RESEARCH ARTICLE

Role of Antioxidants in the Treatment of Unexplained Infertility

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Abstract

Aim: To evaluate the role of free radical induced oxidative stress in the etiopathogenesis of unexplained infertility by biochemical estimation of oxidants and antioxidants levels.

Objectives:

- To evaluate the levels of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione reductase before antioxidant supplementation.
- To compare the effect of antioxidants (vitamin C 790 mg/day, vitamin E 15 mg/day, zinc 10 mg/day, Se 55 µg/day) given for 6 months on fertility outcome in patients with unexplained infertility by evaluating the levels of MDA, SOD, and glutathione reductase post supplementation.

Materials and method: This prospective study is carried out on 100 patients in LLR and associated hospital, Department of Obstetrics and Gynaecology, Kanpur, during the period from January 2014 to October 2015. Specific investigation of lipid peroxidation product MDA, SOD, and glutathione reductase (GR) was done initially at the 1st visit (baseline level) and then after supplementation of antioxidants for 6 months. Patients with other causes of infertility were excluded.

Results: Out of 100 patients, 30 were controls with spontaneous conception, and 70 were patients with unexplained infertility. Out of these 70 patients, 36 were given antioxidants from outside. Levels of MDA were significantly higher, and levels of GR and SOD were significantly lower in patients with unexplained infertility when compared with patients with spontaneous conception. There was an improvement in pregnancy outcome by supplementation with antioxidants from outside.

Conclusion: An imbalance in the level of oxidants and antioxidants leading to oxidative stress can affect the quality of gametes and can be the cause of unexplained infertility. Antioxidant supplementation from outside can improve fertility outcomes in these patients.

Keywords: Antioxidants, Oxidative stress, Unexplained infertility.

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Introduction

Unexplained infertility is infertility that is idiopathic in the sense that its cause remains unknown even after an infertility workup.[1] It accounts for 20–35% of the infertile couples.

It is hypothesized that reactive oxygen species (ROS) are released during follicular rupture by inflammatory cells (such as macrophages and neutrophils) present in the ovary at the time of ovulation. Gametes are sensitive to damage by ROS. Antioxidants and glutathione reductase protect gametes from these ROS by converting them into harmless compounds.[2]

An imbalance between antioxidants and ROS results in oxidative stress leading to cellular damage.[3] To suppress oxidative stress and minimize damage caused by ROS, antioxidative enzymatic system of the body comes into action such as SOD and GR.[4]

It is seen that levels of antioxidants in patients with unexplained infertility are lower than those in fertile patients, and levels of MDA, a lipid peroxidation end product, a reactive oxygen species, in the peritoneal fluid were higher in the infertile patient than in fertile patient.[5]

Materials and Methods

This study was a prospective case-control study. This study included 100 patients attending OPD of the Department of Obstetrics and Gynaecology, UISEMH, in collaboration with central Pathology, GSVM Medical College, Kanpur, during the period from January 2014 to October 2015. We recruited the controls after exclusion of the criteria as mentioned below and after taking informed consent. Women with spontaneous conceptions in the age group 20–35 years and in the gestation age group 20–24 weeks coming on the Monday and Thursday OPD's were taken sequentially as per the OPD sequence. This group of antenatal women formed controls. Similarly, the cases of unexplained infertility in the age group 20–35 years getting worked up and attending the OPD's of different units were

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asked regarding the participation in the study after explaining the details, were enrolled in the study. Those who accepted the therapy included in group A. Those who refused the treatment because of the cost or any other reason were included in group B. Thus, in our study:

- Control group—Consisted of 30 normal pregnant patients in the gestational age group 20–24 weeks.
- Study group—Consisted of 70 patients in the age group 20–35 years with unexplained infertility (both primary and secondary). This group was further subdivided into (a) cases and (b) control group.
  - Group A – consisted of 36 patients with unexplained infertility who were supplied antioxidants from outside.
  - Group B – consisted of 34 patients with unexplained infertility who were not supplied antioxidants from outside.

Those patients with endocrinal, hormonal, tubal, uterine, cervical factors, diabetes, hypertension, thyroid disorder, or any explained causes of infertility such as PCOS, endometriosis, tubal blockage due to PID, previous surgery, pelvic disease, hypothalamic dysfunction ovarian insufficiency, and hyperprolactinemia were excluded from the study.

All the patients selected for the study, both from control and study groups were studied in detail with regard to the clinical history, general examination, local examination, and basic investigations and special investigations which included T3, T4, TSH, serum prolactin, Mantoux test, ESR, chest X-ray, single/serial ultra-sound examination endometrial biopsy on 1st day of menses, HSG on 8th day of menses, husband semen analysis.

Specific Investigation

It was done at the time of starting of the workup of the patient to know the baseline level and then after supplementation of antioxidant, i.e., after 6 months.

- Lipid peroxidation product [malondialdehyde (MDA)]
- Super oxide dismutase (SOD)
- Glutathione reductase

Method of Collection of Blood Samples

Blood sample was collected from the control as well as the study group.

Heparinized 5 mL of blood from each subject would be collected from the median vein of the forearm. Blood would be centrifuged for 10 minutes and 15,000 rpm in a refrigerated centrifuge machine (0–5 degree centigrade) so as to collect plasma and plasma-free packed cell volume (RBC, etc.) and processed immediately for preparation of hemolysate.

Preparation of Hemolysate

Plasma removed from packed cell volume was washed three times with normal saline. Cells were lysed by adding 10 mL chilled distilled water for 10 minutes and then shaken vigorously for 2 minutes, with 0.5 mL chloroform added as a preservative. The mixture was centrifuged at 3000 rpm for 20 minutes. The mixture was clearly seen into three layers, lowermost layer was chloroform, the middle of stroma, and the uppermost layer was clear hemolysate.

Precautions during Blood Sampling and Investigations

- Hemolyzed blood was used for estimation.
- Centrifugation of samples was done at desired temperature and speed for the required period only.

Biochemical Investigation

Levels of Lipid Peroxidation

Malondialdehyde (MDA), is the most abundant aldehyde obtained after lipid peroxidation. The lipid peroxide content was estimated according to the method described by Ohkawa et al.[6]

Principle

Acetic acid detached the lipid and protein content of the tissue. The protein in the reaction mixture is dissolved by the addition of sodium dodecyl sulphate. 2-Thiobarbituric acid (TBA) reacts with lipid peroxide, hydroperoxide, and oxygen labile double bond to form the color adducts with absorption, maximum at 532 nm.

Reagents

- Sodium dodecyl sulphate (SDS) 8.0%—8.0 gm of SDS in 100 mL of double-distilled water.
- Acetic acid 20%—20% acetic acid solution was made by diluting glacial acetic acid accordingly.
- 2-Thiobarbituric acid 6.8%—900 mg of TBA suspended in 20 mL double-distilled water. The pH was adjusted to 7.0 by 0.1 N NaOH. This TBA was dissolved and the volume was adjusted to 100 mL with distilled water.

Procedure

From the prepared plasma, 0.2 mL was taken for the estimation of LPO. To the taken plasma, 0.2 mL of 8% SDS, 1 mL of 20% acetic acid, and 1.5 mL of 0.8 TBA were added. The pH of the mixture was adjusted to 7.0 using a concentrated NaOH solution. After adjusting the pH of the mixture, the volume was made into 4 mL by adding 1.1 mL of distilled water. Then the reaction mixture was kept in boiling water bath for 1 hour. After incubation for some time, pink color develops with particles in the reaction mixture. Then the mixture was centrifuged at 10,000 rpm for 15 minutes and the optical density (OD) of the resultant supernatant was taken at 532 nm in the spectrophotometer.

An appropriate standard made up of malondialdehyde 2.5 µmoL was run simultaneously.

Standard absorbance of MDA (25 µmoL) was used to calculate the amount of lipid peroxides in the samples, and the results were expressed as nmoL of MDA/mL plasma.

Estimation of Superoxide Dismutase (SOD)

Superoxide dismutase was determined by the modified method of McCord and Fridovich.[7]

Principle

Superoxide anions were generated in a system comprised of NADH and phenazine methosulphate. These superoxide anions reduce the nitroblue tetrazolium, forming a blue formazan, which was measured at 560 nm.

Superoxide dismutase inhibited the reduction of nitroblue tetrazolium, and thus the enzyme activity was measured by monitoring the rate of decrease in optical density at 560 nm.[7]

Reagents

- Ammonium sulphate
- 20.4 mL of tetrasodium pyrophosphate (454.9 mg of $Na_4P_2O_2$ $10H_2O$) was dissolved in distilled water. The volume was made up to 50 mL, and the pH was adjusted to 9.2

- 2.34 mL of nicotinamide adenine dinucleotide (NADH) (18.29 mg of NADH) was dissolved in 10 mL of pyrophosphate buffer, 0.93 mL phenazinemethosulphate and 2.9 mg of methosulphate were dissolved in 100 mL pyrophosphate buffer (pH 9.2), and the volume was made up to 25 mL in a volumetric flask.
- 1.5 mL of nitroblue tetrazolium (32.138 mg of nitroblue tetrazolium) was dissolved in pyrophosphate buffer (pH 9.2) and the volume was made up to 25 mL in a volumetric flask with buffer.
- Acetic acid

Procedure

A total of 2 mL of RBCs were hemolyzed with distilled water and dispensed in a centrifuge tube. The tubes were placed in a refrigerated centrifuge and spun at 10,000 × *g* for 15 minutes.

To the supernatant from the each sample of 313 mg/mL of ammonium sulphate was added to the final concentration of 50%. The tubes were shaken thoroughly and kept for 4 hours at 4°C.

The supernatant sample was dialyzed against triple distilled water with three changes, each change after 3 hours interval. The contents of the dialysis bags were subsequently used as enzyme sources. Later, the two reaction setups were run in parallel.

The tubes in first setup (experimental) received 0.3 mL nitroblue tetrazolium (NBT), 0.2 mL phenazinemethasulphate (PMS), 1.0 mL pyrophosphate buffer, 1 mL triple distilled water (TDW), and 0.2 mL enzyme source.

The tubes in the second setup (reference) received all the above reagents except the enzyme source.

The reaction were started simultaneously in the two acid sets by the addition of 0.2 mL NADH. After an interval of 90 seconds, 1 mL of glacial acetic acid was added to each tube for checking the reaction. The absorbance in these tubes was read at 560 nm on a spectrophotometer against a blank (NBT + PMS + Buffer + TDW).

Calculation

The unit of enzyme activity was defined as the amount of enzyme required to inhibit the optical density at 560 nm of NBT reduction by 50% in 1 minute under the assay conditions. Results were expressed as units/mg protein.

$$\frac{\text{Diff. between optical density (Exp. \& Ref.)} \times 2000}{\text{Experimental Reading} \times \text{Protein in mg}} = \text{units/mg Protein}$$

Estimation of Glutathione Reductase

Glutathione Reductase (GR) catalyzes the reduction of oxidized glutathione (GSSG) by NADPH to reduce glutathione (GSH) according to the following equation:

$$GSSG + NADPH + H + GR\ NADP_2 + GSH$$

The activity of the enzyme will be measured by following the decrease in optical density/minute during oxidation of NADPH spectrophotometrically at 340 nm.

Statistical Analysis

Data were analyzed statistically using appropriate tools of statistics '*t*' test—paired and unpaired, Fischer test, Odd's ratio using Medcalc software, Graph pad prism.

Results and Analysis

The maximum number of patients belonged to the age group of 20–25 years in both cases and controls, and the mean age group in cases was 25.5 + 4.1 years, and in the control group was 25.7 + 3.8 years.

Also, maximum number of patients in the study belonged to the age group of 20–25 years in both cases with intervention (55.6%) and cases without intervention (50%).

Most of the patients in the study belonged to the lower socioeconomic strata, both in cases (50%) and controls (50%) as well as in cases with intervention (47.2%) and in cases without intervention (52.9%).

Most of the patients in the control group had a mean BMI of 21.57 + 2.97, and in the cases, the mean BMI was 22.88 + 3.37, whereas the mean BMI for cases with the intervention was 23.59 + 3.45 and for cases without intervention was 22.13 + 3.17.

Table 1 depicts the difference in the levels of MDA, SOD, and GR in both the groups and it was found that there was a statistically significant difference in the levels of MDA, SOD, and GR in cases and controls, i.e., the levels were significantly higher in patients with unexplained infertility (p <0.0001).

Paired *t*-test was used, and no statistically significant difference was found in the MDA, SOD, and GR levels measured in cases without intervention before and after 6 months ($t = 0.6$, $p = 0.5$; $t = 0.8$, $p = 0.4$; $t = 1.3$, $p = 0.2$ for MDA, SOD, and GR levels, respectively).

But the levels of MDA measured after supplementation were significantly low in patients with unexplained infertility ($t = 6.8$, p <0.0001), whereas the levels of SOD and GR measured

Table 1: Comparison of levels of MDA, SOD, and GR in cases and controls

	Cases (70)	*Controls (30)*	*t-value*	*p-value*
MDA levels in study patients				
Mean with SD nmoL/mg of plasma	8.23 ± 1.79	4.44 ± 0.62	11.256	<0.0001
95% confidence interval (nmoL/mg of plasma)	7.8070–8.6639	4.2083–4.6717		
SOD levels in study patients				
Mean with SD unit/mg of protein	0.35 ± 0.14	0.65 ± 0.15	9.288	<0.0001
95% confidence interval (unit/mg of protein)	0.3185–0.3864	0.5899–0.7035		
GR levels in study patients				
Mean with SD unit/mg of protein	33.82 ± 10.07	62.43 ±7.24	14.07	<0.0001
95% confidence interval (unit/mg of protein)	31.42–36.22	59.72–65.13		

Table 2: Comparison of levels of MDA, SOD, and GR in cases before and after supplementation

	Number	*Cases before supplementation*	*Cases after supplementation*	*t-value*	*p-value*
MDA levels in cases with intervention after supplementation					
Mean with SD nmoLes/mg of plasma	36	8.64 ± 1.43	6.47 ± 1.89	6.8	<0.0001
95% confidence interval (nmoL/mg of plasma)	36	8.1613–9.1276	5.8290–7.1055		
SOD levels in cases after supplementation					
Mean with SD unit/mg of protein	36	0.32 ± 0.81	0.48 ± 0.14	6.98	<0.0001
95% confidence interval (unit/mg of protein)	36	0.2969–0.3517	0.4370–0.5300		
GR levels in cases after supplementation					
Mean with SD unit/mg of protein	36	30.85 ± 4.59	45.04 ± 12.97	5.7	<0.0001
95% confidence interval (unit/mg of protein)	36	29.2943–32.4063	40.6467–49.4255		

Table 3: Comparison of levels of MDA, SOD, and GR in cases with intervention and cases without intervention after 6 months

	Number	*Cases with intervention after 6 months*	*Cases without intervention after 6 months*	*t-value*	*p-value*
MDA levels in cases with intervention and cases without intervention after 6 months					
Mean with SD nmoL/mL of plasma	36	6.47 ±1.89	7.86 + 1.77	3.174	0.002
95% confidence interval (nmoL/mg of plasma)	36	5.8290–7.1055	7.2393–8.4731		
SOD levels in cases with intervention and cases without intervention after 6 months					
Mean with SD unit/mg of protein	36	0.48 ± 0.14	0.38 ± 0.18	2.675	0.009
95% confidence interval (unit/mg of protein)	36	0.4370–0.5300	0.3214–0.4479		
GR levels in cases with intervention and cases without intervention after 6 months					
Mean with SD unit/mg of protein	36	45.04 ± 12.97	36.88 + 12.98	2.628	0.011
95% confidence interval (unit/mg of protein)	36	40.6467–49.4255	32.3527–41.4090		

Table 4: Effect of antioxidant supplementation on fertility outcomes of cases

	UPT positive	*UPT negative*	*Total*
Cases with supplemention	07 (a)	29 (b)	36
Cases without supplemention	03 (c)	31 (d)	34
Total	10 (a + c)	60 (b + d)	70
Odd's ratio = a × d/b × c = 7 × 31/29 × 3 = 2.49			

The statistical analysis concluded that the Odd's are 2.5 times in favor of occurrence of pregnancy when antioxidant supplementation is given

after supplementation were significantly high in patients with unexplained infertility ($t = 6.8$, $p < 0.0001$; $t = 5.7$, $p < 0.0001$ for SOD and GR levels, respectively), as depicted in Table 2.

It was observed that the levels of MDA measured were significantly low in patients with unexplained infertility with intervention ($t = 3.174$, $p = 0.002$), and the levels of SOD ($t = 2.675$, $p = 0.009$) and GR ($t = 2.628$, $p = 0.011$) measured were significantly high in patients with unexplained infertility with intervention after 6 months as compared to values in cases without intervention after 6 months as shown in Table 3.

The statistical analysis in Table 4 concluded that the Odd's are 2.5 times in favor of the occurrence of pregnancy when antioxidant supplementation is given.

Discussion

Oxidative stress caused by an elevated level of reactive oxygen species affects the quality of gametes and thus can be a cause of both male and female infertility.

The present study was carried out on 100 patients, 70 patients of unexplained infertility, and 30 patients were controls (patients with spontaneous conception), the results were compared between control and cases regarding the value of MDA, SOD, and GR.

In our study, the age group between cases with and without intervention was matched and comparable. Maximum patients in both groups were in the age group 20–25 years. Hence, age was not a confounding factor in our study. This result was similar to the study conducted by Youssef et al.[8]

On comparing socioeconomic status between cases and controls, maximum patients belong to the lower socioeconomic status in both cases (50%) and controls (50%). Also, between cases with and without intervention maximum number of patients in both groups were in the lower socioeconomic status. Hence, socioeconomic status is not a confounding factor.

The distribution of BMI was studied in control group, cases with intervention, and cases without intervention. All the patients in our study were found to be in normal range. Hence, BMI was not a confounding factor, and this result was similar to a study conducted by Youssef et al.[8]

In present study, level of MDA was measured in unexplained infertility patients (70) and normal fertile patients (30). The mean value of MDA level was 4.44 nmoL/mL in control and 8.23 nmoL/mL in cases. The difference in mean value was highly significant (*p*-value –0.0001). Thus, infertile female cases have higher oxidative stress.

Similar results were found in studies done by Agarwal and Allamaneni, Agarwal et al., as well as Veena et al.[9–11]

Similarly, SOD enzyme activity was estimated in both groups, the mean was 0.65 U/mg of protein in the control group and was 0.35 U/mg of protein in cases and the difference of mean value was highly significant. This result was in accordance with studies done by Murphy et al., Shanti, and Liu et al.[12]

Glutathione reductase enzyme activity was estimated in both groups.[13,14] The mean value was 62.43 U/mg of protein in control and 33.82 U/mg of protein in cases, and the difference of mean value was highly significant. This result was similar to the study conducted by Naseer Mukheer and Agarwal et al.[15] Hence, the controls with spontaneous conception had good antioxidant status.

Cases with intervention were supplied with antioxidants from outside for a period of 6 months, and levels of MDA, SOD, and GR were re-measured and compared with the baseline level.

Levels of MDA, SOD, and GR were also re-measured in cases without intervention and compared with previous values.

In our study, the mean level of MDA was lower (6.47 nmoL/mL of plasma) after 6 months of antioxidants supplementation as compared to the baseline mean level (8.64 nmoL/mL of plasma). Paired *t*-test was applied and was found to be 6.8 and the difference of mean was highly significant. Thus, supplementing infertile patients with antioxidants might reduce oxidant stress.

The mean value of SOD in cases with intervention before 6 months was 0.32 units/mg of protein, and after 6 months was 0.49 units/mg of protein. A *t*-test value was 6.98, and the difference of mean value was highly significant (*p* value <0.0001).

The mean level of GR in cases with intervention before 6 months was 30.85 units/mg of protein and after 6 months was 45.04 units/mg. A *t*-test was applied and was found to be 5.7, and the difference of mean value was highly significant (*p* <0.0001). Therefore, supplementing antioxidants might improve the outcomes in infertile patients.

There was no significant difference in mean value of level MDA, SOD, and GR in cases without intervention before and after 6 months.

There was a significant difference in the mean value of the level of MDA, SOD, and GR in cases with intervention and in cases without intervention (MDA levels were lower and SOD and GR levels were higher in cases with intervention). Thus, showing an increase in antioxidant capacity and decrease in the level of lipid peroxidation product after antioxidant supplementation.

In our study, the effect of antioxidant supplementation was also seen on fertility outcomes of cases with and without intervention. It was found that 3 patients out of 34 cases who were not supplemented from outside became pregnant, whereas 7 out of 36 cases who were given antioxidant supplementation from outside became pregnant. Odd's ratio was found to be 2.494 (*p* value-0.3), i.e., there are 2.5 times more chances of getting pregnant after antioxidant supplementation but statistical significance could not be established due to the small sample size and short period of follow-up.

The present study was in accordance with the studies of Hosseini et al., Pyari, Wang et al., and Agarwal et al. as they demonstrated in their study that oxidants level MDA was high and antioxidant level was low in women with unexplained infertility.[10,13–15]

Whereas there are studies like the one conducted by Youssef et al., which concluded that oral antioxidants in the form of multivitamins and minerals did not improve pregnancy rates in women with unexplained infertility.[8]

Conclusion

- Mean value of MDA level in idiopathic infertility patients was significantly higher, and the mean value of SOD and GR was found to be significantly lower when compared to the control group.
- Antioxidant supplementation from outside can improve fertility outcomes in cases of unexplained infertility.

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