ORIGINAL RESEARCH PAPER

Biochemistry

THE ROLE OF FOLLICULAR FLUID ANTIOXIDANT ENZYME ON FEMALE INFERTILITY

KEY WORDS: Female Infertility, Oxidative stress, In-vitro fertilization, Follicular fluid, Superoxide dismutase

Dr. Hina Kausar F. A. Ansari. Assistant Professor, Department of Biochemistry, MGM Medical College And Hospital, Aurangabad, Maharashtra.

Dr. D. V. Bhale. Head & Professor, Department of Biochemistry, MGM Medical College and Hospital, Aurangabad, Maharashtra.

Dr. Farah Kausar Ansari. Assistant Professor, Department of Obstetrics and Gynaecology, F H Medical College and Hospital, Agra, UP. *Corresponding Author

Dr. M. D. Hivre. Assistant Professor, Department of Biochemistry, MGM Medical College and Hospital, Aurangabad, Maharashtra.

ABSTRACT

Reactive oxygen species (ROS) are generated as by-products during ovarian physiological metabolism, and antioxidants can maintain the balance between ROS production and clearance. A disturbance in this balance can induce pathological consequences in oocyte maturation, ovulation, fertilization, implantation, and embryo development, which can ultimately influence pregnancy outcomes. The balance between the generation and elimination of ROS is required for almost every metabolic function. In humans' pathological consequences of decreased antioxidant defence systems include many reproductive diseases, such as polycystic ovarian syndrome (PCOS), endometriosis, and unexplained infertility, as well as complications during pregnancy, such as early miscarriage, recurrent pregnancy loss, and preeclampsia. The objective of the study was to correlate antioxidant level in follicular fluid of infertile women undergoing in vitro fertilization and their pregnancy outcome. For this study women undergoing in vitro fertilization were included and divided in two groups and compared in terms of follicular fluid superoxide dismutase levels and pregnancy outcome. Study result showed that pregnant cycles were associated with significantly higher follicular fluid superoxide dismutase levels as compared to another group (P=0.001). These findings suggest that elevated follicular fluid superoxide dismutase level may have a positive impact on In-vitro fertilization outcome.

INTRODUCTION:

Fertility is the ability to conceive and produce offspring. Following regular and frequent unprotected sexual intercourse, about 84% and 92% of couples in general population are expected to conceive within one year and two years respectively. When a few fails to conceive even after two years of regular frequent coitus and there is no obvious reproductive pathology, the couple may be considered infertile.[1]

Incidence of infertility has increased since last few decades, regarding to social phenomenon, such as women being more carrier oriented and the tendency for marriage at a later age, late child bearing, increasing use of contraception especially intrauterine device and liberalized abortion. The prevalence varies widely, more in developing countries as compare to developed countries as there are limited resources available for investigation and treatment in developing countries.[2]

In India, the prevalence of primary infertility is estimated to be 10-20%. Male factor alone accounts for infertility in about 40% cases, female factor alone in 40% of the cases and in 20% cases, there is combined male and female factor.[3] Evaluation of infertility usually starts after 12 months; however, it may be indicated earlier. The absolute number of couples seeking infertility services has increased dramatically. Although infertility is a common problem, treatment is sometimes inadequate because the etiology is not fully understood.

Assisted reproductive techniques have allowed many infertile couples to fulfil their dream of having biological offspring. In vitro fertilization (IVF) is one of the most widely used treatments for infertility and is a process by which oocytes are fertilized by sperm in vitro.[4] Although IVF has resulted in numerous pregnancies and healthy deliveries, total failure of fertilization after IVF occurs in 10-25% of cycles even in cases where sperm and oocytes seem to be normal. Therefore, embryologists pay utmost attention to assess the quality of oocyte in human in vitro fertilization.[5] In IVF, oocyte selection and the identification of the best oocytes, would help to limit embryo overproduction and to improve the results of oocyte cryostorage programs.

For the development of oocytes, follicular fluid (FF) plays an important role. Follicular fluid is a product of both the transfer of blood plasma constituents that cross the blood follicular barrier and of the secretary activity of granulosa and theca cells.[6]

Follicular fluid surrounding the oocyte play vital role in determining oocyte quality and ability of oocyte to achieve fertilization and embryo development. Therefore, analysis of follicular fluid components may also provide information about metabolic changes and the fate of the egg coming from that specific follicle. Many researchers suggest that imbalance between reactive oxygen species and antioxidants in follicular fluid has significant role in infertility.[7]

Reactive oxygen species (ROS) has a double role as it affect multiple physiological as well as pathological processes involving the female reproductive tract.[8] Oxidative stress (OS) caused by the imbalance between ROS and the biological antioxidant systems that can lead to oxidative modification of DNA, lipids, and proteins.

The antioxidant enzymes neutralize and protect the oocyte and embryo from excessive ROS.[8] Antioxidant mechanisms present in every organism, which enable them to deal with oxidative environments and repair the damage caused by ROS. These are divided into nonenzymatic and enzymatic mechanisms. Enzymatic antioxidants include catalase, superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione oxidase (GPX). SOD, catalase, and GPX are the three most common enzymatic antioxidants, and they play critical roles in removing the harmful oxygen products.[10] ROS may originate either directly from gametes and embryos or from their surroundings. Therefore, the prevention against ROS formation must be

linked both to internal and external protection.[11]

SOD is one of the major antioxidant enzymes that catalyses the conversion of superoxide radicals to hydrogen peroxide.[12] The presence of higher total antioxidant capacity (TAC) in FF reflects the antioxidant activity of granulosa cells. Elevated TAC levels would be a marker of mature follicles resulting in the expansion of high-quality oocytes.[13]

Materials And Methods:

The present study was administered in Department of Biochemistry in our institute; in collaboration with Center for Reproductive Medicine, Fertility and Stem cell. The study was approved by Institutional Ethical and Research Committee. Informed consent was taken from patient. The study was conducted from November 2012 to July 2014. 60 Infertile females between the age group of 22-42 years were included in this study.

Inclusion Criteria:

Infertile female producing oocytes between the age group of 22-42 years and having their normo-spermic male partner, consistent with WHO 2010 guidelines for semen analysis and those undergoing their first IVF treatment cycle were selected.[14]

Exclusion Criteria:

Following patients were excluded from the study:

1. Female patients not producing follicles which having ovarian failure and poor ovarian reserve
2. Female patients with any systemic disease like hypothyroidism, hyperthyroidism, diabetes mellitus, hyperprolactinemia
3. Female patients whose partners have Sperm count < 15 million /ml, Motility < 60% and Grade 4 motility (Grade A) < 33%

Procedure:

The standard procedure was followed as per Richard T. Scott *et al* (1994) [15]study.

Infertile female reporting for IVF embryo transfer, was stimulated with a gonadotropin releasing hormone (GnRH) agonist from mid luteal phase onwards and when optimally down regulated, were stimulated with recombinant follicular stimulating hormone (FSH). Follicular size was monitored regularly by ultrasound scan. Subcutaneously human chorionic gonadotropin (hCG) was administered when average diameter of the leading follicles reached a minimum of 18mm. Follicles having 18-20 mm diameter on ultrasound were selected.

Transvaginal oocyte retrival was performed under ultrasound guidance, 36 hr after hCG administration. Microscopic examination of follicular fluid was performed by embryologist, to ascertain the maturity of oocyte. Mature oocytes have second polar body and clear cytoplasm; whereas immature oocytes have no polar body. Oocytes were separated and placed into media, whereas follicular fluids were collected into separate tubes. Only uncontaminated follicular fluids were retained for further determinations. After that mature oocyte inseminated with spermatozoa. Embryo quality was assessed before embryo transfer by double inverted microscope and a maximum of two grade A embryo were transferred to patients undergoing IVF procedure, approximately 48hr (4-cell stage) after insemination.[15]

Follicular Fluid Processing:

After collection, follicular fluid samples were centrifuged at 2000 rpm for10 minutes to get rid of cellular components and the clear supernatant transferred to sterile tubes and kept at -80c for not more than 1 week.

Laboratory Analysis:

Aliquots of the FF were thawed at room temperature and follicular fluid Superoxide dismutase (SOD) level was assessed by method of S Marklund and G Marklund(1974), modified by Nandi and Chatterjee.[16,17]

The chemicals and reagents used for the procedure were of analytical grade.

Pregnancy tests were performed on day 14 post embryo transfer, employing a commercial urinary kit (UPT kit).[18] Women with positive urinary pregnancy test were classified into the successful pregnancy group, while the women with negative urinary pregnancy test were considered the unsuccessful pregnancy group.

Statistical Analysis:

Mean and standard deviation were figured out for estimating the levels of follicular fluid SOD, in patients of female infertility. In order to compare these parameters unpaired '*t*' tests were used for group comparisons and the '*p*' values (probability values) were obtained. '*p*' value less than 0.05 was considered as statistically significant. The data was analyzed using SPSS version 20.

Result:

60 Infertile females included in this study were divided into two groups i.e., Group I (pregnancy positive group) and Group II (pregnancy negative group), as shown in Figure No. 1. Out of 60 infertile females, 27 females were in group I and 33 females were in group II. Mean age (mean ± SD) of Group I and Group II patients were 29.96 ± 4.55 years and 32.66±4.88 years respectively which were statistically significant ($p<0.05$). The patients in group I was significantly younger than that of group II as shown in Table No. 1.

Figure 1: Graphical Representation Of Number Of Patients Studied

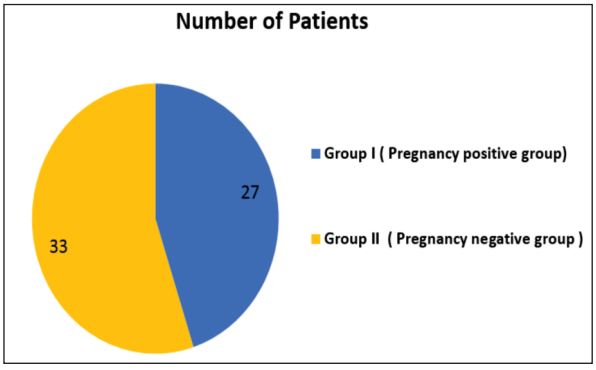

Table No. 1: Mean Age Of Group I And Group II Patients.

Variable	Group I (n=27)	Group II (n=33)	*t*-value	*p*-value
Age (Years) (Mean ± SD)	29.96 ± 4.55	32.66 ± 4.88	2.19	0.032*

(*p*<0.05, Statistically significant)

Mean (mean ± SD) follicular fluid SOD level in group I and group II patients were 12.25 ±0.90 U/ml and 10.15 ± 0.92 U/ml respectively as shown in Table No.2 also presented graphically in Figure No.2. The statistical analysis by unpaired *t*-test showed that there was significant increase in SOD level in group I as compared to group II patients ($p<0.001$).

Table No. 2: Mean Follicular Fluid SOD Level In Group I And Group II Patients

Variable	Group I (n=27)	Group II (n=33)	*p*-value
SOD (U/ml) (Mean ± SD)	12.25 ± 0.90	10.15 ± 0.92	0.001*

(*p*<0.05, Statistically significant)

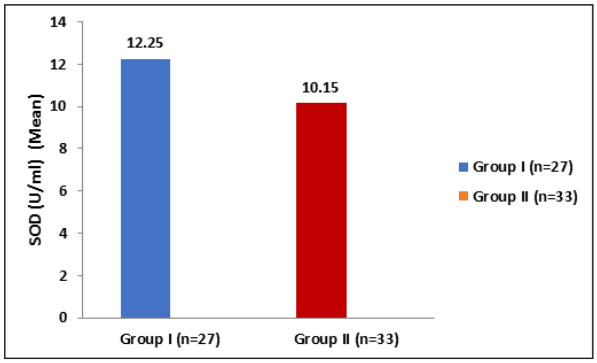

Figure 2: Comparison Of Mean Follicular Fluid SOD Level Between Group I And Group II Patients

DISCUSSION:

Infertility is a problem with a large magnitude. Assisted reproductive technologies offer excellent opportunities to infertile couples for achieving pregnancy. Free radicals like ROS influence oocytes, spermatozoa, embryos and their environments. The microenvironments associated with follicular fluid, hydro salpingeal fluid and peritoneal fluid have a direct effect on oocyte quality, sperm–oocyte interaction, sperm-mediated oocyte activation, implantation, and early embryo development.

ROS exert their cytotoxic effects by causing per oxidation of membrane phospholipids, which results in increase in membrane permeability, loss of membrane integrity, enzyme inactivation, structural damage to DNA and cell death. Oxidative stress can have detrimental effects on female fertility by affecting ovulation, fertilization, embryo development, and implantation. Thus, OS is considered as a cause of female infertility.[19]

In female reproductive tract, macrophages, neutrophils and granulosa cells in Graffian follicles are a source of ROS which are balanced by antioxidants. During follicular maturation oocytes are well protected against the toxic injury due to OS by important antioxidants such as catalase, superoxide dismutase (SOD), glutathione transferase, paraoxanase, heat shock protein 27 and protein isomerase.[20]

Follicular fluid contains high concentrations of antioxidants, which protects oocytes from ROS-induced damage. It is possible that an imbalance within the pro-oxidant /antioxidant systems operated in the follicular fluid may cause the abnormal development of oocytes and impaired fertility as well as damage to the oocytes DNA, cytoskeleton or membrane .The cytoskeleton helps ensure that meiosis occurs in the oocyte, which is a pre requisite before fertilization for formation of haploid gamete.[21] The concentration of these oxidative stress markers are lower in follicular fluid then the serum, indicating follicular fluid contain high concentration of antioxidant system, which helps in protecting an oocyte from oxidative damage , thus pregnancy outcome in an IVF progaramme could be affected by many variables of oxidative stress.[21]

Antioxidants protect the embryo from damage caused by pro-oxidants, which thereby aids in the establishment of a successful pregnancy. SOD is one of the major antioxidant enzymes that catalyzes the conversion of superoxide radicals to hydrogen peroxide.[22]

In our study we found that there was a statistically significant difference in follicular fluid SOD level between group I (pregnant) and group II (non-pregnant) patients. Mean (mean ± SD) follicular fluid SOD level in group I and group II patients were 12.25±0.90 U/ml and 10.15±0.92 U/ml respectively. The statistical analysis by unpaired *t*-test showed that there was significant increase in SOD level in group I subjects as compared to group II subjects (p=0.001). Our results are in accordance with the result of Oyawoye *et al* (2003) [9], who evaluated total antioxidant capacity (TAC) using FF collected from 63 women undergoing oocyte retrieval for IVF after controlled ovarian stimulation. In their study they found baseline TAC was significantly higher in FF samples of oocytes that achieved successful fertilization, suggesting that higher TAC may predict increased fertilization potential.

The observation that higher follicular fluid total antioxidant capacity is associated with successful fertilization is consistent with the findings of Paszkowski *et al* (1995),[23] who observed higher mean GSHPx activity in follicles yielding oocytes that were successfully fertilized compared to follicles with non-fertilized oocytes. Our results are supported by the study done by Paszkowski *et al* (1995).[23] This shows that increase in the antioxidant capacity helps in increasing the fertilization rate by protecting the embryos from the effects of oxidative stress which cause damage to the oocytes and quality of embryo.

CONCLUSION:

Assisted reproductive technologies are being increasingly used to help infertile couples realize their dream of having a biological child.

In our study we found that there was high level of SOD in group I subjects as compared to group II subjects. Baseline SOD concentration were higher in follicles whose oocytes fertilized successfully. However, SOD concentrations are lower in the follicles that produced embryos but were not capable of surviving transfer. Thus, we conclude that the ROS scavenging ability of antioxidants is related to have protective effect in fertilization outcomes.

Recently, there has been growing interest in the role of antioxidants in female reproductive activities. Antioxidant products and ROS balance are shown to be closely associated with female subfertility or infertility. Thus, it is necessary to emphasize the role of antioxidants in the development and survival process of follicles and in follicle responsiveness to gonadotropins as well as in steroidogenesis.

Further studies are required to be done with more sample size for evaluating the levels of antioxidant protective effect with more TAC Markers. This will improve the quality of embryo and will be beneficial for healthy pregnancy outcome.

Conflict Of Interest:

All the authors have declared that they have no conflict of interest

REFERENCES:-

1) Ayaz K, Miah MT, Ahasan HN, Raihan MR, Islam MA, Siddque AA. Male infertility-a review. J Med 2012;13(2):190-199.
2) Kamel RM. Management of the infertile couple: an evidence-based protocol. Reprod Bio Endocrinol 2010;21(8):1-8.
3) Jajoo S, Kalyani KR. Prevalence of abnormal semen analysis in patients of infertility at a rural setup in Central India. Int J Reprod Contracept Obstet Gynecol 2013;2(2):161-64.
4) Kovacs P, Kovats T, Sajgo A, Szollosi J, Matyas S, Steven G. J Assist Reprod Genet. 2011 January;28(1):49–54.
5) Das S, Chattopadhyay R, Ghosh S, Goswami SK, Chakraverty BN, Chaudhary K. Reactive oxygen species level in follicular fluid embryo quality marker in IVF? Hum Reprod Advance Access 2006 July;22:1-5.
6) Fortune JE. Ovarian Follicular Growth and Development in Mammals. Biology of Reproduction 1994;50:225-32.
7) Ruder EH, Hartman TJ, Blumberg J, Goldman MB. Oxidative stress and antioxidants: exposure and impact on female fertility. Human reproduction Update 2008 Jul-Aug;14(4):345-57.
8) Moreira da Silva F, MarquesA and Chaveiro A. Reactive Oxygen Species: A Double-Edged Sword in Reproduction. Open Vet Sci J 2010;4:127-33.
9) Oyawoye O, Gadir AA, Garner A, Constantinovici N, Perrett C, Hardiman P. Antioxidants and reactive oxygen species in follicular fluid of women undergoing IVF: relationship to outcome. Human Reproduction 2003; 18 (11): 2270-74.
10) I.Fridovich. "Superoxide radical and superoxide dismutase," Annual Review of Biochemistry, vol. 64, pp. 97–112, 1995.
11) Carbone MC, Taton C, Monache D, Marci R, Caserta D, Colonna RF. Amicarelli Antioxidant enzymatic defences in human follicular fluid: characterization and age-dependent changes. Molecular Human Reproduction 2003;9(11):639-43.
12) Wallace DC, Melov S. Radicals r'aging. Nat Genet 1998; 19:105–106.

13) Paszkowski T, Traub AI, Robinson SY, McMaster D: Selenium dependent glutathione peroxidase activity in human follicular fluid. Clin Chim Acta 1995; 236:173-80.
14) World Health Organization WHO. Laboratory manual for the examination of human semen and semen-cervical mucus interaction, 4th edi. Cambridge, UK7C Ambridge University Press, 1999 pp 4-23.
15) Richard T. Scott, Daniel Navot. Enhancement of ovarian responsiveness with microdoses of gonadotropin-releasing hormone agonist during ovulation induction for in vitro fertilization. Fertility and Sterility vol61, Issue 5, May 1994, pp880-885
16) Marklund S, Marklund G. Involvement of the Superoxide Anion Radical in the Autoxidation of Pyrogallol and a Convenient Assay for Superoxide Dismutase Eur. J. Biochem. 1974; 47:469-474.
17) Nandi A, Chatterjee IB. Assay of superoxide dismutase activity in animal tissue. J Biosci 1988; 13(3):305-15.
18) Salha O, Nugent D, Kaufmann S, Levett S, Jenner L, Sharma V. The relationship between follicular fluid aspirate volume and oocyte maturity in in-vitro fertilization cycles. Human Reprod 1998; 13(7):1901–06.
19) Agarwal A, Gupta S. Sharma R. Oxidative stress and its implications in female infertility –a clinician's perspective. Reprod Biomed 2005; 11(5):641–50.
20) Agarwal A, Gupta S, Sikka S. The role of free radicals and antioxidants in reproduction. Curr Opin Obstet Gynecol 2006; 18:325-32.
21) Jozwik M, Wolczynski S, Jozwik M, Szamatowicz M. Oxidative stress markers in preovulatory follicular fluid in humans. Mol Hum Reprod 1999; 5:409-13.
22) Wallace DC, Melov S. Radicals r'aging. Nat Genet 1998; 19:105–106.
23) Paszkowski T, Clarke RN. Antioxidative capacity of preimplantation embryo culture medium declines following the incubation of poor-quality embryos. Hum Reprod 1996; 11:2493-95.