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Clinical Significance of Assessment of Spermatozoal Creatine Kinase (CK) Enzyme Activity as an Indicator of Spermatozoal Oxidative Stress (OS) on Teratozoospermiac In Infertile Patients After Use of Simvastatin as Antioxidant Drug

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ABSTRACT

Despite the presence of numerous tests of sperm quality and function, no single laboratory test can determine with accuracy and precision whether a man is fertile or not. It is important to evaluate the oxidative stress (OS) in the male and female reproductive tract, especially because the results have diagnostic and prognostic value in the management of infertility. Out of total number, the present study is carried out on a total of (55) patient with primary male factor infertility in Maternity and Childhood Teaching Hospital in Najaf Province and Cancer Research Unit in Kufa Medical College between April 2004-June 2005. The distribution of 55 patients in group I were classified into 8 subgroups according to pathological cause of infertility:

1. Asthenozoospermia (A),n=7
2. Asthenonecrozoospermia (AN),n=5
3. Asthenoteratozoospermia(AT),n=6
4. Asthenoteratonecrozoospermia(ATN),n=5
5. Oligoasthenoteratozoospermia (OAT).n= 8
6. Oligoasthenotetronecrozoospermia (OATN),n=9
7. Oligoasthenozoospermia (OA),n= 7
8. Teratozoospermia (T),n=8

And 35 normal proven fertility volunteer men served as a control group II. All patients were given simvastatin (simlo) tablets Ipca, Ipca laboratories Ltd Mubai: India at dose 20 mg twice daily for a period of 3 months. Seminal creatine kinase (CK) was assessed before and after treatment. The results showed that highly significant decrease ($p<0.05$) of CK activity are shown in A , AT ,OAT and T while, the most influenced subgroup in the decrease of CK was T subgroup that showed about (2.5) fold lower than similar pretreated subgroup patients . The study suggests that simvastitin might be use as antioxidant treatment and CK activity assessment could be use as an indirect index of oxidative stress status of infertile patients.

Keywords: oxidative stress, spermatozoa, antioxidant, simvastatin, creatine kinase.

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INTRODUCTION

Infertility is common in couples of childbearing age, approximately half of these cases of infertility are caused by a male factor [1]. An important factor contributing to the development of sperm abnormalities appear to be the disruption of spermatogenesis, leading to the release of excess residual cytoplasm by the differentiating spermatozoa [2].

Creatine Kinase (CK) (E.C.2.7.3.2) catalyses the reversible phosphorylation of their ADP to ATP or creatine to creatine phosphate, thus maintaining an immediately accessible energy reservoir in the cell and cells requiring high energy such as spermatozoa are characterized by high creatine kinase activity [3]. A study by Dandikar [4] reported that the presence of isoform CK-MB was found in human sperm samples and was measured spectrophotometrically and presence of an isoenzyme of CK-MB was also observed in the study and measured spectrophotometrically at 340 nm based on the principle on that CK- MB being composed of two moieties CK-MB and CK-BB in human spermatozooa CK-BB is located in mitochondria of midpiece region by direct immunoglobin staining and CK-BB isoenzyme is found in seminal plasma [5, 6]. Azoospermic ejaculates showed presence of CK-BB while the greater portion of isoenzyme separable of isoenzyme CK-MB was observed and also measured in normal ,oligospermia and azoospermia samples and was found to be highest in azoospermic patients [7]. Semen with low sperm counts showed higher CK detecting sperm quality of sperm men [8]. However, creatine kinase levels predict sperm quality better than conventional semen analysis [9,10].

A number of independent studies have indicated that defective sperm function is associated with elevated levels of certain key enzymes such as creatine kinase CK [11-13). Eventhough, these enzymes are not directly responsible for loss of sperm function, they act as a biochemical markers of sperm differentiation, there by reflecting the presence of exfoliated, precursor germ cells and retention of excess residual cytoplasm during the final stages of spermatogenesis [14]). Creatin Kinase levels in human sperm are an objective biochemical marker of sperm maturity and fertilizing potential [15]. Immuno cytochemical studies of CK levels in individual spermatozooa have demonstrated that increased CK concentrations reflect residual cytoplasm in sperm that was extruded during late spermatogenesis and an interest in CK has been stimulated by studies suggesting that defective sperm function is associated with defects in spermatogeresis that leads to the release of immature spermatozoa from the germinal epithelium [16].

Oxidative Stress (OS) is a condition associated with an increased level of cellular damage induced by oxygen [17] and oxygen – derived oxidants commonly, known as reactive oxygen species (ROS) have been implicated in over a hundred of disease states which range from arthritis and connective tissue disorders to carcinogenesis , aging , toxin exposure, physical injury infection and acquired immune deficiency syndrome, the role of oxidative stress in infertility and methods for counteracting its impact on reproductive tissue with antioxidant are still in it's infancy [18]. A study mentioned that oxidative stress (OS) arises as a consequence of excessive production of ROS and impaired antioxidative defective mechanisms [19] and also proposed that OS precipitates the range of pathologies that currently are thought to afflict the reproductive function [20]. Spermatozoa are particularly susceptible to damage induced by excessive ROS because their plasma membranes contain large quantities of polyunsaturated fatty acids and their cytoplasm contain low concentration of scavenging enzymes [21].

Infertility and sexual dysfunction indicate that seminal oxidative stress tests has diagnostic and prognostic capabilities beyond these of conventional tests of sperm quality or dysfunction and OS can accurately discriminate between fertile and infertile men and identifying patients with a clinical diagnosis of male factor infertility who are likely to initiate patients with a clinical diagnosis of male infertility in which OS is a significant factor and who may benefit from antioxidant supplementation in corporation of such tests into routine andrology laboratory practice may be of particular importance to the future management of male infertility [22].

Simvastatin is an 3 hydroxy 3 methyl –glutayl, coenzyme 3 (HMG-CoA) reductase competitive inhibitor that is derived synthetically from fermentation of *aspergilus terreus*, it exerts a hypocholesferolemic action by stimulating an increase in LDL receptors on hepatocyte membrane thereby increasing the clearance of LDL from circulation. Recent evidence suggests that the beneficial effects of HMG CoA reductase inhibitors on endothelium function and cardiovascular ischemic events may be attributed not only to their lipid lowring effect but also to cholesterol lowering independent (direct) effect on atheroseclerotic vessel wall and this

indicates that simvastatin treatment preserved the endothelial function associated with decrease in markers of oxidative stress, these beneficial endothelial effects of simvastatin are likely to occur independently of plasma lipid concentratims and to be mediated by its antioxidant action [23]. Similar adoption had been proposed and applied in the present study, that simvastatin might serve with antioxidant properties in the field of male infertility in preserving sperm function parameters, this study was designed with following aims: (1) To identify the clinical significance of simvastatin as antioxidant drug on spermatozoal oxidative stress in the field of male infertility (2) to establish an assay for accurate and reliable assessement of spermatozoal oxidative stress.

PATIENTS AND METHODS

Data collection: The present study was carried out on a total of 243 patients with primary male factor infertility (barren marriage for more than one year). Infertile patient were selected after their referral to the Infertility Unit at Maternity and Childhood Teaching Hospital in Najaf Province at the period between April 2004 and June 2005, and Cancer Research Unit in Kufa Medical College. Out of the total number of patients, only 55 patients are submitted for this study and 35 normal proven fertility volunteer men served as control group for comparison purpose.

Selection of study patients: The referred infertile patients with proven abnormal sperm parameters (motility, morphology and dead-live%) are exclusively registered in this study on the basis of their spermiogram disruptive spermatogenesis and with normal sexual function while patients with infertility that will interfere with infertility-related origin excluded from the study viz: hypopitutarism hypogonadism, diabetes mellitus, testicular varicocale, venereal disease, leukocytopermia, other allied exclusions that interfere with fertility were also obtained by history namely: drug and hormonal therapy, heavy smoking and heavy drinking, below and beyond age group 20-50 year and any patient with erection dysfunction, impotence and who had difficulties in semen collection by masturbation or coitus interrupts. All patients participating in the study were accept with verbal consent, and patients included in treated group were given simvastation a(simlo) tables, Ipca, Ipca laboratories ltd, Mumbai, India at dose 20mg twice daily for a period of 3months. All assays were carried out before giving any treatment and reviewed after termination of the period of 3months.

Design of study: The distributions of 90 men in the study are fallen into 2 main groups, the first group composed of:

55 patients were classified into 8 subgroups according to the pathological cause of infertility of sperm parameter, this had been considered in regard to scores of WHO criteria [6].

1. Asthenozoospermia (A),n=7
2. Asthenonecrozoospermia (AN),n=5
3. Asthenoteratozoospermia(AT),n=6
4. Asthenoteratonecrozoospermia(ATN),n=5
5. Oligoasthenoteratozoospermia (OAT),n= 8
6. Oligoasthenotetronecrozoospermia (OATN),n=9
7. Oligoasthenozoospermia (OA),n= 7
8. Teratozoospermia (T),n=8

The second group (control) consists of 35 healthy normal with proven fertility volunteers (donors) initiated a successful pregnancy within the last 12 month.

Semen Collection: Samples of ejaculates were collected from married patients by masturbation technique or coitus interrupts after3-5 days of sexual abstinence [22]. Ejaculate samples were collected in clean transparent plastic cups with wide opening and precise sealing after ejaculation, the specimen was placed in an incubator at 37C^{o} for 30 min to allow liquefaction. The specimen was examined according to Zaneveld and Polakoski techniques [(22)] and seminal leukocytes counts by positive myeloperoxidase staining (Endtz test) [23].

Sperm preparation: All masturbated semen samples liquefied after 30 minutes at room temperature, spermatozoa were separated from seminal plasma by centrifugation at 500x rpm for 30 minutes. The supernatant was precisely measured by a graduated centrifuge test tube and discarded. Homogenized buffer

consisted of (11.9 gms of menthol, 4.8 gms of sucrose, 0.09 gms of EDTA in 250 ml of distilled water adjusted the pH to 7.4 with tris-base). Homogenized buffer was kept in refrigerator at 4c°. The samples were hand homogenized and subsequently centrifuged for 10 minutes at 3000 rpm. Cooled, 0.9 ml of triton x-100 (0.1 %) was added to each 0.1 ml of pallets obtained from the sample. The samples were centrifuged again at same rpm for half an hour in a centrifuge; the supernatant was used for CK measurements [24].

Randox creatine kinase (CK) with catalogue no:CK 1673, randox laboratories ltd, Ardmore, Co, antrim United Kingdom enzymatic colorimetric method was applied to assay CK with spectrophotometer cecil-1011 England .

Principle

Creatine Kinase utilizes Creatine phosphate as substrate to act as the initial catalyst for a series of reactions resulting in the formation of NADPH as outline in the coupled enzyme assay. NADPH producted, is proportional to CK activity and is used to reduce nitroblue tetrazolium (NBT), in the presence of diaphorease, to give the blue violet color of diformazan which has an absorption maximum around 560nm.

Procedure

Wave length 560nm
Cuvatte 1 cm path length
Temperature 37c°
Measurement: against reagent blank

	Sample	Standard	Reagent blank	Sample blank
Color Reagent	0.5 mL	0.5 mL	0.5 mL	0.5 mL
Incubate for 30 minute at 37c°				
Incubate for 10 minute at 37c°				
0.1N HCL	0.5 mL	0.5 mL	0.5 mL	0.5 mL

Seminal plasma

The absorbance of the sample (A sample), standard (A standard) and sample blank (A sample standard) are measured against the reagent blank at 500 Mn.

Calculation

Creatine activity in sample $\left[UL = \frac{\text{A Samle}}{\text{A standard}} \text{ x Standard Value} \right]$.

In this study, all enzymetic assays and values including CK values were assessed and converted form U/L as fixed on kit instructions to the wanted values U/10^8 sperm to be compared to international values.

Data were analyzed using inbuilt function within the statistical SPSS UK Serrey UK. Least significant difference (LSD) had been applied for difference between mean at level of significance 0.05 considered statistically significant.

RESULTS

Table (1) depicted clearly the highest means of abnormal sperm morphology % that high statistically significant decrease ($p<0.05$) in AT, OATN and T subgroups the results were (48.45±1.67, 65.00±5.00 and 52.71±2.68 Vs 32.22±1.28). Whereas, A, ATN and OA subgroups show significant values (39.00± 2.56, 43.50 ±4.69 and 50.00±10.00 Vs 32.22±1.28) insignificant statistical results ($p>0.05$) are showed in AN and OAT subgroups (39.00±11.00 and 46.57±3.06) respectively. The high significance value are attributed to simvastatin treatment while, the cause of insignificance is due to effectiveness of simvastatin treatment that cause a raise in values in comparison with control value. It is important to point out that the pathological levels of ROS

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caused by morphologically abnormal sperm that appears to be the disruption of spermatogenesis leading to decrease in the release of excess residual cytoplasm (cytoplasmic mid-piece surplus) are caused by ROS.

Table (2) demonstrated the high statistically significant differences ($p<0.05$) of Creatine Kinase fall in A, AT and T subgroups. While the subgroups that did not show significant difference ($p>0.05$) fall in AN, ATN, OATN and OA the post- treated value is 2.5 fold lower than pre treated value in T subgroup.

Table (3) presented the results of CK that showed a high statistically significant result ($p<0.05$) in all subgroups and without any exception, mean value of CK level were less than mean value in T, A and OA subgroups respectively.

Table 1: Mean of sperm abnormal morphology % of infertile patients after Simvastatin 40mg daily for 3 months treatment with control group of normal fertile volunteers (n=35) versus each pathological subgroup. Data are presented as mean ±(SEM).

Pathological subgroups (n=55)	Post Simvastatin treatment		P value
	mean	±SEM	
Asthenozoospermia (n=7)	39.00	±2.56	0.01*
Asthenonecrozoospermia (n=5)	39.00	±11.00	0.10
Asthenoteratozoospermia (n=6)	48.45	±1.67	0.00**
Asthenoteratonecrozoospermia(n=5)	43.50	±4.69	0.01*
Oligoasthenoteratozoospermia (n=8)	46.57	±3.06	0.01*
Oligoasthenoteratonecrozoospermia (n=9)	65.00	±5.00	0.00**
Oligoasthenozoospermia (n=7)	50.00	±10.00	0.01*
Teratozoospermia (n=8)	52.71	±2.68	0.00**
Control group (n=35)	32.22	±1.28	

** Highly significant value P < 0.05 when value 0.00 (2-tailed)
* Significant value less than P < 0.05
Insignificant value P > 0.05

Table 2: Effect of simvastatin (40mg daily for 3 months) on CK ($U/10^8$) in infertile patients in each pathological subgroup (n=55). Data are presented as mean ±(SEM).

Pathological subgroups (n=55)	CK $U/10^8$ sperm		
	Pre	Post	P value
Asthenozoospermia (n=7)	8.46 ±0.23	4.36 0.46	0.00**
Asthenonecrozoospermia (n=5)	8.41 ±0.59	4.43 ±1.79	0.34
Asthenoteratozoospermia (n=6)	9.05 ±0.21	5.25 ±0.30	0.00**
Asthenoteratonecrozoospermia (n=5)	8.24 ±0.26	5.66 ±0.80	0.65
Oligoasthenoteratozoospermia (n=8)	8.95 ±0.27	5.85 ±0.42	0.00**
Oligoasthenoteratonecrozoospermia (n=9)	9.03 ±0.30	4.81 ±0.89	0.17
Oligoasthenozoospermia (n=7)	8.21 $\pm 9.50 \times 10^{-2}$	6.89 ±0.91	0.41
Teratozoospermia (n=8)	8.41 ±0.48	3.66 ±0.72	0.00**

** Highly significant value P < 0.05 when value 0.00 (2-tailed)
* Significant value less than P < 0.05
Insignificant value P > 0.05

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Table 3: Mean of Creatine Kinase U/10^8 of infertile patients after Simvastatin 40mg daily for 3 months treatment with control group of normal fertile volunteers (n=35) versus each pathological subgroup. Data are presented as mean ±(SEM).

Pathological subgroups (n=55)	Post Simvastatin treatment		P value
	mean	±SE	
Asthenozoospermia (n=7)	4.36	±0.46	0.00**
Asthenonecrozoospermia (n=5)	4.43	±1.79	0.00**
Asthenoteratozoospermia (n=6)	5.25	±0.30	0.00**
Asthenoteratonecrozoospermia(n=5)	5.66	±0.80	0.00**
Oligoasthenoteratozoospermia (n=8)	5.85	±0.42	0.00**
Oligoasthenoteratonecrozoospermia (n=9)	4.81	±0.89	0.00**
Oligoasthenozoospermia (n=7)	6.89	±0.91	0.00**
Teratozoospermia (n=8)	3.66	±0.72	0.00**
Control group (n=35)	0.61	±5.71 x10^{-2}	

** Highly significant value P < 0.05 when value 0.00 (2-tailed)
* Significant value less than P < 0.05
Insignificant value P > 0.05

DISCUSSION

The results of simvastatin on sperm abnormal morphology depicted a shiny picture since most sperm pathological subgroups are either highly significant or significantly decreased (p<0.05) table (1). In our belief, that are many contributing factors might be collectively contribute this result [1] simvastatin decrease the process that results in decrease reactive oxygen species production [2]. Decrease in abnormal sperm morphology as a source of reactive oxygen species production as well, from this point of view our results are supported by other studies that mentioned increase in ROS production result in an increase of morphologically abnormal morphology (cytoplasmic surplus) [22, 26] however, our study suggested such contribution of variables with co-existence of this relationship be attributed to simvastatin treatment.

The relationship between abnormal sperm forms and significant decrease of creatine kinase are shown in tables (2 and 3).

The present study displayed that this relation will initiate a key that creates and opens an interest in the study of creatine kinase. Several studies suggesting that defective sperm function is associated with defects in spermiogenesis that induce release of immature spermatozoa from germinal epithelium which are regarded as a source of ROS generation [11,14,16, 26- 29].

In our opinion, that the action of simvastatin results in decrease abnormal sperm morphology that create reactive oxygen species [30]. This may give antioxidant activity causing a significant reduction in CK activity that is suggested in the present study as an indirect index of oxidative stress status of spermatozooa and this might participate in dual purpose first to minimize spermatozoal oxidative insult second: to reinforce and discriminate our strategy in management of infertile patients particularly in field of infertility due to spermatozoal oxidative stress.

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