Original Article

Study of oxidants and antioxidants in subtypes of female infertility in blood samples

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Abstract

Background: numerous studies have shown the role of oxidative stress in male factor infertility. The aim of present study is to evaluate the role of oxidative stress in female infertility again in various subtypes of female infertilities. **Materials and Methods**: oxidative stress parameters like Nitric oxide and Malonaldehyde were assayed as oxidants whereas Superoxide dismutase, reduced glutathione and Vitamin E were assayed as antioxidants both in blood. Statistical analysis was performed using Z test and One Way ANOVA. **Result**: Oxidants Nitric oxide and Malonaldehyde showed increase in blood. On the other hand antioxidants Superoxide dismutase, reduced glutathione and vitamin E showed decrease in blood. **Conclusion**: our approach clearly indicates that oxidative stress can be one of the major factors affecting fertility in females. As oxidative stress was present in many subtypes of infertility, it can be one of the factor responsible for causing tissue damages in related conditions like endometriosis,tubal factor etc.

Keywords: infertility, oxidative stress, endometrial tissue, nitric oxide, malonaldehyde, reduced glutathione, superoxide dismutase.

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Received Date: 14/09/2016 Revised Date: 12/10/2016 Accepted Date: 23/11/2016

Access this article online	
Quick Response Code:	Website: www.statperson.com
	DOI: 01 December 2016

INTRODUCTION

In present medical era, diagnosis and treatment of infertility is very costly, time-consuming, and invasive, and they can place immense stress on marital and family relations. Infertility diagnosis also faces problems because couples merely present themselves as infertile only after they embrace parenthood as a desired social role. Although relatively little is known about factors affecting fertility and early pregnancy loss, there is sufficient emerging evidence to hypothesize that dietary antioxidants and oxidative stress may influence the timing and maintenance of a viable pregnancy.[1] A couple may be considered infertile if, after one year of regular sexual intercourse, without contraception, the woman has not become pregnant.[2] In general, an estimated 84% of couples conceive after 1 year of intercourse, and 92% of the couples conceive after 2 years.[3] According to recent studies by the World Health Organization (WHO), approximately 50 to 80 million people are facing some kind of infertility problem.[3] The 1981 census of India estimated infertility to be in the range of 4% to 6%. An imbalance between oxidants and antioxidants in favour of oxidants potentially leading to damage is termed as oxidative stress.[4,5] The association between Oxidative Stress and infertility is hypothesized to exist, but a true cause and effect relationship has yet to be established. Further as indirect evidences reflects, the oxidative stress results in luteolysis, and antioxidant supplementation, for example vitamin C and vitamin E, has been shown to have beneficial effects in preventing luteal phase deficiency and resultant increased pregnancy rate.[6,7]There is growing understanding about the role of oxidative stress in human infertility and its causative factors like, endometriosis, unexplained infertility and tubal factor infertility.[8-11] Intensive research since last couple of decades suggests that ROS can modulate cellular functions, and oxidative stress can impair the intracellular milieu, resulting in diseased cells or endangered cell survival.[12] A review of the existing literature demonstrates the role OS plays in modulating a gamut of

How to site this article: Ashwini Kamble, Kanchan Mohod, Satish Kumar. Study of oxidants and antioxidants in subtypes of female infertility in blood samples. *International Journal of Recent Trends in Science and Technology*. December 2016; 21(2): 100-105 http://www.statperson.com (accessed 03 December 2016).

physiological functions and its role in pathological processes affecting the female reproduction. The oxidative stress modulates a host of reproductive pathologies affecting natural fertility in a woman's life and also menopausal transition and post menopausal years Considering all these things, we performed a study regarding estimating the role of oxidative stress in female infertility. In this study we had estimated Superoxide dismutase (SOD), Vitamin E and reduced Glutathione(GSH) as antioxidants and Nitric oxide (NO) and Malonaldehyde (MDA) as oxidants. Both oxidants and antioxidants were measured in blood samples of infertile females.

MATERIAL AND METHOD

The present cross sectional, observational and non interventional, case-control study was carried out during 2010-2011 after attaining the approval from institutional ethical committee and after taking informed consent. Any women who does not conceived during 1 year of unprotected intercourse (without use of any contraceptive device and with/without previous pregnancy status) was taken as cases whereas woman with prolapsed uterus who were fertile (having at least one or more children) in their reproductive life came for dilatation and curetting were considered as control. About 10ml of blood was collected from anticubital vein from each individual after taking informed consent. About 5ml of blood was stored in EDTA and rest 5ml in plain bulb for serum. Plasma and serum was separated by centrifuging at 3000rpm for 10 minutes and stored separately at -20^{0}C after adding 5% sodium azide 20µl/ml as preservative. The plasma was used to estimate Vitamin E, Nitric oxide and GSH while the serum was used to estimate MDA and SOD concentrations. NO was assayed by Griess Reagent assay[13] and MDA by Thiobarbituric acid method[14]. SOD was assayed by pyrogallol autoxodation method [15]whereas GSH and Vitamin E were estimated by Beutler procedure[16] and Emmerie Engel[17] procedure respectively. Endometrial tissue protein levels were assayed by Erba Kit method.

RESULTS

In present study, oxidative stress was assessed in blood so as to examine oxidative stress as a presumed systemic cause of infertility. We recruited 100 infertile females as cases and 100 diagnosed cases of uterine prolapse were assayed for their comparative levels of oxidants and antioxidants. For Oxidants, parameters assayed were Nitric Oxide (NO), and Malonaldehyde (MDA) while for antioxidants Superoxide Dismutase (SOD), Reduced Glutathione (GSH), Vitamin E. In blood Oxidants (NO and MDA) mean levels were expressed in µM and nmol/ml respectively and those of antioxidants, levels of SOD were expressed in units/ml while those for GSH and Vitamin E were expressed in mg/dl. Results have shown that, primary infertility accounted for 79% and rest 21% comprised secondary infertility cases. Ovarian factor contributed as a predominant presenting cause amounting to 62% (49 of primary and 13 of secondary) of overall cases, followed by unexplained infertility 22% (14 of primary and 8 of secondary), while tubal and peritoneal factors like endometriosis were noted in only 16% cases. Indeed all the 16% cases of later were classified under primary infertility. Table1 shows the distribution of primary and secondary diagnosed cases of infertility according to the causal factors. On prima facie primary infertility accounted for 79% and rest 21% comprised secondary infertility cases. Ovarian factor contributed as a predominant presenting cause amounting to 62%(49 of primary and 13 of secondary) of overall cases, followed by unexplained infertility 22%(14 of primary and 8 of secondary), while tubal and peritoneal factors like endometriosis were noted in only 16% cases. Indeed all the 16% cases of later were classified under primary infertility.

Table 1: Distribution of Female Infertility cases according to subtypes

Causal Factors	Number of Patients	Primary infertility	Secondary infertility
Ovarian factor	62	49	13
Unexplained Infertility	22	14	8
Tubal and peritoneal factor	16	16	0
Total	**100**	**79**	**21**

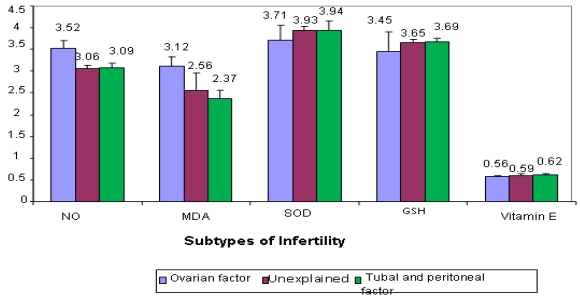

Figure 1: Analysis of Oxidants and Antioxidants in subtypes of Infertility in blood samples

NO-Nitric oxide, MDA-Malonaldehyde, SOD-Superoxide dismutase, GSH-reduced glutathione

We found that highest level of NO as well MDA were notably seen in cases of ovarian factor patients (3.52±0.18,3.12±0.21) respectively contributing for infertility and their levels were falling steadily for NO in tubal and peritoneal factors (3.09 ± 0.08) followed by unexplained infertility (3.06±0.07) and in the reverse way for MDA i.e. unexplained infertility (2.56 ± 0.41), and tubal peritoneal factors (2.37 ± 0.18) as reflected in the figure 1. Similarly, lowest expressed levels of SOD, GSH and Vitamin E were observed in the cases of ovarian factor (3.71±0.35±0.45 and 0.56±0.03) respectively. In contrast to ovarian factor, tubal and peritoneal factor patients showed lowest observed blood levels (SOD 3.94±0.21GSH 3.69±0.07, Vitamin E 0.62±0.02) of all the measured antioxidants as seen from the figure 1. This overall suggests that patients of ovarian factors accounting for clearly high propensity for estimated oxidative stress. Further, analysis of variance for the level of oxidants and antioxidants in blood using one way ANOVA showed significant group difference was observed between and within the groups in the blood levels of all the parameters (Table 1). Least amount of variation in the set of data within the group is observed with Vitamin E followed by nitric oxide whereas maximum variance was shown by NO.

Table 2: Levels of enzymes in subtypes of infertility according to diagnosis (in blood) One Way ANOVA

Variable		Sum of Squares	df	Mean Square	F	p-value
NO	Between Groups	4.65	2	2.32	94.98*	<0.05
	Within Groups	2.37	97	0.02		
	Total	7.02	99			
MDA	Between Groups	10.02	2	5.01	71.13*	<0.05
	Within Groups	6.83	97	0.07		
	Total	16.85	99			
SOD	Between Groups	1.12	2	0.56	6.41*	<0.05
	Within Groups	8.48	97	0.08		
	Total	9.61	99			
GSH	Between Groups	1.12	2	0.56	4.14*	<0.05
	Within Groups	13.13	97	013		
	Total	14.25	99			
Vitamin E	Between Groups	0.05	2	0.02	15.73*	<0.05
	Within Groups	0.15	97	0.001		
	Total	0.20	99			

NO-Nitric Oxide, MDA-Malonaldehyde, SOD-Superoxide Dismutase, GSH-reduced Glutathione, (* significant p value)

Analysis of Oxidants and Antioxidant in Blood Samples to examine the oxidative stress as a systemic cause of infertility in infertility Cases and prolapsed Controls: Table 5.4.1 provides us with the clear comparisons of oxidants and antioxidants levels in the blood samples of cases and controls. NO mean levels for control was (1.31 ±0.13) while that for cases was (3.35 ± 0.26) MDA levels, in blood was (0.90 ± 0.27) for control group while (2.88 ± 0.41) for case groups. Compare to controls MDA levels were found to be more than 3 times in the case groups (0.90 vs 2.88) while those for NO were marginally close to less than 3 times in cases compared to control (3.35 vs 1.31) Both these oxidant levels were found to be significant ($p<0.05$). Similarly antioxidant SOD levels decrease was found to be clearly more pronounced in the cases (3.80 ± 0.31) i.e., almost twice less than those for in controls (6.50 ± 0.30) and the decremental trend in the comparative value of GSH (cases 3.53 ± 0.37.,control 5.65 ± 0.33) i.e. almost 1.6 times and Vitamin E (case 0.57 ± 0.04., control0.87 ± 0.07) i.e. almost 1.5 times were noted in the blood samples of cases and controls respectively and the difference was found to be significant in all of them ($p<0.05$). Thus in blood oxidant MDA and antioxidant SOD showed maximum significance.

Table 3: Levels of Oxidants and Antioxidant in Blood Samples of Cases and Controls

		No analysed	Cases	Control	p value
OXIDANTS	NO(µM)	100	3.35 ± 0.26	1.31 ±0.13	<0.05*
	MDA(nmol/ml)	100	2.88 ± 0.41	0.90 ± 0.27	<0.05*
ANTIOXIDANTS	SOD(units/ml	100	3.80 ± 0.31	6.50 ± 0.30	<0.05*
	GSH(mg/dl)	100	3.53 ± 0.37	5.65 ± 0.33	<0.05*
	Vitamin E(mg/dl)	100	0.57 ± 0.04	0.87 ± 0.07	<0.05*

NO-Nitric Oxide, MDA-Malonaldehyde, SOD-Superoxide Dismutase, GSH-reduced Glutathione * Significant p value

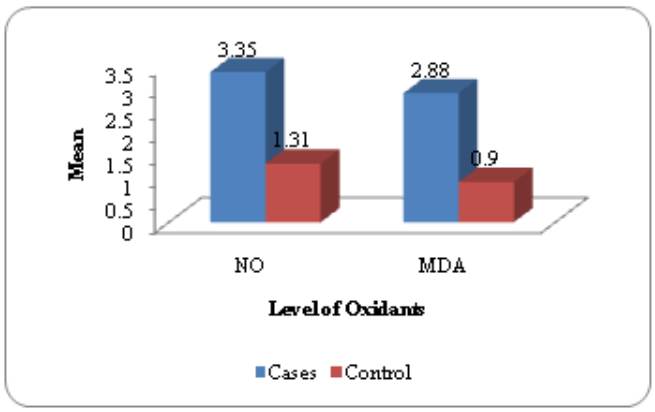

Figure 2

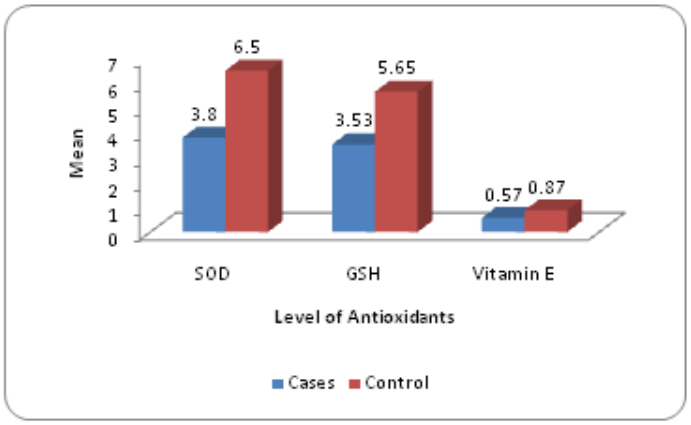

Figure 3

Legend

Figure 2: Levels of Oxidants in infertility cases and control in Blood Samples NO-Nitric Oxide, MDA-Malonaldehyde (Significant p value, $p < 0.05$)

Figure 3: Levels of Antioxidants in infertility cases and control in Blood samples SOD-Superoxide Dismutase, GSH-reduced Glutathione (Significant p value, $p < 0.05$)

DISCUSSION

Researchers are becoming convinced that during normal metabolism free radicals and other ROS are continuously produced in all cells and also in a wide variety of disease processes, varying from ischemic heart disease and cerebrovascular disorders to infertility.[18] Till now, several studies have estimated the role of ROS mainly in tissue fluid. Polak G *et al*[19] in the year 2000 tried to explore the activity of an extracellular superoxide dismutase (EC SOD) and total antioxidant status in peritoneal fluid and plasma unexplained female infertility and patients with tubal infertility. The authors noted that total antioxidant status (TAS) was significantly lower in peritoneal fluid from women with unexplained infertility compared to patients with tubal infertility. Plasma TAS did not differ significantly between the groups. In the year 2001 Dong M and collegues[20] had estimated the nitric oxide in peritoneal fluid is associated with endometriosis and infertility. They found that there were raised concentrations of nitrate/nitrite in both infertile females (42.02+/- 12.98 mmol/L) and patients with endometriosis (41.75 +/- 16.42 mmol/L) were significantly higher than that in controls (33.96 +/- 13.07, p<.05 for both). Polak *et al*[21] had subsequently assessed the concentration of Plasma Glutathione Peroxidase (pIGPx) in the peritoneal fluid (PF) of patients with unexplained infertility and infertile women with minimal and mild endometriosis. They then found that the pIGPx concentration was significantly (p=0.04) lower in PF from women with unexplained infertility (846 +/- 177 ng/ml) compared to the reference group (1023 +/- 238 ng/ml), but did not differ significantly (p=0.25) between women with endometriosis (918 +/- 81 ng/ml) and patients with tubal infertility. Their work thus suggests that low peritoneal PIGPx concentration may play a role in the pathogenesis of infertility. In present study, we assessed oxidative stress in blood sample presuming as systemic cause. We observed notably highest levels of NO and MDA in the blood samples of cases with ovarian factor (3.52+0.18., 3.12+0.21 respectively) as reflected in the Figure 1. Among two oxidants in blood, MDA levels were more increased. On further analysis, NO and MDA levels in infertility patients were significantly higher ($p<0.05$) in blood sample compared to controls. Jaiswar SP *et al* in 2006[22] have documented the oxidative stress in infertile females. Their result showed that infertile women had significantly ($p<0.001$) high MDA levels and significantly ($p<0.001$) low Catalase (CAT) and SOD levels in both blood as compared to those in controls. Unexplained infertility group of patients further had significantly ($p<0.001$) high levels of oxidant (MDA) while antioxidant (CAT and SOD) levels were significantly low ($p<0.001$). However in their study, the authors only assessed MDA levels for oxidants along with two antioxidants and therefore need to identify the spectrum of other oxidants in human subtypes of infertility remained. There are various possible ways for explaining oxidative stress causing infertility**.** NO due to unpaired electron is highly reactive free radical and induces adverse alterations in the structure of proteins, carbohydrates, nucleotides and lipids and it also has a role in cell and tissue destruction, sterile inflammation and formation of adhesions.[23]Although low concentration of NO in follicular fluid were associated with follicles containing mature oocytes that eventually become fertilized[24] but increased NO levels in the fallopian tubes

which are cytotoxic to the invading microbes and also maybe toxic to spermatozoa.[25] MDA, another oxidant,is a lipid peroxidaton product obtained due to ROS attack on polyunsaturated fatty acids on cell membrane. Kuscu *et al* [26]and Sabuncu *et al* [27] MDA levels were found to be raised in patients with PCOS (anovulatory cause of infertility) compared to controls. Mechanism of MDA which attributes to possible generation of infertility could be that antibodies are formed against MDA, which leads to stimulation of more mononuclear phagocytes in red blood cells, endometrial cells, and peritoneal cells, thus perpetuating cycle of oxidative damage.[28-31] In our study the levels of SOD, GSH and Vitamin E were observed to be quite reduced in the cases of ovarian factor (3.71 ± 0.35., 3.45 ± 0.45 and 0.56 ± 0.03 respectively) in blood. followed by unexplained infertility and tubal and peritoneal factor respectively (Figure 1).This overall suggests that patients of ovarian factors accounting for clearly high propensity for estimated oxidative stress followed by unexplained infertility as seen from our results. Further, (Table 2) show the analysis of variance for the level of antioxidants using one way ANOVA. Significant group difference was observed between the groups in the blood levels of all the parameters of antioxidants. The role of antioxidants in fertility is very important. Superoxide dismutase is present in the theca interna cells of the antral follicles [26]and it is found out that the theca interna cells may protect the oocyte from excess ROS. SOD also released at the time of sperm-oocyte fusion by Spermatozoa and oocyte to prevent excessive production of ROS.[32] Glutathione (g-glutamylcysteinylglycine, GSH) acts as an enzyme cofactor, antioxidant and antitoxin. GSH in mature oocytes is interestingly thought to be a highly relevant biochemical marker for the viability of mammalian oocytes [33-34] and Kim IH [35] observed the positive effect in the in vitro fertilization of exogenous supplementation of glutathione on of bovine oocytes.The antioxidant vitamin E is very important as NADPH oxidase-mediated generation of superoxide anion is inhibited by Vitamin E.[36] Murphy *et al*[37] have carried out study for estimating levels of Vitamin E in patients with endometriosis and normal females. His study reported the low levels of Vitamin E in peritoneal fluid and in plasma of endometriosis females as compared to their normal control groups and so he suggested the role of Vitamin E in endometriosis. At the end,the study carried out by us our study has showed the increased concentration of oxidants: NO and MDA and decreased concentrations of antioxidants: SOD, GSH and Vitamin E in different subtypes of infertility both in blood sample. Our understanding of OS and its role in pathologies has given rise to several new treatment modalities, now being investigated to improve both male and female infertility. Although many new antioxidants are available to improve infertility, a major concern about their usage remains due to lack of scientific evidence supporting their effectiveness.

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Source of Support: None Declared
Conflict of Interest: None Declared