Original Article

Levels of enzymes in subtypes of infertility in endometrial tissue of infertile females

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Abstract

Background: Many studies have implicated the role of oxidative stress in infertility. Most of the studies have estimated the oxidative stress in blood and mostly in male counterpartes. The aim of this study was to assess the effect of oxidative stress on local endometrial tissues leading to infertility in females. **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 in endometrial tissue of infertilities females. Statistical analysis was performed using Z test and One Way ANOVA. **Result**: antioxidants Superoxide dismutase, reduced glutathione and vitamin E showed decrease in endometrial tissue. On the other hand. Oxidants Nitric oxide and Malonaldehyde showed rise in endometrial tissue. **Conclusion**: our approach clearly indicates that oxidative stress was present at endometrial tissue level as in females with infertility. Our study is to our knowledge become the first documented assessment in considering GSH and Vitamin E as affected antioxidants in female infertility cases especially emphasizing the direct local endometrial tissue levels.

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

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INTRODUCTION

Infertility is a life crisis with invisible losses, and its consequences are manifold.[1] Series of studies have demonstrated that childless women experience stigma and isolation. Oxygen the very essence of life can itself become a toxin and endanger cellular components through formation of its free radicals. Tetravalent reduction of oxygen produces water, but univalent reduction produces a series of reactive radicals and non radicals, which are collectively known as Reactive Oxygen Species (ROS).[2] These ROS are capable of damaging compounds of all biochemical classes; including nucleic acid, proteins, lipids, lipoproteins, carbohydrates and connective tissue micro molecules.[3] Under normal conditions a variety of antioxidant mechanisms operate to detoxify or scavenge these ROS. An imbalance between oxidants and antioxidants in favors of oxidants potentially leading to damage is termed as oxidative stress.[4] The association between Oxidative Stress and infertility is hypothesized to exist, but a true cause and effect relationship has yet to be established. 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.[5-7] 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.[8] Therefore In the present study, oxidative stress was assessed in the endometrial tissue to estimate the possible role and impact of oxidative stress in local causes of infertility from Indian women. The levels of Nitric oxide and Malonaldehyde as oxidants and Superoxide dismutase, Reduced Glutathione and Vitamin E as antioxidants were measured in the endometrial tissue of infertile females and differentially compared to those in the samples of the control fertile group.

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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 1gm of endometrial tissue sample from infertility patients in operation theatre undergoing dilatation and curettage was taken. Tissue samples washed in normal saline and homogenized in phosphate buffer containing 0.05 M KH2PO4 and 1 mM EDTA, pH7.8 (1 g tissue per 2 ml buffer) in a glass homogenizer. Aliquoted and frozen at -70°C for 20 h in order to disrupt cell membranes. Homogenates were vortexed 1 min and centrifuged at 8600 g, for 20 min at 4°C After addition of ethanol/chloroform extraction reagent (62.5/37.5 vol/vol) to completely remove haemoglobin interference, samples were centrifuged at 6000 g for 20 min, at 4°C. NO was assayed by Griess Reagent assay [9] and MDA by Thiobarbituric acid method [10]. SOD was assayed by pyrogallol autoxodation method [11]whereas GSH and Vitamin E were estimated by Beutler procedure[12] and Emmerie Engel[13] procedure respectively. Endometrial tissue protein levels were assayed by Erba Kit method.

RESULTS

In present study, oxidative stress was assessed in endometrial tissue as local 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. In endometrial tissue Oxidants (NO and MDA) mean levels were expressed in µM /mg of protein and nmol/ mg of protein respectively whereas GSH and Vitamin E were expressed in mg/ mg of protein while SOD in Units/ mg of protein. All levels were expressed as Mean ± standard deviation. Cases in present study were of reproductive age group while that of prolapse control was presented at the age more than 45 years. Table 1 shows the distribution of primary and secondary diagnosed cases of infertility according to the causal factors.

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**

Analysis of Oxidants and Antioxidants in subtypes of Infertility in Endometrial tissue samples

Figure1 shows the blood levels of oxidants (NO and MDA) and antioxidants (SOD, GSH and Vitamin E) in different subtypes of infertility viz. ovarian factor, unexplained infertility and tubal and peritoneal factors. We found that highest level of NO as well MDA were notably seen in cases of ovarian factor patients (3.14$\pm$ 0.21., 3.59 $\pm$ 0.23) respectively. Followed by those in the group of unexplained infertility (NO 2.87 ± 0.14, MDA 2.81 ± 0.16) and further followed by Tubal and peritoneal factor infertility group (NO 2.75 ± 0.17 and MDA 2.64 ± 0.17). Similarly least levels (1.32± 0.07) of SOD were seen in the group of tubal and peritoneal factor. However GSH and Vitamin E were notably obtained in the group of ovarian factor infertility (GSH1.17 ± 0.02, Vitamin E 0.12 ± 0.10) again followed by decreasing trend in unexplained (GSH1.31 ± 0.05, Vitamin E 0.12 ± 0.10) and tubal and peritoneal factor infertility (GSH 1.34 ± 0.04 Vitamin E 0.16 ± 0.01) respectively. Table 2 shows the analysis of variance for the level of oxidants and antioxidants in endometrial tissue using one way ANOVA. Significant group difference was observed between the groups in the endometrial tissue levels of all the parameters except Vitamin E and they were found to be as follows. NO ($F= 33.93$, $df= 2, 97$, $p<0.05$), MDA ($F= 193.35$, $df = 2, 97$, $p<0.05$), SOD ($F= 11.74$, $df = 2, 97$, $p<0.05$), GSH ($F= 185.76$, $df = 2, 97$, $p<0.05$) and Vitamin E ($F= 2.8$, $df = 2, 97$, $p>0.05$).

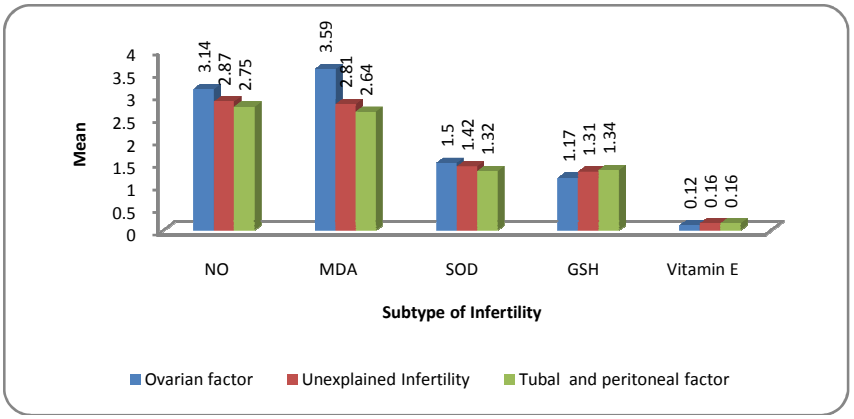

Figure 1: Levels of Oxidants and Antioxidants in different subtypes of infertility in Endometrial Tissue

NO-Nitric Oxide, MDA-Malonaldehyde, SOD-Superoxide Dismutase, GSH-reduced Glutathione.

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

		Sum of Squares	df	Mean Square	F	p-value
NO	Between Groups	2.62	2	1.31	33.93*	<0.05
	Within Groups	3.75	97	0.03		
	Total	6.37	99			
MDA	Between Groups	17.33	2	8.66	193.35*	<0.05
	Within Groups	4.34	97	0.04		
	Total	21.68	99			
SOD	Between Groups	.48	2	0.24	11.74*	<0.05
	Within Groups	1.99	97	0.02		
	Total	2.47	99			
GSH	Between Groups	0.55	2	0.27	185.76*	<0.05
	Within Groups	0.14	97	0.001		
	Total	0.70	99			
Vitamin E	Between Groups	0.03	2	0.01	2.81	<0.05
	Within Groups	0.67	97	0.006		
	Total	**0.71**	**99**			

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

Analysis of Oxidants and Antioxidants in Endometrial Tissues to examine the oxidative stress as local causes of infertility in Cases and Control groups

Levels of oxidants and antioxidants in endometrial tissue both in case and control groups are depicted in table3 and are further schematically represented in the bar diagrams showed in figure 2. In control group NO and MDA levels were 0.15 ± 0.03 and 0.27 ± 0.04 respectively. Levels in cases were 3.02 ± 0.25 for NO and 3.27 ± 0.46 for MDA. Both these oxidant levels were found to be significant ($p<0.05$) in endometrial tissue. Compare to controls NO levels were found to be almost 20 times in the case groups (3.02 vs 0.15) while those for MDA were marginally close to 12 times in cases compared to control (3.27 vs 0.27). Similarly Mean levels of SOD, GSH and Vitamin E were 1.46 ± 0.15, 1.22 ± 0.08 and 0.14 ± 0.08 respectively in infertile case groups. But Control groups mean levels were significantly higher ($P<0.05$) for SOD (5.48 ± 0.39), GSH (4.47 ± 0.24) and Vitamin E (0.89 ± 0.08) (table 3). Vitamin E levels were found to be 6 times higher in controls compared to cases followed by SOD levels and further followed by GSH levels and the difference were found to be significant in all of them ($p<0.05$). Thus in endometrial tissue NO as oxidant and Vitamin E as antioxidant were showing highest levels of significance.

Table 3: Levels of Oxidants and Antioxidants in Endometrial Tissues of Cases and Controls

		No analysed	Cases	Control	p value
OXIDANTS	NO(µM /mg of protein)	100	3.02 ± 0.25	0.15 ± 0.03	<0.05*
	MDA(nmol/ mg of protein	100	3.27 ± 0.46	0.27 ± 0.04	<0.05*
ANTIOXIDANTS	SOD(units/ mg of protein)	100	1.46 ± 0.15	5.48 ± 0.39	<0.05*
	GSH(mg/ mg of protein)	100	1.22 ± 0.08	4.47 ± 0.24	<0.05*
	Vitamin E (mg/ mg of protein)	100	0.14 ± 0.08	0.89 ± 0.08	<0.05*

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

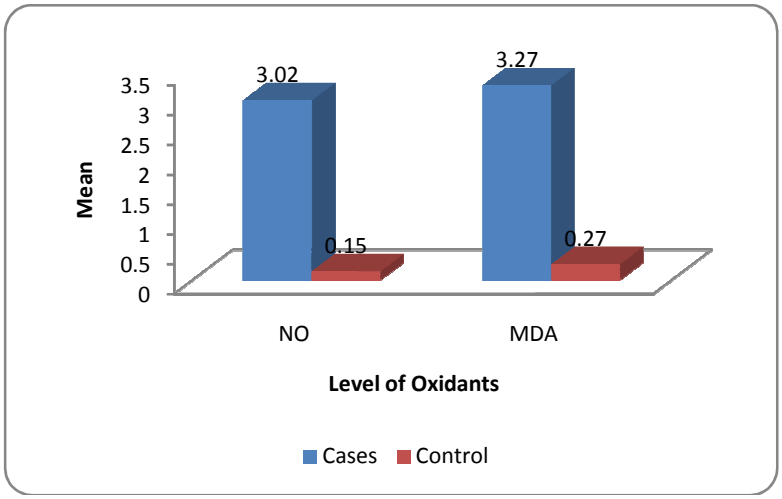

Figure 2: Levels of Oxidants in infertility cases and control in Endometrial Tissue

NO-Nitric Oxide, MDA-Malonaldehyde (Significant p value, $p < 0.05$)

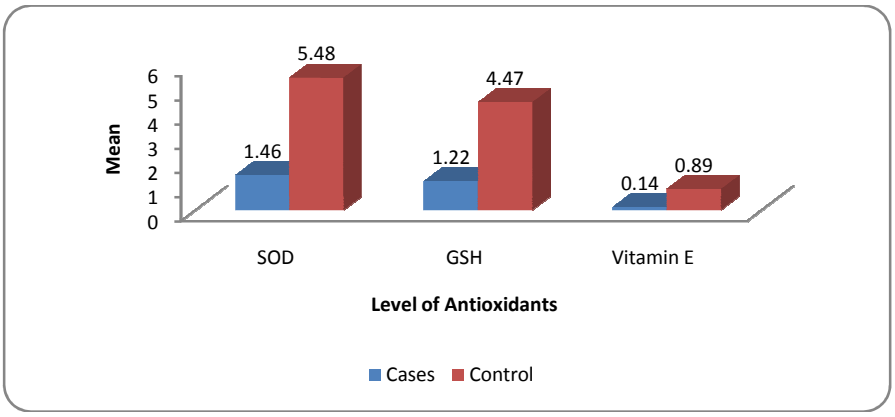

Figure 3: Levels of Antioxidants in infertility cases and control in Endometrial Tissue

SOD-Superoxide Dismutase, GSH-reduced Glutathione (Significant p value, $p < 0.05$)

DISCUSSION

Oxygen brings life into every cell of our body but unfortunately it is one of the chemical elements frequently involved in free radical formation. Antioxidant defence system acts as defender against the reactive oxygen species (ROS) for its inactivation and removal. Once ROS are present in high concentrations, it is probable that their overabundance is a product of oxidative damage, which further triggers DNA damage and increases cell apoptosis, also referred to as cellular death.[14,15] Human infertility is a common problem affecting about 8% to 10% of couples worldwide.[16,17] Lack of proper knowledge about aetiological factors responsible for infertility is a common problem faced for treating infertility cases especially in primary and unexplained infertility where oxidative stress can be a hidden culprit.[18] A number of studies have proclaimed that controlled oxidative stress (OS) plays a modulatory role in a variety of physiological processes in the female reproductive system like oocyte maturation, physiological follicular atresia, ovulation, fertilization, luteal regression, and luteal maintenance in pregnancy. Polak G *et al*[19] tried to explore the activity of an extracellular superoxide dismutase (EC SOD) and total antioxidant status in peritoneal fluid and plasma from 10 women with unexplained infertility and 10 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. In one of the related study by Polak *et al*[20] further made an effort to determine whether impairment of the antioxidant systems of peritoneal fluid might be a factor responsible for infertility. The authors had interestedly noted that total antioxidant status was significantly lower in peritoneal fluid from women with unexplained infertility (0.49+/-0.21 mmol/l) compared to

both fertile patients (0.67+/-0.24 mmol/l, p=0.02) and women with tubal infertility (0.76+/-0.26 mmol/l, p=0.001). Peritoneal fluid total antioxidant status did not differ significantly between patients with endometriosis (0.61+/-0.2 mmol/l), tubal infertility and the fertile group (p>0.05). Their results suggest that low antioxidant status in peritoneal fluid may play a role in the pathogenesis of infertility. Apart from these prospective studies, a remarkable retrospective study done by Szczepanska M and colleagues[21] from University Medical Hospital, Poland assess the total antioxidant potential of women with endometriosis-associated infertility, women with idiopathic infertility, and fertile controls. Their results showed that mean activity of superoxide dismutase, glutathione peroxidase, and total antioxidant status was lowest and lipid peroxide level was highest among infertile patients with endometriosis. Women with idiopathic infertility, in contrast, had the highest superoxide dismutase, glutathione peroxidase, and total antioxidant status activity and the lowest lipid peroxide level. Surprisingly their result were paradoxical compared to previously published studies as they concluded that high antioxidant potential is not a contributing factor in women with idiopathic infertility. In present study, we assessed oxidative stress in endometrial tissue as local causes of infertility. Earlier studies, while assessing the local oxidative stress, used peritoneal fluid rather than actual endometrial tissue. However, in the present study we attempted to draw a better parallel support for identifying role of OS. Endometrial tissues, NO and MDA levels were also shown to be maximum (3.14 ± 0.21 and 3.59 ± 0.23 respectively) as reflected in the figure 1 in infertility cases with ovarian factor. Our findings were in accordance with the previously published study carried out by Ming-Yih Wu *et al*[22] who showed the presence of higher concentration of nitric oxide at endometrial tissue levels in females with endometriosis which is one of the cause for female infertility (peritoneal cause of infertility). His findings were in conformity with those of ours and by Dong M *et al*[23] who had shown that in endometriosis and idiopathic infertility, there were elevated levels of Nitric Oxide Synthase (NOs) and NO in peritoneal fluid. What could be the possible reason for such causal associations? Evidences are confirming that NO is a local factor involved in the autocrine and paracrine modulation of ovarian folliculogenesis and steroidogenesis[24]. On the other hand, another oxidant MDA which is a lipid peroxidation product obtained due to ROS attack on polyunsaturated fatty acids on cell membrane. There are documented studies carried out by Polak G[25] and Liu, Y[26] in peritoneal fluid showing elevated levels of MDA in peritoneal fluid of patients with endometriosis. In another study by Kuscu *et al*[27]and Sabuncu *et al*[28] MDA levels were found to be raised in patients with PCOS (anovulatory cause of infertility affecting 6-10% of premenopausal women[29]) 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 a cycle of oxidative damage.[30-33] Antioxidants levels SOD, GSH and Vitamin E were also studied in 100 infertile female in endometrial tissue and compared with respective control groups.. endometrial tissue GSH means level was minimum in ovarian factor Infertility (1.17 ± 0.02) whereas SOD mean levels was found to minimum in tubal and peritoneal factor (1.32 ± 0.07) while Vitamin E levels did not differ much among all three subtypes of infertility. 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 **(Fig 1).** Further our study is to our knowledge become the first documented assessment in considering GSH and Vitamin E as affected antioxidants in female infertility cases especially emphasizing the direct local endometrial tissue levels rather than in systemic circulation. Let us also understand the implicated mechanism in perpetuating infertility by antioxidants. Antioxidants help protect the embryo from damage caused by oxidants, which thereby aids in the establishment of a successful pregnancy. Superoxide dismutase is present in the theca interna cells of the antral follicles [34] and it is found out that the theca interna cells may protect the oocyte from excess ROS during its maturation due to presence of SOD. Likewise, Glutathione (g-glutamylcysteinylglycine, GSH) acts as an enzyme cofactor, antioxidant and antitoxin. According to Duke 1996[35] and Nobel 1995 [36] depletion of GSH in cell is the suicide of cell what is known as apoptosis. Further, GSH in mature oocytes is interestingly thought to be a highly relevant biochemical marker for the viability of mammalian oocytes and Kim IH[37] observed the positive effect in the in vitro fertilization of exogenous supplementation of glutathione on of bovine oocytes. Finally, the role of Vitamin E has been found to be crucial as NADPH oxidase-mediated generation of superoxide anion is inhibited by Vitamin E.[38] Non-enzymatic antioxidants including Vitamins E, is dietary supplements that aid the female body's oxidant defence system.. Murphy *et al* 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.[39] Since our study is only observational in nature rather than interventional direct comparison with them may not be possible**.** However, these studies clearly indicate the importance of supplementation on antioxidants to a certain extent helps in overcoming the problem of infertility in females. In other words these are the indirect evidences suggesting the contribution of oxidative stress to infertility in females. To sum up, our study clearly reveals the presence of increased concentration of oxidants: NO and MDA and decreased concentrations of antioxidants: SOD, GSH and Vitamin E in different subtypes of infertility in endometrial tissue.

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