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# Isoenzyme Creatine Kinase Mi as a Possible Indicator of Spermatozoa Maturity

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## ABSTRACT

**INTRODUCTION**: The assessment of creatine kinase (CK) in human sperm cells is an unbiased indicator of sperm maturity and fertilization potential. Elevated CK values are associated with an increased number of functional abnormalities and increased cytoplasmic residues. The CK-Mi isoenzyme in human sperm is of mitochondrial origin. Increased spermatozoal CK activity is associated with high CK cell levels. The objective of the present study was to compare the biochemical marker CK with morphological changes of the head, neck, and tail. The authors also investigated the assessment of CK activity in human sperm to obtain an objective biochemical marker of sperm maturity and fertilization potential.

**METHODS**: The activity of CK was assessed for seminal plasma-deprived spermatozoa in 126 men. The participants were divided into 2 groups. Patients in group 1 (n = 64) had reduced spermatozoa count. They were subdivided into: group 1a (n = 28) patients with moderate oligospermic characteristics (spermatozoa count 5.1 - 20 x $10^6$/mL), and group 1b (n = 36) patients with severe oligospermic characteristics (spermatozoa count < 5 x $10^6$/mL). Group 2 (n = 62) was a comparison group of patients with normospermic characteristics (spermatozoa count > 20 x $10^6$/mL; motility > 0.30). Semen analysis was performed according to World Health Organization guidelines. The CK-Mi isoenzyme was separated from CK-B using DEAE Sephadex™ A-50 columns, and agarose gel electrophoresis was used for separating the CK-Mi isoenzyme. The total CK activity was assessed with a spectrophotometer.

**RESULTS**: CK was significantly higher in the group with severe oligospermia (mean [SD] = 1.9 [2.2] UI/$10^8$ sperm cells) than in the group with normospermia (0.097 [0.026] UI/$10^8$ sperm cells) ($P$ < .01). The group with oligospermia had a significantly lower CK-Mi/CK ratio (0.16 [0.10]) than the group with normospermia (0.36 [0.12]) ($P$ = < .01).

**DISCUSSION**: The concentration of CK and synthesis of CK-Mi isoforms reflect normal spermatogenesis and can be used for predicting human sperm maturity and fertilization potential. CK appears to be a sensitive indicator of spermatozoa quality and maturity for men with male-factor infertility.

**KEYWORDS**: Oligospermia; Normospermia; Creatine kinase; Isoenzyme CK-Mi

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**Abbreviations and Acronyms**
CK = creatine kinase
CK-Mi = CK of mitochondrial origin

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original study

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## INTRODUCTION

The ability of sperm to fertilize the egg is of exceptional importance in cases of inexplicable infertility in men, when clinical studies and clinical analyses of sperm do not show the presence of any disorder. Therefore, it is important to identify cell markers of sperm quality that, together with other diagnostic methods, can facilitate the identification of specific deficits associated with sperm function. One of those markers is the creatine kinase (CK) enzyme.

Spermatozoa are sperm cells that require high-energy supply (ie, CK enzymatic activity) due to their dynamic movements. CK is the key enzyme in energy synthesis and transport. The role of CK in mitochondria is that of the catalyser in the process of creatine phosphorylation to creatine phosphate [1,2]. The CK in the spermatozoa tail catalyzes rephosphorylation of adenosine diphosphate (ADP) into adenosine triphosphate (ATP).

Sperm CK is found in the form of 2 isoenzymes: CK-B and CK-M. The isoenzyme CK-M is also marked as CK-Mi because of its mitochondrial origin and because it differs from the serum CK-M that originates from the cytosol. This difference is observed in the electropherogram obtained by the separation of the CK isoenzyme in the agarose gel [3].

A previous study [4] showed a negative correlation between sperm cell count and CK activity; that is, the metabolic properties of sperm in patients with oligospermia differ from those in patients with normospermia. A cytochemical study [5] showed that the CK values were elevated for immature sperm in which the presence of cytoplasmic residue could be observed.

Normal, mature sperm cell forms have regularly shaped complete cytoplasmatic extrusion in the neck region and a properly developed tail. This form is correlated with low CK values. A new sperm CK isoform (CK-Mi) is identified in those sperm cells. It has electrophoretic characteristics similar to CK-M, with the CK-Mi and CK-B ratio that could be used for predicting the ratio of mature to immature sperm in the sample [6]. The predictive value of this ratio is based on the results of studies showing that immature spermatozoa with cytoplasmic residue are not involved in binding to the pellucid area of human oocytes [7]. With the loss of cytoplasmic mitochondrial residue in the sperm neck during spermatogenesis, the CK-Mi isoform is produced with simultaneous modeling of the spermatozoa plasma membrane and formation of the site for spermatozoa binding to the oocyt pellucid zone. Such changes can be recorded by the assessment of CK activity in cases with spermatogenetic disorders, shown in the occurrence of cytoplasmatic residues or in an increased number of pathological spermatozoa forms [8,9].

The objective of the present study was to compare the biochemical marker CK, the concentration of which is associated with the degree of spermatozoa maturity, with morphological changes of the head, neck, and tail. The authors also investigated the assessment of CK activity in human sperm to obtain an objective biochemical marker of sperm maturity and fertilization potential. If the raised CK values point to the presence of cytoplasmic residue that has not been lost in the late spermatogenesis period, the assessment of CK can be used for a late spermatogenesis marker.

## METHODS

### *Participants*

The activity of CK was assessed for seminal plasma-deprived spermatozoa in 126 men over a period of 20 months. The participants were divided into 2 groups, according to World Health Organization (WHO) guidelines [10]. Patients in group 1 (n = 64) had reduced spermatozoa count. They were subdivided into: group 1a (n = 28) patients with moderate oligospermic characteristics (spermatozoa count 5.1 - 20 x $10^6$/mL), and group 1b (n = 36) patients with severe oligospermic characteristics (spermatozoa count < 5 x $10^6$/mL). Group 2 (n = 62) was a comparison group of patients with normospermic characteristics (spermatozoa count > 20 x $10^6$/mL; motility > 0.30).

### *Procedures*

The analysis of the specimen was conducted according to WHO guidelines [10]. The test samples were collected through masturbation following sexual abstinence for 3 to 7 days. Spermatozoa characteristics of count, motility, and morphology were assessed 30 minutes after liquefaction at 37°C.

CK values were assessed in the seminal fluid and after spermatozoa preparation [11] using a kinetic test (CK test OSR 6279) by Olympus© (Center Valley, PA, USA). Spermatozoa were prepared by rinsing the ejaculated semen with three 250-µL aliquots of imidazole buffer (0.15 mol/L NaCl and 0.03 mol/L imidazole, pH 7.0) to remove the plasma. The precipitate on the last rinse was centrifuged to dryness and resuspended in 0.1% Triton™ X-100 (Dow Chemical, Midland, MI, USA) solution with mixing on the vortex for 20 seconds. The sample was recentrifuged and the CK activity was assessed using the supernatant. The result was expressed as IU/100 million sperm cells. The separation of the isoenzyme CK-Mi from the isoenzyme CK-B in the supernatant (obtained with the


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Table 1. Means and Standard Deviations (SD) for Number, Motility, and Morphology of the Spermatozoa (N = 126).
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| Variable | Normospermia (n = 62) | | Oliogospermia (n = 64) | | | |
|---|---|---|---|---|---|---|
| | | | Moderate (n = 28) | | Severe (n = 36) | |
| | Mean | SD | Mean | SD | Mean | SD |
| Sperm count (n x $10^9$/L) | 58.7 | 31.6 | 11.7 | 4.4 | 1.41 | 1.07 |
| Progressive motility | 0.28 | 0.09 | 0.14 | 0.07 | 0.07 | 0.06 |
| Morphology | | | | | | |
| Head | 0.12 | 0.04 | 0.16 | 0.05 | 0.18 | 0.06 |
| Neck | 0.10 | 0.03 | 0.12 | 0.05 | 0.15 | 0.06 |
| Tail | 0.08 | 0.04 | 0.11 | 0.06 | 0.16 | 0.08 |

previously described procedure) was conducted in 2 ways: (1) by agarose gel electrophoresis, and (2) by using a DEAE Sephadex™ (Pfizer Inc., NY, NY, USA) A-50 column. The CK-Mi isoform was separated from the total spermatozoal CK enzyme using the agarose gel electrophoresis. The isolated CK isoforms were detected by standard method.

The isoenzyme CK was isolated by an ion-exchange column. The column was prepared by swelling in 1 g of Sephadex™ in 250 mL of buffer at room temperature. The swollen Sephadex™ was rinsed 3 times and the obtained content was kept in the refrigerator at 4°C. One hundred mL of the prepared spermatozoa supernatant was added to 50 µL of Sephadex,™ contained in the Eppendorf centrifuge cell [11]. The contents were mixed well at vortex for 1 minute. The cell was placed on ice for 5 minutes and centrifuged for 5 minutes at 2500 x g. Seventy-five µL of the supernatant was used for the assessment of CK activity of the CK-Mi form. The activity of CK-B was assessed by subtracting the activity of CK-Mi from the total spermatozoa CK activity. The obtained activity was expressed as U/100 million sperm cells. The ratio CK-Mi/CK was expressed as 1.

*Statistical Analysis*

The nonparametric Mann-Whitney test was used to detect differences between patient groups. Correlations were analyzed by the Spearman correlation coefficient. Statistical significance was evaluated using analysis of variance (ANOVA). A value of $P < .05$ was considered significant.

## RESULTS

The study was conducted with 126 patients who were referred to the urological clinic because of infertility. Their spermatogram findings, based on the initial tests of the spermatozoa count and motility, are presented in Table 1. The distribution of the data was similar across groups; in particular, the groups had similar spermatozoa motility ($P > .05$).

The CK values, presented in Table 2, were obtained by the assessment of the CK activity in the seminal plasma. There was no statistically significant difference in CK activity between the groups. There was no significant correlation between the seminal plasma CK activity and standard spermogram parameters (Spearman $r = 0.1$; $P > .05$). However, Table 3 shows

Table 2. Means and Standard Deviations (SD) for Sperm Count and Creatine Kinase (CK) in the Seminal Fluid of Investigated Patients (N = 126).
doi: 10.3834/uij.1944-5784.2010.06.08t2

| Variable | Normospermia (n = 62) | | Oliogospermia (n = 64) | | | |
|---|---|---|---|---|---|---|
| | | | Moderate (n = 28) | | Severe (n = 36) | |
| | Mean | SD | Mean | SD | Mean | SD |
| Sperm count (n x $10^9$/L) | 58.7 | 31.6 | 11.7 | 4.4 | 1.41 | 1.07 |
| CK (U/L) | 1092.2 | 614.3 | 949.5 | 735.2 | 854.5 | 623.8 |

Table 3. Creatine Kinase (UI/$10^8$ Sperm) Levels in Correlation with Sperm Count and Morphology (N = 126). doi: 10.3834/uij.1944-5784.2010.06.08t3

| Variable | Normospermia (n = 62) | Oliogospermia (n - 64) |
|---|---|---|
| **Head** | | |
| **< 0.1** | 0.040 | 0.37* |
| **> 0.1** | 0.070 | 0.27* |
| **Neck** | | |
| **< 0.1** | 0.045 | 0.29* |
| **> 0.1** | 0.075 | 0.41* |
| **Tail** | | |
| **< 0.1** | 0.042 | 0.25* |
| **> 0.1** | 0.077 | 0.41* |

*Spearman $r = 0.23$; $P < .01$

that there was a significant correlation between CK levels (UI/$10^8$ Sperm) and sperm count and morphology (Spearman $r = 0.23$; $P < .01$).

The values for CK, CK-Mi, and the ratio CK-Mi/CK are presented in Table 4. CK was significantly higher in the group with severe oligospermia than in the group with normospermia ($P < .01$). The ratio CK-Mi/CK for the group with oligospermia was significantly lower than the ratio for the patients with normospermia ($P = < .01$).

Electrophoretic separation of CK in the prepared spermatozoa samples produced electropherograms with different enzyme distribution in the seminal plasma and sperm cells than in the serum, when compared with electropherograms obtained with serum separation. These differences are shown in Figure 1. Line (a) shows that 3 isoforms (CK-B, CK-MB, and CK-M) are observed in serum. Line (b) shows that only the CK-B isoform is observed in the seminal plasma. Line (c) shows that patients with normospermia have both the CK-B isoform and CK-Mi isoform, the latter of which is observed in a high percentage. This mitochondrial M form shows a somewhat longer distance than the cytosolic M serum form. Line (d) shows that the CK-Mi isoform occurs in a smaller percentage in patients with oligospermia.

## DISCUSSION

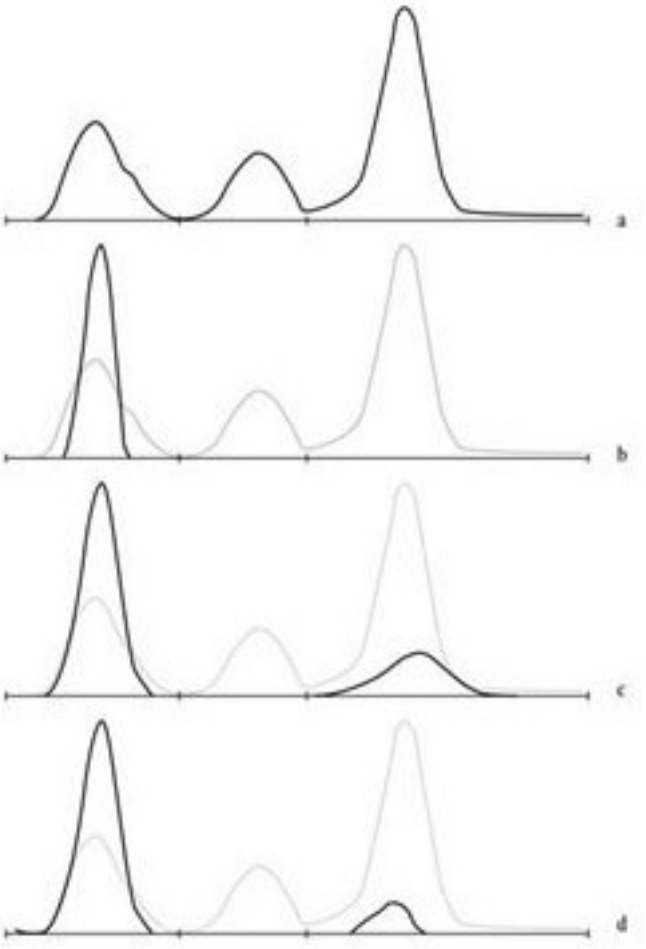
Creatine kinase in human sperm is a marker of cytoplasmic retention and, thus, diminished sperm maturity [6]. In the seminal fluid, CK is found in the form of coenzyme CK-B [11]. The values many times higher that those in serum show that CK is also produced by the local excretion of the genital system glands. Rolf et al [12] showed that there was no significant difference between the CK value in the seminal fluid and spermatozoa count, motility, and morphology. Similar results were obtained in the present study.

CK values were significantly higher in the patients with oligospermia when compared with patients with normospermia, as shown in Table 4. The inverse correlation between the CK spermatozoa activity and spermatozoa count was confirmed, which also shows that there was a metabolic difference between the sperm cells of the patients with oligospermia and the patients with normospermia [13]. These findings point to a disorder of sperm maturity. CK is a known predictor of sperm maturity and fertilization potential. In the course of spermatogenesis, the mature sperm cells are released from the cytoplasmic residue, which is rich in the mitochondrial material that is the source of CK. The presence (in immature sperm) or absence (in mature sperm) of the cytoplasmic residue is the reason for the difference in CK activity between the groups. Immature cells with cytoplasmic retention were not able to bind to the zona pellucida, suggesting that the zona-binding sites are part of the membrane remodeling process [14]. Mature spermatozoa which bind to the hyaluronic acid do not show cytoplasmatic retention. Those cells are of normal morphology; they have high DNA integrity and low frequency

Table 4. Creatine Kinase (CK) (UI/$10^8$ Sperm) Levels, CK-Mi (UI/$10^8$ Sperm) Levels, and the Ratio CK-Mi/CK (Expressed as 1) in Patients with Different Sperm Counts (N = 126). doi: 10.3834/uij.1944-5784.2010.06.08t4

| Variable | Normospermia (n = 62) | | Oliogospermia (n = 64) | | | |
|---|---|---|---|---|---|---|
| | | | Moderate (n = 28) | | Severe (n = 36) | |
| | Mean | SD | Mean | SD | Mean | SD |
| **CK (UI/$10^8$ sperm)** | 0.097 | 0.026 | 0.46 | 0.30 | 1.90 | 2.20 |
| **CK-Mi (UI/$10^8$ sperm)** | 0.041 | 0.015 | 0.11 | 0.07 | 0.18 | 0.10 |
| **CK-Mi/CK (1)** | 0.360 | 0.120 | 0.16 | 0.10 | 0.10 | 0.05 |

Figure 1. Electrophoresis of the Creatine Kinase (CK) Isoenzyme. doi: 10.3834/uij.1944-5784.2010.06.08f1



In the serum (a), there are 3 isoforms of CK: CK-B, CK-MB, and CK-M; in the seminal plasma (b) there is only a CK-B isoform. In the patients with normospermia (c), there CK-B and a high percentage of the form CK-Mi. In the patients with olygospermia (d), the percentage of CK-Mi forms is smaller than in patients with normospermia.

of chromosomal aneuploidies [15].

The samples with a reduced spermatozoa count show frequent abnormalities in spermatozoa morphology, which have a direct negative impact on the fertilization potential. Several papers [16,17] demonstrate that, in addition to the reduced spermatozoa count, high CK activity is associated with an increased incidence of pathological changes in the neck and tail regions. These changes reveal a close correlation between biochemical and morphological abnormalities and impaired spermatogenesis. Furthermore, mature spermatozoa that are able to bind hyaluronic acid significantly differ in all tail, head, and tail:head long axis ratio parameters when compared with the spermatozoa that are not able to bind [18].

The activity of the spermatozoa CK is induced by two isomeric CK forms: CK-M and CK-B. The predictive activity of the CK-M ratio (CK-M/CK) was tested in the present study. The authors found that CK-Mi values were significantly higher in the patient group with low CK and high sperm count and morphology, when compared with the patients with normospermia.

Mature sperm cells contain high levels of the CK-Mi form of CK. The high CK-Mi values show that plasma membrane remodeling and cytoplasmic residue elimination were completed in mature sperm cells and may be used as a maturity marker. Concurrently, as the formed sperm cells come to caput epididymis, their maturation is completed and the CK and CK-Mi values remain unchanged during their passage into the descendent part of the epidydimus [19]. The relative proportions of the subpopulations of mature and diminished maturity spermatozoa in the ejaculate are important in defining why men with similar sperm counts have different chances of reproductive success. Additionally, it has been reported that the CK levels in cancer patients were similar to those of normal donors. This finding suggests that the final phase of spermatogenesis may not be altered in men with cancer. However, semen from these men should be banked to ensure fertility after cancer treatment [20].

The primary beneficiaries of the CK test are men with oligospermia who, by conventional semen parameters, would be classified as having diminished fertility. By utilizing the sperm biochemical parameters, these men can be tested to determine if their sperm have adequate maturity. If maturity is confirmed, the infertility focus could be shifted to the female partner's condition. Therefore, the CK measurement can aid in the selection of the most efficient treatment for couples with male-factor or unexplained infertility.

**Conflict of Interest**: None declared

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