertility group. This is supported by the lack of similar increase in antioxidant levels- in the form of FRAP, thus providing way for oxidative damage. Since obtaining peritoneal fluid is an invasive procedure on the patient, it would be more beneficial if the same ROS are estimated in serum obtained when blood is collected for other hormonal estimations, as was done in the present study.

It has been postulated that, PRL regulates the local production of proopiomelanocortin (POMC) derived peptides, which includes β -endorphins. A study done on rats showed that these β -endorphins inhibit prostaglandin release and nitric oxide (NO) production thus blocking ovulation, suggesting that the anti-ovulatory effect of PRL might be mediated via stimulation of ovarian production of endogenous opioids (24, 25). The role of nitric oxide and their metabolites- nitrate and nitrite ($\text{NO}_3^-/\text{NO}_2^-$) in ovulation, control of oocyte maturation, ovarian blood flow and prostaglandin production-which are believed to be the critical signals for achieving follicle rupture (26, 27, 28).

Fernanda Polisseni et al suggested that the inhibitory effect of PRL on ovarian prostaglandin release may not involve the

NO pathway, as no change was induced either on NO synthase activity or NO metabolite accumulation in PRL treated ovaries (29). In infertility due to both partners a positive correlation was observed between nitrite and prolactin. This thus suggests that in this group an increase in ROS might be contributing to the infertility. Thus it is suggested to estimate their ROS levels prior to treatment. A positive correlation between FRAP and PRL in unexplained infertility is suggestive of increase in the antioxidants to overcome the oxidative damage induced by ROS.

Since the body's complex antioxidant system is influenced by dietary intake of antioxidant vitamins and minerals such as vitamin C, vitamin E, selenium, Zinc, taurine, hypotaurine, glutathione, beta-carotene and carotene (30), the present study emphasizes on supplementation of diet rich in the antioxidants to women, irrespective of the etiology of infertility.

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