Estimation of serum and follicular fluid Malondialdehyde among women undergoing IVF: Association with age, duration of subfertility and other IVF outcome parameters.

ABSTRACT

Background: Oxidative stress affects various functions in female reproduction and its role in female infertility is proposed. Hence it was of interest to analyze serum and follicular fluid (FF) Malondialdehyde (MDA) among women undergoing IVF and determine its association with IVF outcome determinants (age, subfertility duration, type of stimulation protocol used, fertilization potential of oocytes, embryo quality and post-IVF pregnancy).

Materials and Methods: FF collected by transvaginal ultrasound guided oocyte rupture and IV blood from 28 women (20-40years) undergoing IVF/ICSI, were analyzed for MDA following TBARS method and its association with IVF outcome parameters were compared.

Results: Mean MDA was significantly higher in serum than FF among all women tested (2.98 ± 0.14 Vs 2.23 ± 0.11; *P*=0.0001), IVF group (2.88 ± 0.16 Vs 2.25 ± 0.16; *p* =0.008) and ICSI group (3.10 ± 0.23 Vs 2.20 ± 0.15; *P*=0.005). No significant association was seen between MDA and other parameters except embryo quality (r=0.378, *p*=0.047).

Conclusion: Low MDA in FF compared to serum evidences that FF has an effective antioxidant mechanism to overcome oxidative stress. The importance of ROS in female reproduction is evidenced by positive correlation between MDA and embryo quality.

Key words:*Malondialdehyde, oxidative stress, serum, Follicular fluid.*

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INTRODUCTION

Oxidative stress (OS) causes defective embryo development andresults when balance between reactive oxygen species (ROS) and antioxidants is disrupted due to ROS surpassing the ability of antioxidants to neutralize them (1, 2). Eventhough physiological amounts of ROS are necessary for healthy body functions, they are involved in the pathophysiology of several diseases of the female reproductive system like endometriosis, polycystic ovary syndrome, preeclampsia, maternal diabetes and recurrent fetal loss. Detrimental effects of ROS on fundamental building blocks of life like fats, lipids, nucleic acids and proteins have been studied. A number of biomarkers to measure the end product of ROS induced damage such as lipid peroxidation (LPO), oxidation of proteins and DNA damage is studied in various human body fluids such as peritoneal fluid, follicular fluid (FF), amniotic fluid and hydrosalpingeal fluid (2). An association between FF OS biomarkers and the outcome after assisted reproductive technology (ART) has been established, (1) but its exact impact on oocyte maturation, fertilization and pregnancy is not fully understood yet (3). Further,Malondialdehyde (MDA), a by-product of LPO damage has been used to monitor the degree of peroxidation on cells (4). This study was, therefore initiated to measureThiobarbituric acid reacting substances (TBARS) in FF and serum of women undergoing IVF and find outany associationwith various IVF outcome measures like age,

duration of subfertility, type of stimulation protocol used, fertilization potential of oocytes, embryo quality and fetal outcome.

MATERIALS AND METHODS: Twenty eight women (age 20-40 years) attending the infertility clinic with varied reasons for infertility and selected for IVF at Manipal assisted reproductive center (MARC), Department of Obstetrics and Gynecology, Kasturba Medical College Hospital (KMCH),Karnataka were included in this study. Biochemical analyses were conducted in the department of Biochemistry, Kasturba Medical College, Manipal, India following necessary institutional permission and ethical committee approval fromKMCH.

Of the total, 14 women were considered for ICSI due to male infertility and rest 14 for IVF-ET due to female infertility. Samples were obtainedonly afterwritten informed consent from the subjectswas taken prior to enrolment for this study.

Collection of Serum and FF:

With all aseptic precautions, 4ml of whole blood was collected 48 hours prior to oocyte retrieval and serum was analyzed for MDA immediately after separation.

Controlled Ovarian Hyperstimulation (COH) was initiated on the 2nd day of menstrual cycle using GnRH analogs preceded by down regulation starting from the late luteal phase of the previous cycle (long protocol) or from the follicular phase of present cycle (antagonist cycle). Follicle stimulating hormone (FSH) was administered IM on the day of drawing blood sample (48 hours prior to oocyte retrieval) adjusted to age, type of infertility and response to previous initial gonadotrophin stimulation starting with 300 IU and gradually tapering up or down depending on the dose response relationships during previous cycles. COH was monitored regularly by ultrasonography andserum estradiol (E_2)estimations. This was followed by

intramuscular administration of HCG (10000 units) unless 3 follicles reached a diameter of 18mm at the least. Transvaginalsonography (TVS) guided needle aspiration of follicles was performed after 35 hours.

FF collected from the largest ovarian follicle was utilized only if the oocyte aspiration was successful during the first attempt with nosignificant contamination by blood or culture medium. Immediately after removal of the oocytes, FF was centrifuged at 3000rpm for 10minutes to remove cellular debris and the supernatant was preserved in liquid nitrogen for not more than a week until further analysis. Levels of OS markers and antioxidants however were not altered by storing it even for more than a week.

[Reagents and MaterialslikeTetramethoxypropanewereobtained from Sigma-Aldrich chemicals, USA and other reagents of analytical gradefrom Merck, India limited, Mumbai and Ranbaxy laboratories ltd, Punjab, India].

Estimation of serum/FF MDA: MDA in serum/FF was determined fluorimetricallyemploying thiobarbituric acid reaction (TBA).Firstly,0.2 ml of serum/FF was taken in a stoppered centrifuge tube, made upto 1ml with 0.9% NaCl aqueous solution, treated with 4ml of N/12 H_2SO_4 and was shaken gently. Then, 0.5ml of 10% phosphotungstic acid was added, mixed well and centrifuged at 3000rpm for 10minutes. Supernatant wasdiscarded and sedimenttreated with 2ml of N/12 H_2SO_4 and 0.3 ml 10% phosphotungstic acid, followed by centrifugation again for 10 minutes. The supernatant was then discarded and 4ml of distilled water plus 1ml of TBA reagent were added to the sediment. 4ml each of distilled water and 0.5nmol/L of tetramethoxypropane were taken as blank and standards respectively, 1ml of TBA reagent was added to both the tubes and all were incubated at 95ºC in a boiling water bath for 60 minutes. The tubes were removed, cooled and treated with 5 ml of n-Butanol, mixed vigorously and centrifuged at 3000rpm for

15minutes. The upper n-Butanol layer was then taken for fluorimetric measurement at 553nm emission and 515nm excitation to determine TBARS (expressed in terms of MDA as nmol/ml of serum) (5).

Data management and Statistical analysis: The data (clinical and lab reports) were entered and analyzed using Statistical Package for Social Sciences (SPSS/PC: SPSS-9, Chicago, USA). Results were expressed as Mean ± SEM. Paired‘t’ test was applied to analyze parameters between serum and FF in patients undergoing IVF (n=28), Independent‘t’ test was applied for analyzing two different fluids (serum /FF) in patients undergoing either IVF or ICSI. Moreover, paired sample correlation was analyzed employing paired sample statistics.

Pearson correlation was applied to associatepatient’s age, oocyte maturity, fertilization potential and duration of subfertility with MDA. Spearman’s rho non-parametric correlation test was applied to correlate embryo quality, IVF outcome as positive pregnancy test and the type of protocol used to stimulate ovary with MDA.

When Spearman’s correlation was applied to study the effect of type of protocol, IVF outcome and embryo quality on MDA:No.“1” was assigned to women who underwent antagonist cycle, failed to conceive with negative pregnancy test and had grade-1 embryos;and No “2”to those who underwent long protocol, had positive pregnancy and good grade-2 embryos.

A value of $p< 0.05$ (CI 95%) was considered to be statistically significant.

RESULTS

Serum and follicular fluid were analyzed for various parameters in patients undergoing ART (IVF+ICSI) (n=28) (Table 1).

They were further grouped into those undergoing ART due to male infertility- hence undergoing ICSI (n=14) or female infertility- hence undergoing IVF (n=14) and the parameters were studied in serum and follicular fluid of both groups (Table 1).

Overall mean MDA was significantly higher in serum than FF in the entire group of 28 women (2.98 ± 0.14 Vs 2.23 ± 0.11; *p*=0.0001), IVF group (2.88 ± 0.16 Vs 2.25 ± 0.16; *p*=0.008) andICSI group (3.10 ± 0.23 Vs 2.20 ± 0.15*;* *p*=0.005). A positive correlation existed between MDA in FF and embryo quality (r=0.378, *p*=0.047). MDA in FF was not affected by patient's age, duration of subfertility and type of stimulation used. Moreover it did not have any influence on oocyte maturity, fertilization potential of oocytes or pregnancy following IVF.

DISCUSSION

Free radicals play a key role in modulating various reproductive functions by their influence on oocytes, sperm and embryos in their microenvironments like FF, hydrosalphingeal fluid and peritoneal fluid. The quality of oocytes, sperm oocyte interaction, implantation and early embryo development are also reported to be affected by changes that take place in such microenvironments (6). However, certain levels of ROS arerequired for normal fertilization and a fine balance between ROS and antioxidants for successful reproductive outcome is crucial both *in vitro* and *in vivo*(7, 8).

Jozwick et al found lower levels of OS markers in FF compared to serum, no significant difference in parameters between pregnant and non-pregnant women and found no relationship between fertilization rate and OS markers (9). Contrarily, Oral O et al demonstrated low FF MDA levels in pregnancy positive group concluding that MDA levels can be used as a predictive marker of ART success compared to other parameters like age, basal FSH and E2 level on HCG administration day (10).Similarly, low MDA in FF compared to serum in all the study groups

(combined or separately) that we observed remains consistent with the afore mentionedreports thus emphasizing existence of effective antioxidant defense mechanisms in the oocyte microenvironmentirrespective of infertility contributed by male factor or female factor.

Revelli A et al commented that substances that could be used as reliable markers of oocyte competence to fertilization, embryo development and pregnancy were not available (11).On the other hand, Fujimoto VY et al stated that lipid peroxidation products and antioxidant enzyme activities in FF were not associated with the quality of embryos (12),contrary toPasqualotto EB et al who observed lipid peroxidation as a good marker of metabolic activity within the follicle, suggesting some amounts of LPO is necessary to establish pregnancy (13). We found a positive correlation between MDA and embryo quality, which indicates that MDA can be used as a marker of expecting possible development of an oocyte to turn into a good quality embryo.

Oyawaye et al studied ROS levels in FF of IVF cases and demonstrated lower OS marker levels in successfully fertilized and transferred oocytes (14).Bedaiwy et al evidenced that lower FF ROS were associated with pregnancy after ICSI (1). However our study failed to produce any association between MDA and fertilization potential, oocyte maturity or pregnancy after IVF. No effect of type of stimulation used, age of patients or duration of subfertility on MDA in FF was demonstratedin our study as well.

Despite highly positive results that we observed in our study, we alsohave had certain limitations too; our sample was lower in number and we collected serum48 hours before obtaining FF.

The current study thus recommends evaluation of the same parameters in a large patient group as well as to compare and correlate parameters analyzed in serum and FF obtained on the same

day.Further large scale multicenter trials are needed to acquire added knowledge base in this field of IVF research.

CONCLUSION

Lower MDA in FF as this study revealed, evidences that effective antioxidant mechanism remains active in the FF environment. OS has a definitive role in female reproduction with some amounts necessary to establish a pregnancy. MDA showed positive correlation with embryo quality suggesting some amount of ROS is essentially required for having an effective IVF outcome.

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Table 1: Comparison of MDA levels between serum and follicular fluid of women undergoing IVF.

	MDA(nmol/L)Measurements		
Study populations (Groups)	**Serum (± SD)**	**Follicular Fluid (± SD)**	**Significance levels**
Combined group (n=28)	2.98 ± 0.14	2.23± 0.11	*p* =0.0001
IVF group (n=14)	2.88 ± 0.16	2.25 ± 0.16	*p* = 0.008

ICSI group (n=14)	3.10 ± 0.23	2.20 ± 0.15	*p* =0.005

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