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Biochemistry

EVALUATION OF ANTIOXIDANT LEVELS AND VITAMIN E IN WOMEN WITH UNEXPLAINED INFERTILITY

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ABSTRACT **Background:** Unexplained infertility is a perplexing disorder. 30% of infertile couples are diagnosed with unexplained infertility with all standard fertility investigations are normal. Oxidative stress has been shown to have a negative impact on reproductive function in women with endometriosis and (PCOS) polycystic ovarian syndrome. In another study antioxidant supplementation improved OS induced infertility. Many studies have demonstrated that vitamin E as a protective antioxidant in the body with positive effect on the fertility. There is growing evidence linking OS and unexplained infertility. Based on this knowledge the specific group of unexplained infertile women were chosen to assess their levels of serum antioxidant enzymes and Vitamin E **Aim:** To compare the Levels of Antioxidant enzymes and Vitamin E in the serum of women with unexplained infertility and control group. **Method:** Case control study. 70 normal ovulatory women who conceived within 12 months of contraceptive free intercourse, and with no history of miscarriage were recruited in the control group. 70 women with unexplained infertility were recruited as study group. All participants included in the study were between 28 and 38 years of age. Serum levels of Antioxidant enzymes (GST, SOD, Catalase) and Vitamin E concentrations were compared between two groups. Vitamin E concentrations were determined by using High performance liquid chromatography. Antioxidant enzymes were measured by standard spectrophotometric assay. **Results:** Data was analysed using SPSS Software. Continuous parameters were analysed using Mann Whitney U test. There is a significant decrease in the vitamin E levels 3.80 (ug/ml) in the unexplained infertile group as compared to controls 6.0 (ug/ml). This decrease was found to be significant with p value of <0.05 **Conclusion:** The study group have a higher oxidative stress status and low level of antioxidants compared to control group. Serial measurement of oxidative stress biomarkers and their defense system may help to understand the aetiology of unexplained infertility and to enhance their chances of conception

KEYWORDS : Vitamin E, Antioxidants, Inferility

1.INTRODUCTION

Infertility is a multifactorial disorder and life style has an important role in its occurrence. In WHO conducted Demographic Health Surveys from 1990 to 2010, responses from women were evaluated and it revealed one in every four couples in developing countries were affected by infertility. It also reported that the overall burden of infertility, in women from 190 countries was similar and that increasing trend was seen in that decade.[1] This clearly shows that the burden remains high. According to Crosignani and Esteves, the evaluation of unexplained infertility in 30-50% of Couples was based on the following simple criteria:

1. Normal ovulatory function
2. Normal semen analysis for men
3. At least one patent fallopian tube[2]

Studies have shown that Assisted Reproductive techniques such as intrauterine insemination (IUI), In-vitro fertilization (IVF) are not always successful. It is worthwhile to investigate the factors which affect the success of this procedure.[3] Ovary is responsible for production of reproductive hormones and oocytes. The oxidative stress in reproductive organs may play a vital role in preventing conception. K. H. Al-Gubory et al. reported that levels of reactive oxygen species and antioxidants influences the female reproductive function in different phases of menstrual cycle and the physiological process of pregnancy.[4] Wang et al. (1997) and Polak et al. (2001) reported that higher levels of reactive oxygen species were found in reduction in ovulation. Vitamin E has a significant role in reproduction and sufficient levels give a better effectiveness in the treatment of unexplained infertile women and women with idiopathic infertility . It was suggested that peritoneal fluid which enters the Fallopian tubes may cause damage to sperm, and creates oxidative stress (Storey, 1997). Fridovich in his study has shown antioxidant enzymes play critical roles in clearing the toxic products produced by superoxide dismutase. [5]

1.1 Role of vitamin E in fertility

Vitamin E the fat-soluble vitamin is found in the ovary especially in follicular fluid. Many studies have shown its role as an antioxidant within the body[6]. In cells and organelles vitamin E is the first line of defence against lipid peroxidation. Rigotti, A has shown role of vitamin E in RBC flexibility and longevity in immune function, and its positive effects on fertility.[7] Savita, et al. have shown that increased OS is associated with decrease in antioxidants and fertility.[8] Vitamin E directly neutralizes superoxide anion, hydrogen peroxide, and hydroxyl radical. It is called a chain breaking antioxidant because of its ability to terminate a free radical chain reaction. Ruder, E.H. et al have shown that Vitamin E increases the number of embryos developing into the expanded blastocysts and increases the viability of embryos exposed to heat shock. [9] Bayer, R. (1960) suggested that in a human trial, infertile couples given vitamin E have shown a significant increase in fertility.[10] Plasma vitamin E levels were found to be higher in fertile women than in infertile women.[11] Vitamin E supplementation in older mice partially prevent reduction in ovulation[12] Vitamin E has a significant role in reproduction and sufficient levels give a better effectiveness in the treatment of unexplained infertile women.

1.2 Antioxidant status in females with unexplained infertility

Oxidative stress is due to the imbalance between prooxidant molecules and protective antioxidants. OS affects the entire reproductive capacity of a woman in many ways (i.e., oocyte maturation, ovulation, implantation, formation of blastocyst, luteolysis and luteal maintenance in pregnancy).[13] Reactive oxygen species act, both as key signalling molecules in pregnancy and at higher levels cause adverse effect on female reproductive tract. The antioxidants scavenge free radicals, thus protecting the cell structures[14]. Excess ROS in the follicular fluid overpowers the antioxidant defence capacity and damages the oocytes. This will affect the fertilization process. Peritoneal cavity microenvironment when flooded with ROS may allow fertilization but OS-induced apoptosis can cause implantation failure[15]. Elevated ROS levels hinder the endometrial function, which is needed for the growth of the embryo [16]and interferes in luteal regression and hormonal support which is responsible for the continuation of a pregnancy[17]

1.3 Antioxidant enzymes

Gonadotropin accelerate the up regulation of antioxidants such as catalase in the follicles and protects oocytes from ROS during steroidogenesis.[18,19] Strong positive correlation was observed between SOD activity and intrafollicular oestradiol levels, which affects oocyte quality. Studies concluded that the ROS scavenging ability of antioxidant enzymes is related to fertilization outcomes.[20,21] GST is involved in the follicle maturation process by detoxifying harmful substances and enhancing the normal development of the oocyte.[22]

This study aims to establish the association of Oxidative stress and antioxidant defence in unexplained infertility

2. MATERIALAND METHODS

2.1 Study design

Case - Control study.

2.2 Control group-

Pregnant women with normal ovulation who attend the antenatal clinic

in Obstetrics unit. Samples were collected from them at first-trimester of their pregnancy. 70 of these participants who had an uneventful pregnancy were included as control group.

2.3. Inclusion criteria

Women with normal ovulation, aged between 28 and 38 years who have conceived within 12 months of contraceptive free intercourse, and who have an uneventful pregnancy.

2.4. Exclusion criteria

1. Abnormal glycaemic status and thyroid function (TSH and Hb A1c were measured).
2. History of adverse effect during pregnancy.

2.5. Cases -

70 married Women with unexplained infertility were diagnosed in the Reproductive Medicine Unit at Gouri devi institute of medical sciences, Durgapur, when they came for infertility treatment procedure.

2.6. Inclusion criteria

1. Women with unexplained infertility were aged between 28 and 38 years.
2. Women with normal results for the following tests

(a) Tubal patency (hysterosalpingogram and/or laparoscopy documents at least one fallopian tube patent.
(b) Normal ovulatory function (Regular menstrual history/ ultrasound documented ovulatory cycle or mid-luteal Progesterone).
(c) Normal semen analysis for their partners

2.7. Exclusion criteria

Women in whom one or more of the above test results is abnormal

2.8. Sample size

In order to detect this difference as statistically significant with an alpha error of 5% and the power of 90%, we included 70 unexplained infertile women and 70 controls who were normal.

Study approved by IRB and consent was obtained from all participants.

2.9. Statistical analysis

Data was analysed using SPSS Software.
Continuous parameters were analysed using Mann Whitney U test. P value of < 0.05 was considered significant.

2.9.1. Sample collection and preservation Blood samples (5 ml) were collected in serum tubes (red clotted tube) and centrifuged at 3000 rpm for 10 min.

Serum were separated and stored in 0.5ml micro tubes at -20◦C until analysis

2.9.2. Analytical methods Vitamin E- was quantified using High performance liquid chromatography (HPLC).

Chromatographic conditions

1. Stationary phase - Phenomenex analytical column (C18 column, 5mm particle size, 250x4.6mm).
2. Mobile phase - Methanol. Detector- (PDA) photo diode array.
3. Vitamin E Retention Time =12.9 ± 0.092

2.9.4. Calibration Vitamin E Standard stock solution was prepared by dissolving 10mg of alpha-tocopherol in 1ml ethanol working standards 12.5, 25, 50 and100 ug/ml were prepared from stock 10mg/ml tocopherol acetate internal standard stock solution was prepared in ethanol.

2.9.5. Steps in sample preparation

1. 100 µl of serum with 100 µl internal standard were mixed for 15 sec in a vortex mixer
2. Tube kept in ice for 5 min. 1 ml of hexane added and vortexed for exactly 1 min.
3. Centrifuged at 25◦C for 5 min at 1,500 rpm.
4. Upper clear hexane layer was separated and evaporated under nitrogen gas.
5. 100µl of methanol was added and vortexed for 15 seconds.
6. The aliquot was transferred to a HPLC glass vial and loaded for further analysis. he Figures 1, 2, 3 and 4 represents the peak of different concentration of vitamin E standards

2.10. Limit of quantification: (LoQ)

1. The procedure was able to detect up to 3 µg/ml with accuracy.
2. The measurements were made on replicate samples (n=6) during the same laboratory run
3. The values were within ± 2 SD of the mean.

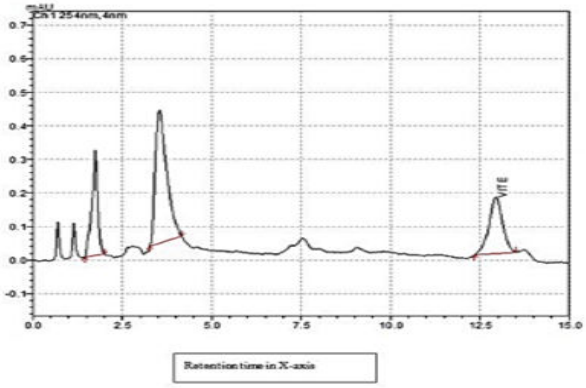

Fig. 1: Standard 12.5ug/ml

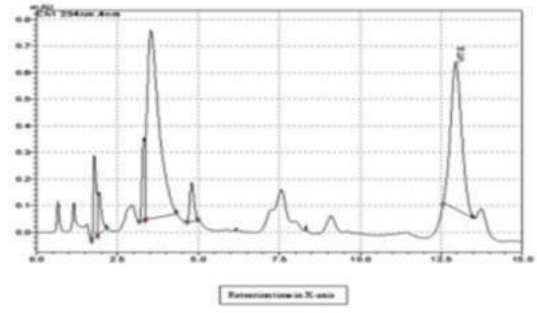

Fig. 2: Standard 25ug/ml

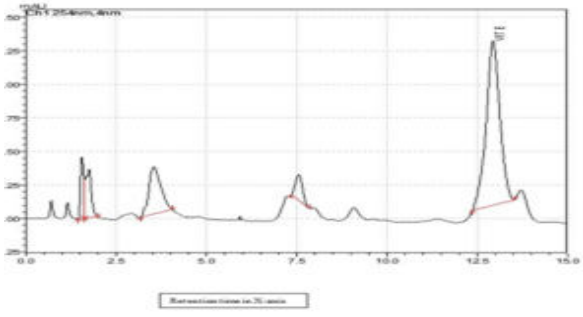

Fig. 3: Standard 50ug/ml

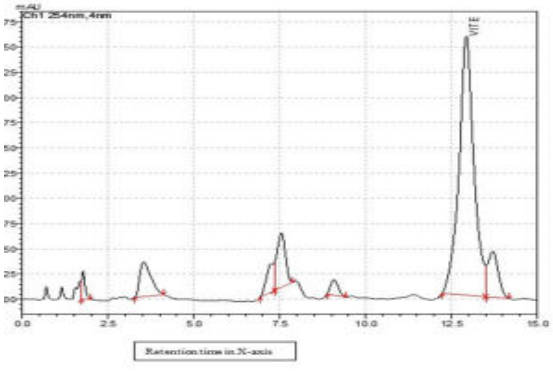

Fig. 4: Standard 100ug/ml

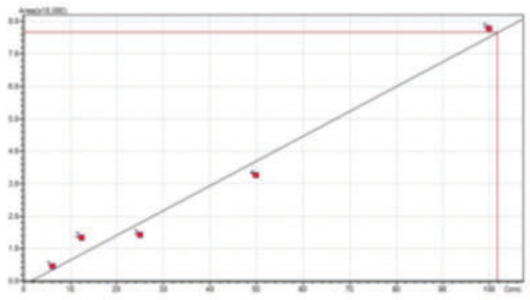

Fig. 5: Calibration graphof Vitamin E standards

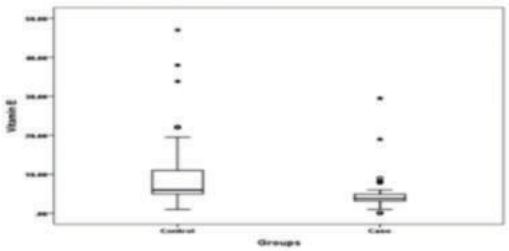

Fig. 7: Boxplot for serum vitamin E((ug/ml) between control and study group

Table I: Baseline characteristics of study subjects

Variables	Groups		P value
	Control(n=70) Mean ± SD / Median (IQR)	Case(n=70) Mean ± SD / Median (IQR)	
Age(yrs)	26.70 ± 4.40	30.39 ± 4.36	< 0.001
Body Mass Index(kg/m2)	23.14 ± 2.41	24.54 ± 4.27	0.02
HbA1C (%)	5.27 ± 0.43	5.28 ± 0.37	0.888
Height(cm)	155.55 ± 5.48	154.53 ± 6.66	0.323
Weight(kg)	55.89 ± 5.49	58.74 ± 10.56	0.049
TSH*	1.56 (1.11, 2.10)	2.08 (1.60, 2.85)	< 0.001

Note: * represents the variables which are reported by Median (IQR)

1. Antioxidant enzymes

1. Catalase – measured by Mahmoud H. Hadwan principle: 2H2O2 Catalase → 2H2O + O2 Catalase activity was measured by incubating the sample in 1.0 ml substrate at 37◦C for 3 minutes

Ammonium molybdate was added to stop the reaction.

Absorbance of the yellow complex of molybdate and hydrogen peroxide is measured at 374 nm against the blank

2. GST – measured by Boyland, E. and Chasseaud using CDNB as a substrate principle Glutathione –SH + → Glutathione –S-CDNB The reaction is assessed by monitoring the conjugation of 1-chloro, 2, 4-dinitrobenzene (CDNB) with reduced glutathione (GSH). This is done by an increase in absorbance at 340nm

3. SOD – measured by Markland's method measured by Markland's method Principle: Superoxide dismutase reacts with pyrogallol and inhibits its autooxidation. The rate of autooxidation of pyrogallol calculated from the absorbance of orange colour compound at 420nm.

3.RESULTS

A total of 140 women, (70 normal and 70 women with unexplained infertility) in the reproductive age group of 28-38 were included in this study.

The mean ages of the participants were 26.70 ± 4.40 and 30.39 ± 4.36 respectively for control and cases. (Table 1)

The antioxidant enzyme activities were significantly lower in case group compared to the control subjects (Table 2).

SOD showed significant decrease in median value in study group compared to control group. p=< 0.001.

Unexplained infertile group showed lower levels of vitamin E in serum compared to control group. This difference were statistically significant.

This is clearly represented in boxplot (Figure 7). Serum vitamin E analysis by HPLC method showed good recovery.(Table 2)

Table 2: Descriptive statistics for antioxidant enzyme and Vitamin E

Variables	Control Case Mean ± SD / Median (IQR)	Case Mean ± SD / Median (IQR) Mean ± SD / Median (IQR)	P value
Superoxide Dismutase* (U/ml)	2.00 (2.00, 3.00)	1.00 (1.00, 2.00)	< 0.001
Catalase* (U/ml)	34.00 (27.00, 45.00)	23.50 (13.00, 35.00)	< 0.001
GST Enzyme (U/ml)	1.87 ± 0.61	1.26 ± 0.53	< 0.001
Vitamin E* (ug/ml)	6.00 (5.00, 11.00)	3.80 (3.20, 4.90)	< 0.001

4. DISCUSSION

Unexplained infertility is a major problem with a significant public health concern. ROS and reactive nitrogen species (RNS) act as signal molecules in physiological and pathological process in female reproductive tract[23]. in this study the mean serum levels of vitamin E were significantly lower in the unexplained infertile group a finding similar to the study by Majid KH et al.[24] The low level of vitamin E in unexplained infertile group shows that there is considerable oxidative damage in this group of women decreasing the possibility of conception.[25,26]

Adequate levels of antioxidants are important for oocyte quality, maturation, fertilization, and implantation. They have a vital role in reducing oxidative stress, a process known to impair conception and its sustenance.[27] Various studies on Antioxidant enzymes in serum levels confirmed decreased levels of catalase, SOD and GST in association with infertility. [28]The results of this study clearly support this finding. Infertile couples were suggested antioxidants in their diet and also adopt a healthy life style.[29] Very few studies on oxidative stress markers like catalase, SOD, GST and MDA were studied collectively in Indian women with unexplained infertile woman. Findings from this study emphasizes the non-invasive protocol including oxidative stress markers

Vitamin E (α-tocopherol) the lipid-soluble antioxidant is considered a "fertility factor". It is said to be a direct free radical scavenger by enhancing the antioxidant enzymes and protects the cell membranes from lipid peroxidation. In this present study, unexplained infertility group have lower concentration of serum vitamin E levels as compared with control group (p=<0.05).

This study points out the importance of vitamin E and its effect on female fertility. Many studies showed low Vitamin E levels in infertile women with reduced total antioxidant status. According to Naseer et al Vitamin E levels in sera and cervical secretions of infertile women with unexplained infertility show a significant decrease when compared with fertile controls.[30]

5.CONCLUSION

The study clearly shows an imbalance in the antioxidant status with, decreased vitamin E and antioxidant enzyme level in the study group as compared to control group.

Vitamin E levels can be measured using this HPLC method and can be titrated to optimal levels. The antioxidant enzymes if included in the fertility workup and found deficient, can be given orally in the treatment protocol.

6.Limitation

Limitations of our study is the small number of subjects taken. A larger group might have been helpful in drawing better correlation between the various parameters. Other OS markers could have been included. Interventional study with antioxidant supplementation and evaluation of OS markers before and after the treatment can be the future study. Measurement of these markers in follicular fluid can also be undertaken.

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8. Conflict of Interest
None

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