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Research Article

PREDOMINANCE OF OXIDATIVE STRESS IN ENDOMETRIAL TISSUE AS COMPARED TO SYSTEMIC CIRCULATION IN VARIOUS SUBTYPES OF FEMALE INFERTILITY

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A B S T R A C T

Background: Many studies have implicated the role of oxidative stress in female infertility. 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 both in blood and in endometrial tissue of infertilitile females. Statistical analysis was performed using Z test and One Way ANOVA.

Result: Oxidants Nitric oxide and Malonaldehyde showed increase both in blood and endometrial tissue but increase was predominantly present in endometrial tissues. On the other hand antioxidants Superoxide dismutase, reduced glutathione and vitamin E showed maximum decrease in endometrial tissue as compared to blood.

Conclusion: our approach clearly indicates that oxidative stress was more pronounced at endometrial tissue level as compared to blood 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.

INTRODUCTION

Childbearing and nurturing of children are very important events in human's life and are some where related with the ultimate goals of completeness, family integration and hence happiness. The presence of infertility is signaled, not by the presence of pathological symptoms, but by the absence of a desired state of 'non-event transition', in the words of Koropatnick.[1] Infertility is a worldwide problem affecting people of all communities, though the cause and magnitude may vary with geographical location and socioeconomic status.[2]

So far the definition of infertility has not been really consistent and defined differently by various disciplines. Infertility primarily refers to the biological inability of a person to contribute to conception. It may also refer to the state of a woman is unable to carry a pregnancy to full term.

Successful initiation of pregnancy requires the ovulation of a mature 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 cause infertility.[3] The follicular fluid microenvironment contains leukocytes, macrophages, and cytokines, all of which are known sources of ROS. Free radicals can influence the oocyte, sperm, and embryos in their follicular fluid, tubal fluid, and peritoneal fluid microenvironments, thus influencing reproductive outcome. 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.[4-7]

Our knowledge of human reproduction has shown phenomenal growth in past decade. A review of the existing literature demonstrates the role OS plays in modulating a gamut of 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. Oxygen radicals ROS play both a physiologic and pathologic role in the female reproductive tract.[8] Literature has documented role of OS in the pathophysiology of infertility and assisted fertility.[9-10]

In the present study, oxidative stress was assessed in the endometrial tissue and in the blood sample of infertile females so as to explore the possible role and impact of oxidative stress in local as well as systemic causes of infertility from Indian women. Nitric oxide and Malonadehyde were assayed as oxidants while Superoxide dismutase (SOD), reduced glutathione (GSH) and Vitamin E were assayed as antioxidants. Oxidants and antioxidants were assayed both in

blood as systemic cause and in endometrial tissue as local causes for infertility.

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.

About 1gm of endometrial tissue sample from infertility and prolapsed 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 KH_2PO_4 and 1 mM EDTA, pH7.8 (1g tissue per 2 ml buffer) in a glass homogenizer. Ethanol / chloroform extraction reagent was added and homogenates were vortexed for 1 min and centrifuged at 8600 g, for 20 min at 4^{0}C. Samples were centrifuged at 6000 g for 20 min, at 4^{0}C and supernatant obtained was taken for estimation.

NO was assayed by Griess Reagent assay [11] and MDA by Thiobarbituric acid method [12]. SOD was assayed by pyrogallol autoxodation method [13] whereas GSH and Vitamin E were estimated by Beutler procedure[14] and Emmerie Engel[15] 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 and endometrial tissue as local cause of infertility in our study primary infertility accounted for 79% and rest 21% comprised secondary infertility cases.

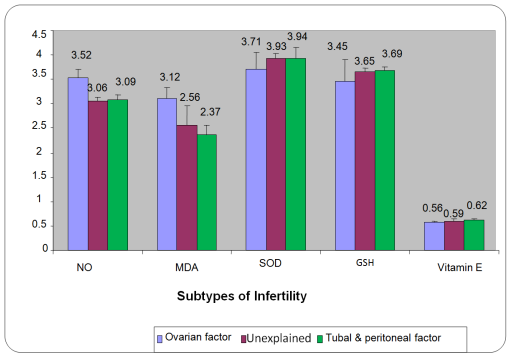

Figure 1 Analysis of Oxidants and Antioxidants in subtypes of Infertility in blood samples

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

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.

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.

Analysis of Oxidants and Antioxidants in subtypes of Infertility in endometrial tissues

We found that highest level of NO as well MDA were notably seen in cases of ovarian factor patients (3.14±0.21., 3.59±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 (Figure 2).

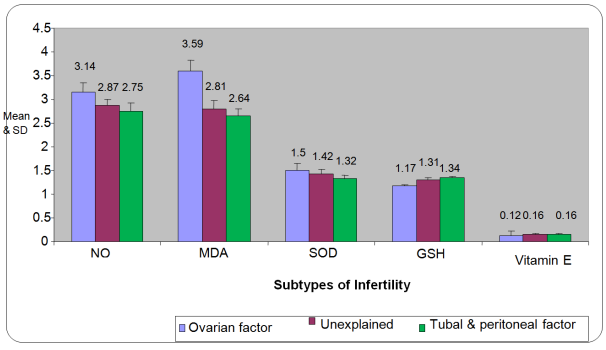

Figure 2 Analysis of Oxidants and Antioxidants in subtypes of Infertility in endometrial tissue samples

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

However, GSH and Vitamin E were notably obtained in the group of ovarian factor infertility (GSH 1.17 ± .02, Vitamin E 0.12±0.10) again followed by decreasing trend in unexplained (GSH 1.31±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 (Figure2).

Also 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 (Table 2).

Both oxidants NO and MDA levels were highest in endometrial tissue as compared to blood i.e. 3.2 times in blood and 12.11 times in endometrial tissue of MDA whereas 2.5 times in blood and 20.13 times in endometrial tissue of NO (Figure 3).

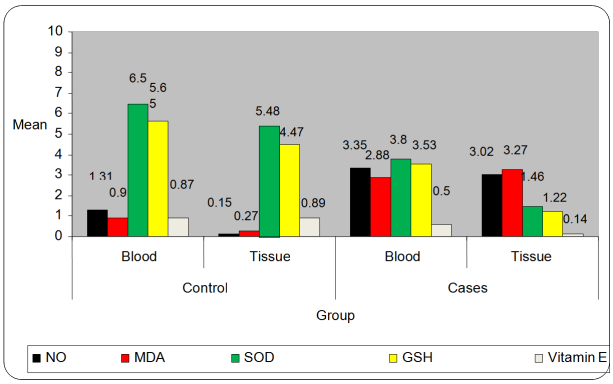

Figure 3 Comparison of Levels of Oxidants and Antioxidants between blood samples as systemic and endometrial tissues as local causes of infertility

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

This clearly shows that rise in MDA levels were more in blood as compared to NO in blood. On contrary in endometrial tissue, rise in levels of NO was more as compared to rise in levels of MDA in same. Inadvertently both the oxidants in tissue and blood in case group showed rise compared to their control ($p<0.05$). On the other hand all the 3 antioxidants on differential comparison showed decreased levels in cases compared to control without any obvious noticeable pattern among blood or endometrial tissue. However decrease was significantly more in endometrial tissue compared to blood levels ($p<0.05$). Vitamin E levels showed maximum decrease in endometrial tissue i.e. 6.3 times whereas SOD and GSH levels were 3.7 and 3.6 times decreased. In blood antioxidants SOD, GSH and vitamin E levels were decreased by 1.7, 1.6 and 1.5 times respectively. Hence endometrial tissue maximum decreased was shown by Vitamin E whereas in blood maximum decrease was seen in SOD levels (Figure 3. So it can be commented that in endometrial tissue oxidant NO showed more increase whereas antioxidant Vitamin E showed more decrease. On the other hand in blood oxidant MDA showed more rise and antioxidant SOD comparatively more decreased.

This overall suggests that local causes for infertility supersede systemic causes for infertility as far as oxidative stress is concerned

DISCUSSION

Though it is proved that, presence of reactive oxygen species is important for normal physiological processes in female reproductive track; excess production can result in generation of oxidative stress. 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 defense 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. [16,17]

After the intensive exploration of molecular mechanism of ROS in pathological and molecular studies in early 70's to mid of 90's , several discrete case control studies estimated the role of ROS either in systemic or local tissue fluid. For instance, Dong M and collegues[18] in 2001 had tried to verify whether nitric oxide in peritoneal fluid is associated with endometriosis and infertility. He found that peritoneal concentrations of nitrate/nitrite in both infertile women (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). No significant difference in peritoneal nitrate / nitrite level was found between primary infertile women and patients with endometriosis ($p>0.05$).

Similarly 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. Polak *et al* [20] 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 by evaluating 8 infertile women with minimal or mild endometriosis, 15 patients with unexplained infertility and 10 patients with tubal occlusion. 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 and 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 and further differentiating its local from systemic causes. 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. Similarly in endometrial tissues, No and MDA levels were also shown to be maximum, (3.14 + 0.21and 3.59+0.23 respectively) as reflected in the Figure 2 in infertility cases with ovarian factor. Among these two oxidants in blood, MDA levels were more increased and contrarily in endometrial tissue NO levels showed more rise as compared to MDA. On further analysis, NO and MDA levels in infertility patients were significantly higher ($p<0.05$) in both blood sample and endometrial tissue

compared to controls however the rise was more pronounced at endometrial tissue or local level(Figure 3).

The first ever compelling evidence systematically documented in human diagnosed cases of female infertility comes from Jaiswar SP *et al* in 2001[21]. Their objective as to develop the baseline data for status of free radicals in different types of infertility. 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 and endometrial 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. In other words, our study to certain extent is an extension and extrapolation of Jaiswar SP *et al* [21] .In this study we included two oxidants, three antioxidants and recruited 100 cases and 100 controls as compared to unequivocal cases and skewed number of controls.

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 causes for female infertility (peritoneal cause of infertility). His findings were in conformity with those of ours and by Dong M *et al* [23] who 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 folliculo genesis and steroidogenesis.[24] Low concentrations of NO in follicular fluid were associated with follicles containing mature oocytes that eventually become fertilized.[25] An endogeneous NO system exists in the fallopian Tubes and some of the studies claimed to have an increased NO levels in the fallopian tubes which are cytotoxic to the invading microbes and also maybe toxic to spermatozoa.[26] on contrary to its physiological role, 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.[6] In addition, NO inhibits steroidogenesis in the corpus luteum and has luteolytic action mediated through increased prostaglandins and by apoptosis,[27,28]. NO exerts deleterious effects on fertility by increasing the amount of OS in the peritoneal fluid, an environment that hosts the processes of ovulation, gamete transportation, sperm-oocyte interaction, fertilization, and early embryonic development [29,30].

Another oxidant MDA which is a lipid peroxidaton product obtained due to ROS attack on polyunsaturated fatty acids on cell membrane. In another study by Kuscu *et al* [31] and Sabuncu *et al* [32] MDA levels were found to be raised in patients with PCOS (anovulatory cause of infertility) compared to controls. But these previous studies neither measured the systemic counterpart nor have they related the levels in peritoneal fluid causing local tissue oxidative stress. Thus, our study again seemed to be more close to the causal association of NO and MDA as oxidant implicating or modulating the physiological and biomolecular processes involved in infertility. 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. [33-36]

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). Similarly in 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(Figure 2). 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 1**and **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. However, in endometrial tissue significant group difference was observed between the groups for other two antioxidants except Vitamin E. Szczepanska M[29] and Polak G[19] have shown the decreased peritoneal fluid SOD concentration in females with infertility. Again except Jaiswar SP *et al* [21] none of the above mentioned studies evaluated SOD levels in endometrial tissue and neither compared local versus systemic differential comparison of SOD.

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 successful pregnancy. Superoxide dismutase is present in the theca interna cells of the antral follicles [31] 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, a metal containing enzymatic antioxidant that catalyzes the decomposition of superoxide into hydrogen peroxide and oxygen, characterized in the theca interna cells in the antral follicles,[38] SOD also released at the time of sperm-oocyte fusion by Spermatozoa and oocyte to prevent excessive production of ROS.[39]

Likewise, 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 [40,41] and Kim IH [42] 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 VitaminE.[43] Non-enzymatic antioxidants including Vitamins E, is dietary supplements that aid the female body's oxidant defence system. Murphy *et al*[44] 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.

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 both in blood sample and endometrial tissue. When compared the severity of oxidative stress in local endometrial tissues (local) vs blood samples (systemic) in infertile females, local oxidative stress was observed more than the systemic, as observed by the high levels of both oxidants and low levels of antioxidants in the endometrial tissues. It could be envisaged that rise in levels of oxidative stress parameters in blood (systemic) may be, to some extent, secondary to increase in endometrial tissue (local) oxidative stress. But this potential paradox in the class of oxidants and antioxidants is difficult to be merely explained by simplistic understanding of rise of oxidants levels in blood secondary to inherent rise in the localized tissues and this clearly needs further exploration for clear conceptual understanding. This reflects the overall relevance and further consolidates the research in the field of infertility and may open up the role / pathways in its comprehensive management.

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.

CONCLUSION

Taken together, the overall results clearly indicate significant increase in the levels of all the oxidants including NO, MDA and significant decrease in antioxidants SOD, GSH and vitamin E in blood samples and endometrial tissues in infertile cases compared to fertile controls .oxidative stress was more pronounced in endometrial tissues as a local cause, which substantiates the propositional hypothesis for oxidative stress as one of the definitive mechanism in etiopathogenesis of female infertility and its management. However, it is not clear that whether the oxidative stress as observed fluctuated oxidants and antioxidants parameter, in blood was secondary to primary rise in local endometrial tissues. This clearly needs further exploration for clear conceptual understanding. Moreover there is still an ongoing debate on the role of antioxidants in modifying the disease outcomes.

Undeniably, the oxidative stress is becoming increasingly important as there is cumulative evidence suggesting that it is involved in conditions such as endometriosis, polycystic ovarian disease, tubal factors, recurrent abortions and hence decreased fertility rats in females. Future, the studies involving an extensive analysis of antioxidants in large group of infertile females will be promising for effective intervention especially in the explicating its role in unexplained infertility and recurrent abortions.

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