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Relationship Between Creatine Kinase Activity and Semen Characteristics in Subfertile Men

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ABSTRACT: Objective—Creatine kinase is an indicator of sperm maturity. We studied whether sperm creatine kinase levels differ between normal healthy donors and subfertile patients and determined the correlation between sperm creatine kinase level and semen quality in subfertile men. **Material and Methods**—Semen samples were obtained from 76 subfertile and 15 healthy normal donors after 48 to 72 hours of sexual abstinence. Sperm characteristics were assessed with a computer-assisted semen analyzer. Morphology was evaluated by Kruger's strict criteria and World Health Organization methods. The thiobarbituric acid assay was used to measure lipid peroxidation; sperm creatine kinase activity was measured using a commercial kit after detergent extraction (Triton X-100). **Results**—Creatine kinase levels were significantly higher ($P < .001$) in subfertile men (median = 0.197 U/10^8 sperm) compared with donors (median = 0.061 U/10^8 sperm). In subfertile men, creatine kinase levels correlated significantly with lipid peroxidation levels ($r = .49$; $P = 0.03$) and sperm concentration ($r = -.70$; $P < .001$), and with normal sperm forms by Kruger's ($r = -.30$; $P = 0.01$) and WHO methods ($r = -.32$; $P < .005$). Creatine kinase levels and spermatozoal characteristics did not correlate significantly in donors. Compared with subfertile normospermic men, creatine kinase activity was significantly higher in oligospermic and asthenospermic men ($P < .001$). **Conclusions**—The inverse relationship between creatine kinase level and sperm concentration and morphological forms suggests that creatine kinase levels can be a reliable marker for semen quality in subfertile men. An elevated creatine kinase level and its correlation with lipid peroxidation levels may reflect biochemically immature spermatozoa. *Int J Fertil* 43(4):192–197, 1998.

KEY WORDS: creatine kinase, lipid peroxidation, sperm, sperm morphology, subfertile men

INTRODUCTION

INFERTILITY IS A SIGNIFICANT PROBLEM for couples of childbearing age. In the United States, one of every six couples of childbearing age may be infertile [1]. About half of these cases of infertility are caused by a male factor [2]. Routine semen analysis, which assesses semen volume, sperm concentration, percentage motile sperm, and abnormal sperm morphology, has limited value in predicting pregnancy [3,4]. Diagnosing defective sperm function is difficult by routine semen analysis because the spermatozoon is a highly specialized cell that expresses a diverse array of biological properties to achieve fertilization. Characterizing the abnormalities and quality of dysfunctional human spermatozoa, therefore, may require alternative biochemical methods, such

as assays for lipid peroxidation, acrosin, hyaluronidase, and creatine kinase [5–7].

Creatine kinase is involved in the synthesis and utilization of energy in sperm [8]. This enzyme does not play any direct role in the process of fertilization. However, despite identical sperm concentration, fertile oligospermic men have lower sperm creatine kinase activity than infertile men [9]. In normal spermatogenesis, spermatozoa shed a majority of their residual cytoplasm as they mature. An elevated creatine kinase level is associated with excessive residual cytoplasm. High creatine kinase levels, unlike sperm concentration, correlate inversely with the fertilizing potential of spermatozoa and indicate the degree of cellular immaturity [10,11]. Another indicator of sperm quality and maturity is the level of lipid peroxidation [11,12]. Human spermatozoa are particularly susceptible to lipid peroxidation, since the cellular and intracellular sperm membranes are rich in polyunsaturated fatty acids; peroxidation can cause irreversible loss of sperm motility [11,13].

The purpose of this study was to determine (1) the difference between sperm creatine kinase levels in normal healthy donors and subfertile patients, and (2) whether the sperm creatine kinase levels correlate with semen quality and lipid peroxidation levels in subfertile men.

MATERIALS AND METHODS

Selection of Subjects

This study was approved by our institutional review board. Seventy-six subfertile men who were referred to our andrology laboratory for evaluation of their semen specimens were included. Healthy normal donors (n = 15) were selected on the basis of a normal semen analysis [14]. Subfertile patients were classified according to sperm concentration as oligospermic (sperm count of less than 20×10^6/mL), or normospermic (sperm count of at least 20×10^6/mL), and by percent motility as asthenospermic (motility of less than 50%) or having normal motility (motility of at least 50%) by WHO criteria [14].

Assessment of Semen Characteristics; Semen Analysis

Semen specimens were collected on site by masturbation into a sterile cup after 48 to 72 hours of sexual abstinence. Specimens were allowed to liquefy at 37°C for 30 minutes before sperm concentration and other semen characteristics were evaluated. The specimens were analyzed on a computer-assisted semen analyzer (CASA: Motion Analysis; Cell-Trak, Model VP110, version 4.22B, Santa Rosa, CA). For each measurement, a 5-μL aliquot was loaded on a 20-μm counting chamber (Micro-Cell, Conception Technologies, Inc., La Jolla, CA) and then analyzed for sperm count and motility. Sperm count and motility were verified manually under an Olympus BH2-S microscope (Olympus; Tokyo, Japan) with a 20x positive phase-contrast objective. Air-dried semen smears were stained with Giemsa stain (Diff Quik, Allegiance Healthcare Corporation, McGraw Park, IL), and scored for sperm abnormalities by both Kruger's strict criteria and World Health Organization (WHO) methods. Midpiece abnormalities included percentage of spermatozoa with swollen midpiece; tail abnormalities included bent and coiled tail [14].

Creatine Kinase Estimation

Creatine kinase was estimated using the procedure described by Huszar and Vigue [2]. An aliquot of the ejaculate was transferred to a 15-mL polystyrene centrifuge tube. Seminal plasma was removed by washing with ice-cold imidazole buffer (0.15 M NaC1 and 0.03 M imidazole at pH 7.0) at a ratio of 1:15 (vol/vol). The supernatant was decanted after centrifugation at 500 g, and the pellet was resuspended in a 0.1% of Triton X-100 detergent solution by vortexing for 20 seconds. The sample was centrifuged again at 500 g, and the supernatant was analyzed for creatine kinase activity using a spectrophotometric method (Sigma Chemical Co., St. Louis, MO). The creatine kinase activity was expressed as units/10^8 spermatozoa.

Lipid Peroxidation Measurement

Lipid peroxidation levels were measured using the thiobarbituric acid method [15]. The washed sperm suspension was incubated with 0.25 mL each of ferrous sulfate (2.5 mM, Sigma) and sodium ascorbate (12.5 mM, Sigma) for one hour in a 37°C water bath. Ferrous sulfate and sodium ascorbate were added as a promoter. Control tubes contained only sperm suspension, and no promoter was added. A

sperm aliquot was added to the control tubes after the incubation period.

To precipitate the proteins after incubation, 500 µL 40% trichloroacetic acid (Sigma) was added. The sample was centrifuged at 500 *g* for 12 minutes; 1 mL of clear supernatant was collected, and 500 µL 2% thiobarbituric acid (Sigma) in 0.2 N NaOH (Sigma) was added to the supernatant. The test tubes were boiled at 100°C for exactly 10 minutes. The samples were cooled in crushed ice and the optical density measured at 534 nm with an Ultrospec III spectrophotometer (Pharmacia Biotech Inc., Piscataway, NJ). To determine the peroxidation level, the malonaldehyde level was calculated from the optical density produced by malonaldehyde standard (malonaldehyde bis-dimethyl acetal, Sigma). The lipid peroxidation level was expressed as nmol malonaldehyde/10^8 sperm/hour.

Statistical Analysis

The results were not normally distributed, and, therefore, a nonparametric test, the Kruskall-Wallis test, was used to compare the creatine kinase values in semen from subfertile patients and normal donors. A P value of <.05 was considered as statistically significant. The Wilcoxon rank-sum test with the Boneferroni correction was used for pairwise comparisons. All results were expressed as median and interquartile range. Spearman's rank correlation was used to determine whether the creatine kinase level correlated significantly with concentration, percentage motility, morphology, and lipid peroxidation. A multiple regression analysis was performed using logarithmically transformed data for creatine kinase activity as the dependent variable and sperm characteristics (such as volume, concentration, motility, curvilinear velocity, linearity, amplitude of lateral head movement, sperm morphology by WHO and Kruger's criteria, and abstinence time) as independent variables. Peroxidation level was not considered on account of a large number of missing values.

The multiple regression analysis used a forward stepwise selection procedure with a P value of 0.15 to enter and 0.05 to remain in the model. We used regression analysis to evaluate the relationship of one or more independent variables (semen characteristics) to a single continuous dependent variable (log creatine kinase), with the purpose of determin-

TABLE I
Comparison of creatine kinase activity in various clinical subgroups.

Group	*Median and Interquartile Range*	*P Value*		
		*Kruskall-Wallis Test**	*Wilcoxon Rank-Sum†*	*Compared to Donors‡*
Normal donors (n = 15)	0.061 (0.056–0.760)	—	—	—
Subfertile men (n = 76)	0.197 (0.080–0.540)	—	—	<.001
Subfertile men				
Normospermic (n = 44)	0.113 (0.061–0.225)	<.001	<.001	0.03
Oligospermic (n = 32)	0.520 (0.225–1.213)			<.001
Subfertile men				
Normal motility (n = 26)	0.073 (0.58–0.208)	<.001	<.001	0.12
Asthenospermic (n = 50)	0.297 (0.137–0.802)			<.001

*Kruskall-Wallis test was used to test the overall group effect; P <.05 considered significant.
†Wilcoxon rank-sum test used for pairwise comparisons between subfertile subgroups with Boneferroni correction; P <.017 was considered significant for all pairwise comparisons.
‡Pairwise comparisons between subfertile subgroups and normal donors.

ing which of several independent variables are important for predicting a dependent variable (log creatine kinase). We also used it to characterize the relationship between the dependent and independent variables to determine the extent, direction, and strength of the association among them. The SAS statistical software package (SAS Institute, Inc., Cary, NC) was used for the data analysis.

RESULTS

Of the subfertile men, 32 were oligospermic; 44 were normospermic; 50 were asthenospermic; and 26 had normal sperm motility (Table I). Significantly higher creatine kinase levels were observed in the subfertile men (0.197 U/10^8 spermatozoa; interquartile range 0.080–0.540) as compared to the normal healthy donors (0.061 U/10^8 spermatozoa; interquartile range 0.056–0.760; P <.001). Similarly, lipid peroxidation levels were significantly higher in subfertile men (28.4 nmol/10^8 sperm/hour; interquartile range 20.9–48.9) than donors (22.6 nmol/10^8 sperm/hour; interquartile range 17.3–25.3; P = 0.03) (Table II). Subgroup analysis of the creatine kinase levels in subfertile men revealed that oligospermic patients had significantly higher creatine kinase levels than did normospermic patients and normal donors (P <.001). Asthenospermic patients had significantly higher creatine kinase levels compared to normal healthy donors and subfertile men with normal motility (P <.001) (Table I).

In subfertile men, creatine kinase activity showed a significant positive correlation with sperm concentration, percentage motile sperm, and lipid peroxidation levels and correlated negatively with normal sperm morphology as determined by both Kruger's and WHO methods, and with mid-piece defects and abnormal tail forms by the WHO method (Table II). In contrast, the spermatozoal characteristics did not correlate with creatine kinase activity in normal donors. A significant association was observed between creatine kinase level and sperm concentration, curvilinear velocity, abnormal tail forms, and normal morphological forms by the multiple regression model (Table III). Creatine kinase activity correlated with sperm concentration (r = –.42; P <.001), mid-piece defects (r = –.40; P <.009), and abnormal tails (r = .44; P <.001).

DISCUSSION

During the final stages of sperm differentiation, spermatozoa undergo a remarkable transformation whereby they lose the cytoplasmic component of the cell during the release of the mature spermatid from the Sertoli cell. Following spermiation, any residual cytoplasm that is associated with the spermatozoa is retained in the mid-piece region as an

TABLE II
Correlation between levels of creatine kinase and sperm characteristics in subfertile men and normal donors.

	Subfertile Men		*Normal Donors*	
	Spearman Correlation		*Spearman Correlation*	
Characteristics	*Coefficient*	P *Value**	*Coefficient*	P *Value**
Sperm concentration	–0.70	0.0001	–0.13	0.65
Motility	–0.47	0.0001	0.15	0.61
Lipid peroxidation	0.49	0.03	0.45	0.11
Sperm morphology:				
Normal forms: Kruger's	–0.30	0.01	0.10	0.77
Normal forms: WHO	–0.39	0.001	0.20	0.52
Mid-piece defect	–0.40	0.0009	—	—
Tail defect				
Coiled	0.32	0.005	–0.06	0.85
Bent	0.08	0.52	—	—

*P <.05 considered significant.
WHO = World Health Organization.

TABLE III
Summarized mutliple regression model for log sperm creatine kinase activity.*

Variable	*Regression Coefficient (R^2)*	*Standardized Regression Coefficient*	*Standard Error*	P *Value*
Intercept	–4.1	0.00	0.67	0.0001
Sperm concentration	–0.009	–0.42	0.0019	0.0001
Curvilinear velocity	0.023	0.24	0.0083	0.006
Normal sperm forms†	0.025	0.22	0.0099	0.01
Abnormal tail forms†	0.058	0.44	0.012	0.0001

*Included both donors and subfertile men (n = 86).
†WHO sperm morphology.
$R^2 = 0.45$.

irregular cytoplasmic mass. If this mass occupies more than one-third the sperm head, it is termed a *cytoplasmic droplet* [14]. We found that subfertile patients had higher creatine kinase activity than normal donors. In our study, creatine kinase activity was higher in oligospermic patients than in subfertile normospermic patients. The majority of normospermic but subfertile patients had normal creatine kinase activity, in agreement with previous findings [2]. The spermatozoa in this group may be considered to be biochemically mature and capable of fertilization, whereas the reverse may be true for oligospermic patients. Elevated levels of creatine kinase activity in a large number of oligospermic patients in our study may explain, in part, why some oligospermic men are able to fertilize, whereas some normospermic men cannot.

Our finding that sperm creatine kinase activity correlates with sperm characteristics in subfertile men further supports the earlier reports [11,16]. Immature sperm with incomplete cytoplasmic extrusion have lower fertilizing ability and high creatine kinase activity. We found that creatine kinase activity shows a significant positive correlation with sperm mid-piece defects and abnormal tail forms, and an inverse relationship with normal sperm forms in subfertile men. Stepwise multiple regression analysis indicated that creatine kinase activity was highly associated with mid-piece defect, abnormal tail forms, and sperm concentration in all subjects studied. A strong association was seen between the creatine kinase levels and mid-piece abnormalities, especially swelling of the mid-piece region [17]. Measurements of the mid-piece area by imaging of the NADH oxidoreductase activity show a high correlation with levels of creatine kinase and glucose 6-phosphate dehydrogenase (G6PDH). Both creatine kinase and G6PDH are biochemical markers of cytoplasmic space [17]. We suggest that excessive residual cytoplasm may be responsible for the loss of sperm function mediated by sperm plasma peroxidative damage [11,17]. This observation suggests that these sperm characteristics may be more representative of sperm maturation and therefore more likely to be affected if spermatogenesis is impaired.

Creatine kinase levels correlated significantly with spermatozoal lipid peroxidation in subfertile men (both creatine kinase and peroxidation levels were elevated). No such correlation was observed in normal donors (both creatine kinase and lipid peroxidation levels were low). These findings suggest that some level of creatine kinase activity and lipid peroxidation may be important for normal physiological function of sperm, such as capacitation and hyperactivation, but higher levels seen in subfertile men may adversely affect sperm function. Increased lipid peroxidation suggests oxidative stress or excessive free radical production and, along with the increased creatine kinase levels, may indicate that the sperm are biochemically immature [11,18]. In pathological situations, the presence of excessive residual cytoplasm increases the cytoplasmic enzyme G6PDH, which accelerates reactive oxygen species (ROS) production, with enhanced availability of NADPH [19,20]. The mid-piece swelling could be involved in the excessive ROS generation by the potential leakage of electrons from damaged

mitochondria [21]. Antioxidant supplementation may be beneficial in such cases.

In conclusion, the inverse relationship between creatine kinase levels and sperm quality as indicated by sperm concentration and percentage of morphological normal forms suggests that some part of spermatogenesis is inhibited in subfertile men. In subfertile men, significantly elevated levels of creatine kinase and its correlation with lipid peroxidation levels reflect a biochemical immaturity of spermatozoa. Thus, the creatine kinase level appears to be a reliable biochemical marker for assessing the quality of semen in men with unexplained infertility.

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