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EVALUATION OF OXIDATIVE STRESS, GONADOTROPHINS AND PROLACTIN IN INFERTILE WOMEN

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ABSTRACT

Background: Reactive oxygen species are implicated in the pathogenesis of many diseases but their role in female infertility has not been extensively established. **Objective:** To determine oxidative stress markers, in infertile and fertile controls. To determine the level of gonadotropins and prolactin in both groups. **Methodology:** The study was a case control study conducted at Usmanu Danfodiyo University Teaching Hospital Sokoto. This study determined serum levels of malondialdehyde (MDA), catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD) using standard methods in 100 females, among whom 50 were infertile and the remaining 50 were fertile (control) subjects matched for age. **Results:** All the infertile subjects showed significantly ($P<0.05$) higher levels of MDA (5.92±0.39 vs 1.35±0.09) with lower levels of antioxidant enzymes compared to the fertile controls (CAT: 24.65±2.20 vs 104.14±2.24; GPx: 7.66±0.13 vs 25.24±0.55) except SOD (0.51±0.03 vs 0.65±0.03). FSH, LH and PRL levels showed significantly lower levels in the infertile subjects (FSH: 17.13±1.23 vs 6.80±0.45; LH: 18.38±1.23 vs 5.76±0.41; PRL: 33.84±4.14 vs 18.38±1.23). **Conclusion:** Women with infertility have high oxidative stress status and low level of antioxidant compared to control. In light of this, antioxidant supplementation to infertile women is recommended as a strategy to enhance their chances of conception.

KEYWORD: Reactive oxygen species prolactin in both groups.

INTRODUCTION

Infertility is the inability of a couple to achieve pregnancy over an average period of one year despite adequate, regular, unprotected intercourse.[1] Worldwide, infertility is generally quoted as occurring in 8-12% of couples.[2] However, the incidence varies from one region of the world to the other, being highest in the so-called infertility belt of Africa that Includes Nigeria.[3] In contrast to an average prevalence rate of 10-15% in the developed countries,[4] The prevalence of infertility has been notably highly variable in sub-Saharan Africa ranging from 20-46%. This has been attributed to high rate of sexually transmitted diseases, complications of unsafe abortions, and puerperal pelvic infections.[5,6] About 30% of infertility is due to female problems, 30% to male problems, and 30% to combined male/female problems, while in 10%, there is no recognizable cause.[5]

Successful initiation of pregnancy requires the ovulation of a matured oocyte, production of competent sperm, proximity of sperm and oocyte in the reproductive tract, fertilization of the oocyte, transport of the conceptus into the uterus, and implantation of the embryo into a properly prepared, healthy endometrium. A dysfunction in any one of these complex biological steps can lead to infertility in females.[7]

Infertility is a problem with a large magnitude and OS has been investigated as a causative factor. Oxidative stress (OS) is the term applied when oxidants (such as reactive oxygen species and free radicals) out number antioxidants.[8] OS induces lipid peroxidation structurally and functionally alters protein and DNA, promotes apoptosis, and contributes to the risk of chronic diseases like cancer and heart disease via effects on redox status and/or redox-sensitive signalling pathways and gene expression.[8] Oxidative stress is hypothesized to pathologically and physiologically contribute negatively to a number of reproductive processes including folliculogenesis, oocyte maturation, sperm DNA damage, necrozoospermia, asthenospermia, endometriosis, and the aetiology of defective embryo development.[9-12] Oxygen species are most commonly formed during the inner mitochondrial membrane's electron transport chain, specifically via the intermediate reduction states of complex I and complex III.[13] Once reactive oxygen species (ROS) are present in high concentrations, it is probable that their overabundance is a product of oxidative stress that triggers DNA damage

and increases cell apoptosis, also referred to as cellular death.[14] A number of ROS commonly generated in the female reproductive tract include the hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2), and the superoxide anion radical (O_2^-).[15]

Infertility is a significant public health problem and diagnosis and treatment are stressful, invasive and costly. The role of oxidative stress in female fertility is understudied and compelling area for investigation. Identifying modifiable factors to decrease oxidative stress in the gynaecologic environment may be an inexpensive and non-invasive therapy for increasing fertility especially in resource poor countries and thus informed this study which aim to study the antioxidant status in infertile female subjects attending gynaecological Clinic of Usmanu Danfodiyo University Teaching Hospital, Sokoto. Specific objectives are to determine oxidative stress markers (glutathione peroxidase, superoxide dismutase, catalase and MDA) in infertile and fertile female controls, to compare the level of biomarkers in both subjects and to determine hormonal concentration of both groups i.e. Follicle stimulating hormone, luteinizing hormone and prolactin.

METHODOLOGY

The study is a case control study conducted at the gynaecological clinic of Usmanu Danfodiyo University Teaching Hospital, (UDUTH) Sokoto. The study included fifty infertile females and fifty fertile females (control) all of reproductive age. The subjects were selected under defined criteria. This study was conducted with newly diagnosed and follow-up patients who had general infertility problem either primary or secondary. New infertility cases were recruited and followed up to know the cause of infertility by tagging their files. The study sample included 50 females attending infertility clinic and 50 healthy fertile female volunteers matched by age with the study group with a history of regular menstrual cycles lasting from 28 – 30 days recruited from the family planning unit at presentation before commencement of family planning. These subjects were referred to the laboratory for pregnancy test at the Family and Planning Unit of the Hospital. Patients with negative pregnancy test were recruited with their consent by filling in questionnaire which was interviewer-administered and venous blood taken. All the subjects had their history taken and physical examination conducted. After obtaining their consent, the relevant clinical findings and results of investigations were documented using a structured questionnaire, which was interviewer-administered. Weight and height of subjects were taken to determine their BMI. Patients gave full consent and the interview was conducted in the native language (Hausa) or English. The study was approved by UDUTH ethical committee and informed consent was obtained from participants.

Chemicals and Reagents

Analytical graded chemicals and reagents were used for this research. Glutathione peroxidase kit (item number: 703102), superoxide dismutase kit (item number: 706002), TBARS assay kit (item number: 10009055) and catalase assay kit (item number: 707002) were all products of Cayman Chemical Company, USA. Prolactin hormone kit (code: 725-300), follicle stimulating hormone (FSH) kit (code: 425-300) and luteinizing hormone (LH) kit (code: 625-300) were all products of Monobind Inc. Lake Forest, USA.

Inclusion and Exclusion Criteria

Inclusion criteria for the study were: Infertile females regardless of cause of infertility (test subject) and those that had history of one or more birth (control/ fertile).

The following were excluded from the study: Pregnant women, Menopausal stage individuals and those with male factor infertility.

Serum Analysis

A venous blood sample (7ml) was collected from each of the case and control subjects under aseptic procedure into a plain vacutainer tubes from antecubital veins of the subjects in the fasting state on day 2 of their menstrual cycle. All blood samples were drawn into tubes and centrifuged at 4000 rpm for 10 min. Serum thus extracted was stored at -20^{o}C for analysis of: Glutathione peroxidase, Super oxide dismutase (SOD), Catalase, Malondialdehyde and Hormones (PRL, FSH and LH).

Determination of Antioxidant Status

Serum glutathione peroxidase (GPX) activity was determined according to the method described by Paglia and Valentine,[16] using hydroperoxide as substrate.

The serum SOD enzymatic activity was determined according to the method reported by Marklund.[17]

Serum MDA levels was determined by the method of Niehans and Samuel.[18] Lipid peroxidation was evidenced by formation of thiobarbituric acid (TBA).

Serum catalase activity was determined using Cayman's catalase assay kit according to Johansson and Borg.[19]

Hormone Assay

The following hormones were determined in serum of each test and control subjects, using Monobind ELISA microwells kit;

Principle

The ELISA method used in determining the three hormones have the same principle. The essential reagents required in the assay are high affinity and specificity antibodies (enzyme and immobilized), with different distinct epitope recognition, in excess and native antigen. The immobilization takes place on the surface of a microplate well through the interaction of streptavidin

coated on the well and exogenously added biotinylated monoclonal anti-(FSH/LH/PRL) antibody.

The complex is simultaneously deposited to the wall through the high affinity reaction of streptavidin and biotinylated antibody. After the attainment of equilibrium, the antibody bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction is directly proportional to the native antigen concentration.

Quantitative determination of FSH, LH and PRL concentration in serum was carried out by ELISA method.

Statistical Analysis

The results were expressed as mean ± SEM by using Instat Software (version 3.0) San Diego, USA. Differences between group means were determined using independent sample t-test. One-way ANOVA, Tukey-Kramer multiple comparison tests was used to check for significant difference in biochemical parameters among the factors identified in female infertility. The significance level was set at $P< 0.05$.

RESULTS

A total of 100 participants, made up of 50 fertile and 50 infertile subjects. All the subjects were within the reproductive age group (18-35years) (Table 1). The mean ages of the fertile and infertile subjects were 27.98 ± 0.96 and 28.36 ± 0.92 (values ± SEM) respectively. The fertile subject had better educational status with 88% of respondents having formal education as compared to 74% with the infertile patients. Parity was higher in the fertility group with only one subject having no live birth as against 21 with the infertility group. The anthropometric index showed that the average BMI of fertile subjects was 26.87 ± 1.04 while that of infertile subjects was 27.74± 0.79 (values ± SEM). Most of the respondents in both fertility and infertility cases fell within the reproductive age bracket.

Table 1: **Socio Demographic Table of Fertile and Infertile Subjects.**

Age of Respondents	Infertile subjects	Fertile (control) subjects
<19	3	2
20 – 24	13	15
25 - 29	19	11
30 - 34	5	11
>35	10	11
Parity (secondary infertility cases were considered in the infertile column);		
Number of children		
0	21	1
1	15	12
2	5	10
3	7	12
4	1	2
>5	1	13
Education Status;		
No formal education	13	6
Primary	3	0
Secondary	15	21
Tertiary	19	23
Anthropometric Index		
Height (cm)	160.714± 1.09	158.46± 1.06
Weight (kg)	71.21± 2.16	65.88± 2.07
BMIkg/m2)	27.74± 0.79	26.87± 1.04

Values expressed as mean ± SEM.

It was observed from this study that secondary infertility had more number of subjects, 62% as compared to 38% of primary infertility. The average BMI of subjects with primary infertility does not differ significantly ($P>0.05$) from those patients with secondary infertility.

Those with ovarian problem as origin of infertility had the highest percent, recording up to 34% of the total.

cases followed by tubal 28% and then uterine, ovarian with 8% each. New cases recorded 18% while ovarian/tubal factor combined had 4%. Those with ovarian/tubal factor combine had the highest BMI average of 31.24± 3.560 kg/m^2 (values± SEM) with mean age (19.00± 1.000) years. Those with cervical problem had BMI average of 29.34± 1.19 kg/m^2 and mean age of 34.00± 2.94 years.

Table 2: Antioxidant Enzyme Activities and MDA levels in Fertile and Infertile Female Subjects.

	Infertile Subject	Fertile (control) Subject	P-value
GPx (nmol/min/ml)	7.66± 0.13*	25.24± 0.55	0.0001
SOD (U/ml)	0.51± 0.03	0.65± 0.05	0.0657
CAT (nmol/min/ml)	24.65± 2.20*	104.14± 2.24	0.0001
MDA (µm)	5.918± 0.39*	1.35± 0.09	0.0001

*values differ from the control significantly at P<0.05. Values expressed as mean ± SEM, GPx-glutathione peroxidase, SOD-superoxide Dismutase.

Activities of antioxidant enzymes and MDA concentration in both fertile and infertile subjects is shown in Table 2. The activities of all the antioxidant enzymes were significantly lower (P< 0.05) in infertile subjects compared to the control subjects. Levels of MDA in infertile subjects were significantly higher than in the fertile subjects.

Table 3: Antioxidant Enzymes in subjects with Primary and Secondary Infertility.

	Primary (n=19)	Secondary (n=31)	Control
GPx (nmol/min/ml)	7.56 ± 0.29*	7.71 ± 0.12*	25.24± 0.55
SOD (units/ug)	0.49 ± 0.05*	0.53 ± 0.04	0.65± 0.05
CAT (nmol/min/ml)	22.75± 2.85*	25.25± 2.94*	104.14± 2.24
MDA (µ/ml)	5.65± 0.50*	5.92± 2.75*	1.35± 0.09

*values differ from the control significantly at P<0.05. Values are expressed as mean ± SEM, GPx-glutathione peroxidase, SOD-superoxide dismutase, CAT- catalase.

Table 3 presents comparison of levels of serum antioxidant enzymes of primary and secondary infertile subjects. There is no significant difference in antioxidant parameters between primary and secondary infertility subjects at P<0.05. In comparison with control (fertile), secondary infertility shows a significant reduction in the antioxidant enzymes as compared to primary infertility.

High activity of GPx was observed in subjects with ovarian problem while those with cervical problem had the lowest activity of the enzyme. Using Tukey-kramer multiple comparisons, there was significant difference (P<0.01) of GPx activity in subjects with tubal and ovarian problem as compared to the other risk factors. SOD did not record any significant difference in the enzyme activity between the factors.

Unpaired t-test was used to compare biochemical parameters in each of the individual factor with the control and all the antioxidant enzymes in various infertility factors show significant reduction when compared to the control with the exception of SOD.

MDA concentration in tubal and uterine factor shows no significant difference with the control while the remaining factors show a significant higher level to the control.

Table 4: Hormone Levels in Fertile and Infertile Subjects.

	Infertile Subjects	Fertile (control) subjects	P-value
PRL (ng/ml)	33.842 ± 4.144*	48.475 ± 6.354	0.0566
LH (mIU/ml)	18.38 ±1.234*	5.756 ± 0.406	0.0001
FSH (mIU/ml)	17.125 ± 1.234*	6.797 ± 0.454	0.0001

*values differ from the control significantly at P<0.05. PRL-prolactin, LH-luteinizing hormone, FSH-follicle stimulating hormone. Values expressed as mean ± SEM

Results from table 4 show hormonal assay in fertile and infertile subjects. Fertile subjects show significantly (P<0.05) higher PRL compared to infertile subjects. In contrast to PRL, FSH and LH were significantly higher in infertile subjects when compared to fertile subjects.

The hormonal levels of primary and secondary infertility subjects as compared to the control subjects shows that both LH and FSH are significantly higher (P<0.05) than in the control. PRL is significantly lower in secondary infertility when compared to the control.

Table 5: Correlation of Prolactin and Antioxidant Parameters Including Body Mass Index.

Antioxidant Parameters	Correlation Coefficient(r)	P-value
GPx	0.11	0.94
SOD	0.25	0.08
CAT	0.34	0.02
MDA	0.04	0.77
BMI	0.10	0.47

P-values less than 0.05 indicates significant relationship, GPx- glutathione peroxidase, SOD- superoxide dismutase, CAT- catalase, MDA- malondialdehyde

All the hormones were correlated with antioxidant parameters all the hormones were positively correlated to GPx and negatively correlated to MDA PRL show a significant (P<0.05) correlation to CAT.

DISCUSSION

Infertility is a common problem with a significant public health concern. In the continent of Africa, infertility is said to constitute up to 65% of gynaecological consultations,[5] although this varies from one region to another. In Sub-Saharan Africa, sexually transmitted diseases, post abortal and post-delivery complications are largely the cause of infertility.[6]

Oxidative stress is an important cause of infertility.[21] Oxidative stress can affect female fertility potential in a number of ways; it may effect ovulation, fertilization, implantation and embryo development.[10,12] Both ROS and reactive nitrogen specie (RNS) act as signal molecules in physiological and pathological process in female reproductive tract.[10,12]

The mean ages of the fertile and infertile subjects were 27.98 ± 0.96 and 28.36 ± 0.92 respectively. The BMI of fertile subjects was 26.87 ± 1.04 while that of infertile subjects was 27.74± 0.79 (Table 1) which forms good bases for comparison of the group even though the groups were matched for age. The predominance (62%) of secondary infertility agrees with other studies in the country.[3,6] This is opposed to the trend in the developed world were primary infertility prevalence is higher.[20] This situation is attributed to poorly manage previous pelvic infections or pelvic inflammatory diseases (PID).[5]

The mean serum levels of MDA was significantly higher in the infertile group compared to control and the mean serum level of the antioxidant enzymes were significantly lower in the infertile group compared to control subjects. (Table 2) this finding was similar to that of previous studies.[12,22,23] The higher MDA level in the infertile group than the fertile control signifies that there is oxidative damage in infertile women which emphasizes on the possibility of increased peroxidative damage in infertile women than fertile women thus decreasing the possibility of conception.[23] The decreased mean serum level of antioxidant enzymes in the infertile group could have been as an effect of a mopping up mechanism due to the high oxidative stress level even though it is suggested that OS is caused by ROS overproduction rather than antioxidant depletion.[23] In the current study the secondary infertility women shows a significant reduction in the antioxidant enzymes compared to those with primary infertility.

There was also no statistical significance in the mean serum level of MDA between primary and secondary infertility. (Table 3) It is likely that ROS overproduction is higher in women with secondary infertility than those with primary infertility. High activity of GPx in primary infertility in comparison to secondary confirms the report of previous study.[24] This could be as a result of infections generating lot of oxidants that constantly depletes GSH, substrate to GPx common to secondary infertility cases. Considerable evidence is available, linking bacterial infections and increase ROS production. Leukocytes are responsible for generating ROS in infectious states.[25] The significant lower GPx activity observed in infertile subjects compromises the antioxidant defence mechanism of the system leading to various pathological issues including infertility.[22]

The prolactin level of infertile group was significantly lower than that of the fertile control group probably because majority of the fertile controls were from the family planning unit and may probably be breastfeeding. It has been postulated that PRL regulated the local production of proopiomelanocortin derived peptide which includes beta endorphins.[23] A study suggested that the anti-ovulatory effect of PRL might be mediated via stimulation of ovarian production of endogenous opioids.[26] FSH and LH were significantly high in the infertile group than the fertile control. (Table 4) All the hormones were positively correlated to GPx and CAT and negatively correlated to MDA in the infertile group. (Table 5) Suggesting oxidative effect in there productive system of the infertile women. The oxidative effect of FSH may be induced through the action of progesterone,[22] since it has been documented to elicit oxidative stress in rats.[25] The protective role of LH may be produced through the action of oestradiol,[22] the beneficial function of oestradiol has been demonstrated in rats.[25] Elevated LH concentrations seems to be a powerful enzyme against oxidative stress,[22] it is correlated negatively with MDA level and positively with GPx level in the current study.

In conclusion; infertility subjects show a significant reduction in antioxidant enzymes as compared to the

control (fertile subjects) and also an increase in MDA was seen in the infertile group compared to the fertile control these results indicate that infertile subjects show a high level of oxidative stress compared to the control groups. The role of OS in female fertility and infertility is an area deserving of continued research and therefore, administration of antioxidants to the infertile subjects may be beneficial in the management of women with infertility.

Author contribution

Panti AA and Umar Ateeque B conceptualised and design the study. Panti AA, Umar Ateeque B, CE Shehu, Tunau KA and Hassan M collected the data. Saidu Y, BIlbis LS and Umar Atteeque B and Rabiu UA conducted the laboratory evaluations. Umar Ateeque B and Panti AA analysed the data. All authors participated in drafting the manuscript, read and approved the final manuscript.

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