Follicular Fluid Oxidative Stress Biomarkers and Intracytoplasmic Sperm Injection (ICSI) Outcome

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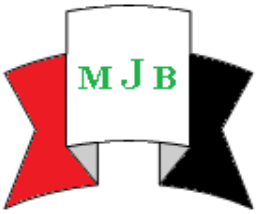

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Abstract

The aim of this study was to evaluate the association between the follicular fluid (FF) malondialdehyde (MDA) levels as a free radical marker and reduced glutathione (GSH) as antioxidant and pregnancy after intracytoplasmic sperm injection (ICSI).

A total seventy-five infertile women aged between 22-45 years(31.43 ± 5.38 years), referred to the fertility clinic in Al-Sadder teaching hospital, , and undergone intracytoplasmic sperm injection throughout period from March 2013 to January 2014,were included in this study. follicular fluid (FF) malondialdehyde was measured by spectrophotometer based on the calorimetric reaction with thiobarbituric acid (TBA) and follicular fluid glutathione was measured by spectrophotometer depends on the action of sulfhydryl groups.

Out of the 75 included patients, 15 (20%) achieved pregnancy after ICSI. Pregnant women were associated with significantly higher MDA level ($P<0.05$) and lower GSH ($P<0.05$) .There was a significant positive correlation between MDA and the number of pronucleus ($r= 0.326$). The best cutoff value of FF.MDA and FF GSH associated with pregnancy were 3.49 µM and 26.90 µM respectively. Higher FF MDA and lower FF GSH levels are associated with pregnancy after ICSI. Oxidative stress parameters may be markers of metabolic activity within the follicle.

Key words: follicular fluid, malondialdehyde , glutathione , intracytoplasmic sperm injection.

علاقة الجهد التأكسدي للسائل الجريبي ونتائج الحقن المجهري

الخلاصة:

كان الهدف من هذه الدراسة تقييم العلاقة بين مستوى المالوندالديهايد في السائل الجريبي كعلامة لمستويات الجذور الحرة ومستوى الكلوتاثيون كعلامة لمضادة للأكسدة ، ونسبة الحمل بعد الحقن المجهري. الطريقة :تضمنت الدراسة خمسة وسبعين من النساء المصابات بالعقم الذين تتراوح أعمارهم بين ٢٢–٤٥ سنة (٣١.٤٣ ± ٥.٣٨ سنة)، واللاتي تم ارسالهن إلى عيادة خاصة للخصوبة في مستشفى الصدر التعليمي في النجف طوال الفترة من مارس ٢٠١٣ إلى يناير ٢٠١٤ تم قياس بين مستوى المالوندالديهايد في السائل الجريبي بواسطة الطيف استنادا الى رد فعل الحراري مع حامض الثايوبيوترك ومستوى الجلوتاثيون في السائل الجريبي بواسطة الطيف اعتمادا على عمل مجموعات سلفهيدريل. وكانت نتائج الدراسة ١٥ مريضة من اصل ٧٥ مريضة حققت الحمل ، (٢٠٪) بعد الحقن المجهري.وكان مستوى المالوندالديهايد في السائل في النساء الحوامل أعلى بكثير مما في النساء غير الحوامل ($P<0.05$) وكان مستوى الجلوتاثيون في السائل في النساء الحوامل أوطئ بكثير مما في النساء غير الحوامل ($P<0.05$). كان هناك علاقة إيجابية ذات دلالة إحصائية بين مستوى المالوندالديهايد وعدد البيوض المخصبة PN ($r = ٠.٣٢٦$). وكانت أفضل قيمة قطع من مستوى المالوندالديهايد في السائل اجريبي في النساء الحوامل و مستوى الجلوتاثيون في السائل المرتبطة بالحمل هي ٣.٤٩ µ و ٢٦.٩٠ µ على التوالي.

ارتبط الحمل بعد الحقن المجهري مستوى العالي للمالوندالديهايد في السائل الجريبي والمستوى الواطئ للكلوتاثيون. قد تكون ارتفاع معامل الاكسدة دلالة على النشاط الأيضي لجريبات المبيض.

Introduction

Infertility is a major problem in developing countries and causes extensive social and psychological suffering . It is an important health issue, affecting approximately 10-15 % of couples worldwide[1]. Fortunately, advances in assistive reproductive technologies (ART) have made many infertile couples to conceive [2]. There are many factors that may affect outcome of ICSI such as age, weight and stress [3]. The recent research in the field of infertility is paying attention on the free radicals, which is suspected to be one of the major factors that affect ART success. In a normal cell there is balance between formation and removal of free radicals (unstable and highly reactive species with one or more unpaired electrons) [4]. However this balance was disturbed, the condition called 'oxidative stress' & can result is serious cell damage if the stress is massive and prolonged [5]. Malondialdehyde (MDA) is generated from lipid peroxidation, and it is one of the most popular and reliable markers that determine oxidative stress in clinical situations [6]. On the other hand Glutathione is responsible for protection against ROS and RNS, and detoxification of various types of endogenous and exogenous toxins [7]. Follicular fluid (FF) is the net result of both the transport of plasma constituents to follicles and the secretary activity of granulosa and theca cells [8]. The oocyte develops within the FF environment and ROS in FF can intimately affects the quality of oocytes and their interaction with sperm, thus affecting embryonic progress, implantation, fertility outcomes, and success of ICSI [9].

Materials and Methods

This prospective cohort study was approved by approved by the Ethics Committee of the University of Kufa and informed consent was obtained from all participants. A total of 75 patients were enrolled in the study from March 2013 to January 2014. All of them were recruited according to the following criteria: Non-smokers; Free from hepatitis and HIV (by screening test); absence of any metabolic or endocrine system-associated diseases, or any other associated condition which could alter the level of free radicals like malignancy and antioxidant therapy. The reasons for the couples' infertility were male factor infertility (n, 28), female factor (n, 41) and unexplained infertility (n, 6). All of these women underwent ovulation induction with either long (N=24) or short (N=51) protocols based on timing, hormonal conditions and ovarian reserve status of the women on the discretion of the clinician. An hCG injection was given to trigger the final stages of oocyte maturation and ultrasound-guided oocyte pick-up was performed 34–36 h later. Fertilization and cleavage was assessed daily and fertilization rate (FR) and cleavage rate was calculated according to following equation respectively.

FR=(NO. of 2PN on day one / Whole NO. of oocytes injected)×100
CR =(NO. of cleaved embryos on day / Total NO. of 2PN)×100.

The embryos were classified according to their morphology and percentage of fragmentation.

The biochemical pregnancies was confirm on the fourteenth day of embryo transfer by measurement of serum B- HCG. The pregnancy rate (PR) is calculated by following equation:

$$PR = \frac{\text{NO. of pregnant ladies after ICSI}}{\text{Total NO. of patients who underwent ICSI}} \times 100\%$$

Oxidative Stress Biomarkers

Follicular fluid (FF) samples were taken from the aspirated FF of the patients during the oocyte pick-up procedure, after removal of oocytes from it.

Measurement of MDA, a secondary product of lipid peroxidation, was based on the calorimetric reaction with thiobarbituric acid (TBA) to form pink color product, which could be measured by spectrophotometer [10].

Determination of GSH depends on the action of sulfhydryl groups [11]. Sulfhydryl group of GSH could reduce disulfide chromogen of 5, 5′-Dithiobis 2. nitrobenzoic acid (DTNB) and change it to an intensely yellow compound which could measure its absorbance directly by spectrophotometer at 412 nm and it was directly proportional to the GSH concentration [12] .

Statistical analysis

Statistical analysis was performed in this study using SPSS (Statistical Package for Social Science; Version 17) program. independent t-test was used to estimate differences between groups in continuous variables . Pearson‘s correlation analysis was used for correlation. The binary logistic regression analysis used to determine the odds ratio for pregnancy as dependant variable. Receiver operating characteristic (ROC) curves were generated to investigate the predictability of OS biomarkers for pregnancy. The sensitivity, specificity were calculated for the optimal OS cut-off levels determined by ROC curve analysis. Results are reported as (mean ± SD). P< 0.05, was considered statistically significant [13].

Results

MDA and GSH were measured in FF of all studied group.

FF.MDA was significantly higher in pregnant women when compared with non pregnant women (P<0.05) as shown in (Table 1); and its levels was insignificantly higher in pregnant women when compared with non pregnant women in respect to infertility cause (P>0.05).

Table 1: Follicular fluid malondialdehyde level in µMM in Pregnant and non pregnant women in each cause of infertility

Causes	F.MDA		P value
	Pregnant (Mean ± SD) µM	Non pregnant (Mean ± SD) µM	
Male factors	3.80 ± 1. 03	3.29 ± 1.18	0.436
Female factors	4.50 ± 2.17	3.25 ± 1.48	0.166
Unexplained factors	-	1.07 ± 0.74	-
Total	4.18 ± 1.70	3.00 ± 1.45	0.041*
P value among groups	0.530	0.046*	

In the other hand FF.GSH levels was significantly lower in pregnant women when compared with non pregnant women (P<0.05) Also its levels was significantly lower in pregnant women with female factor infertility when compared with non

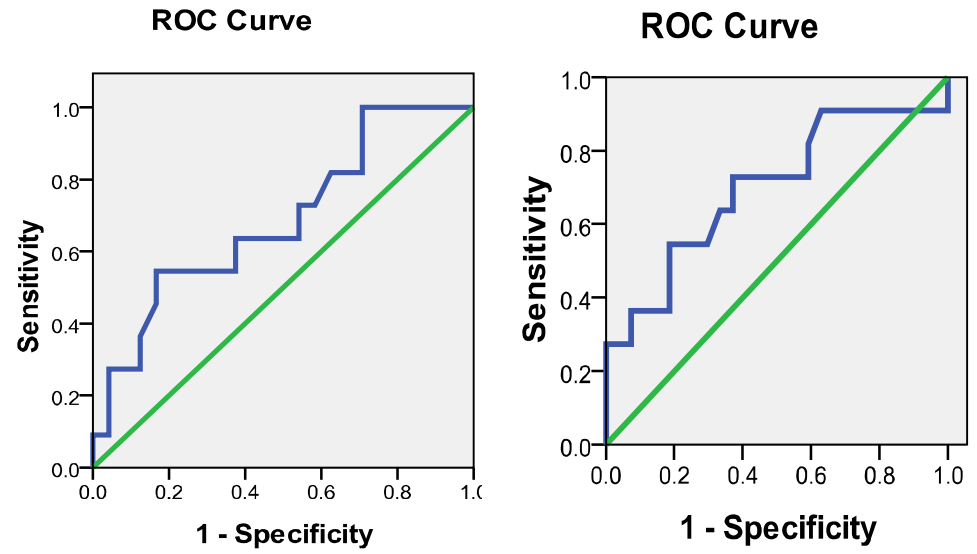
pregnant women (P<0.05) as shown in (Table 2).

Table 2: Follicular fluid glutathione level in µMM in pregnant and not pregnant women in each infertility factor

Causes	F.GSH		P Value
	Pregnant (Mean ± SD) µMm	Non Pregnant (Mean ± SD) µMm	
Male Factors	26.96 ± 11.72	29.86 ± 8.89	0.549
Female Factors	22.02 ± 4.64	31.35 ± 7.30	0.021*
Unexplained Factors	-	35.15 ± 6.36	-
Total	24.71 ± 9.16	30.86 ± 7.97	0.046*
P value among groups	0.402	0.675	

The best cut off point of F.MDA associated with pregnancy was 3.49 µM which detected from ROC curve which represent point with best sensitivity and specificity as shown in figure 1-a.

While the best cut off point of FF.GSH associated with pregnancy was 26.90 µM which detected from ROC curve as shown in figure 1-b.



a **b**

MDA	Area	Cut off point µM	Sensitivity	Specificity	P value	Asymptotic 95% Confidence Interval	
						Lower Bound	Upper Bound
F.MDA	.686	3.49	64%	62%	.082	.494	.877
F.GSH	.697	26.90	73%	63%	.060	.497	.897

Figure (1): ROC curve analysis of follicular fluid Malondialdehyde and glutathione in predicting pregnancy outcome.

Relation of Oxidative Biomarker with ICSI Outcome

Correlation analyses between MDA and ICSI parameters showed that FF MDA level had a significant positive correlation with the PN ($p<0.05$) while FF GSH had insignificant positive correlation with all ICSI parameters ($p>0.05$) as shown in Table 2.

Table (2) : Relation of follicular fluid malondialdehyde and glutathione with ICSI outcome

ICSI Characteristics		FF.MDA		FF.GSH	
		r	p	r	p
No. of retrieved oocytes	GV	.217	.259	.138	.453
	MI	.124	.521	.085	.645
	MII	0.074	0.682	.245	.138
	Total	0.076	0.718	.135	.470
PN		.326*	.040	.105	.529
Fertilization rate (%)		.247	.124	0.1	.408
Cleavage rate		.024	.892	.083	.742
Total embryo		.107	.547	.072	.673
Embryo grading	Grad 1	.249	.155	.030	.862
	Grad2	0.048	.794	.025	.886
	Grad 3	-.249-	.170	.046	.791
	Grad4	-.339-	.058	.066	.701

r: Correlation coefficient; p: Significance

To examine the relationship of measured factors with the positive pregnancy, a binary logistic regression analysis was performed.

In this analysis, FF.GSH was significantly associated with the positive pregnancy. Increased FF.GSH were associated with decreased odds ratio for the positive pregnancy (OR = 0.891, [0.803-0.988] Table (3).While Increased FF MDA was associated with increased odds ratio for the positive pregnancy (OR = 1.471, [0.928-2.330].

Table (3) Binary logistic regression analysis for positive pregnancy as the dependent variable

OS Parameters		P value	Odds Ratio	95% Confidence Interval for Odds Ratio	
				Lower Bound	Upper Bound
Positive	F.MDA	.100	1.471	.928	2.330
Pregnanc	F.GSH	.029*	0.891	.803	.988

Discussion

FF.MDA was significantly higher in pregnant women when compared with non pregnant women ($P<0.05$) as shown in (Table 1); and its levels was insignificantly higher in pregnant women when compared with non pregnant women in respect to infertility cause ($P>0.05$). This result agrees with Appasamy et al. 2008.

In a study by Appasamy et al., it was found that follicular fluid ROS levels had a positive correlation with the pregnancy rate in IVF patients [14]. This observation made researchers think that a restricted amount of oxidative stress may be essential for embryonic development since it is an indicator of metabolic activity.

Yalçınkaya et al., 2013, found that the follicular fluid MDA levels in pregnant women were significantly higher than those in non-pregnant women. This might be explained by the weak positive correlation of MDA with the number of grade I embryos and total number of embryo and PN, which are important predictors of successful IVF outcome [15].

In contrast, Borowiecka et al., who studied the role of proteins carbonyls in FF on IVF outcome and found that non-pregnant women had twice the amount compared to pregnant females [16].

FF.GSH levels was significantly lower in pregnant women when compared with non pregnant women ($P<0.05$) Also its levels was significantly lower in pregnant women with female factor infertility when compared with non pregnant women ($P<0.05$) as shown in Table 2. This result agrees with Gupta, et al., who showed that a higher amount of oxidative stress during ovarian stimulation associated with a concomitant reduction in antioxidant proteins [17].

Gowda et al., demonstrate that the increase in FF ROS is overcome by subsequent increase in antioxidants (GSH) as an adjustment to overcome the damage caused and signify a potent antioxidant environment in and around developing follicle thus decreasing their amount [18].

In contrast to Yalçınkaya et al., who found that there was no significant difference between pregnant and non-pregnant women in terms of follicular fluid GSH levels. This result may reveal the inefficiency of relatively low GSH levels in follicular fluid as an antioxidant compared to intracellular levels [15].

Yalçınkaya et al. 2013, found that ROC curve analysis indicated that the follicular fluid MDA concentration is a good marker for pregnancy with an AUC of 0.746 (it showed 60% sensitivity and 64% specificity.

Oral, et al., found that the most helpful parameters for predicting the probability to conceive using follicular MDA levels, age, 3rd day FSH levels, E2 levels on HCG administration day was follicular fluid MDA levels. The cut-off value for follicular fluid MDA was 5.25 µmol/L, it showed 60% sensitivity and 64% specificity (AUC: Area Under Curve = 0.673) [19].

It is suggested that a certain threshold level of ROS in FF is essential for several reproductive events and may be necessary for a better reproductive outcome in IVF/ICSI cycles [20].

While some studies suggest optimum level of follicular fluid ROS to be a marker for predicting success in IVF cycles [21].

While the best cut off point of F.GSH associated with pregnancy was 26.90 µM which detected from ROC curve as shown in figure 1-b.

Yalçınkaya et al., found that ROC curve analysis indicated that the follicular fluid glutathione concentration showed less sensitivity in estimating pregnancy status [15].

The free radical activity is also associated with parameters of ovarian responsiveness and *in vitro* fertilization (IVF) outcome [22].

Oral, et al. found positive insignificant association between follicular fluid malondialdehyde levels and fertilization rates [19]. In contrast to Das, et al. indicated that high FF ROS levels tend to decrease the fertilization potential of oocytes; besides, a higher percentage of good quality embryos was observed corresponding to ROS levels <100 cps[23].

Song et al., showed protein OS is present in FF of women on IVF-ET and proved that high level of oxidation protein products have adverse effects on the outcome of IVF-ET[24]. Fujimoto et al. stated that lipid perioxidation products and antioxidant enzyme activities in FF were not associated with the quality of embryos [25].

Pasqualotto, 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 [26]. Yalçınkaya et al., found that no significant association was found between follicular fluid GSH levels and fertilization rate and embryo quality [15]. So certain levels of ROS are required for normal fertilization and a fine balance between ROS and antioxidants for

successful reproductive outcome is fundamental both in vitro and *in vivo*.

In our study, patients who became pregnant had higher levels of MDA than those who did not. Therefore, we conclude that elevated levels of MDA in the follicular fluid do not impair oocyte and embryo development and quality and may reflect the metabolically active ovaries.

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