Sperm Creatine Kinase Activity in Normospermic and Oligozospermic Hungarian Men

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Purpose: *Our purpose was to measure sperm creatine phosphokinase (CK) activity, which reflects cytoplasmic retention in immature spermatozoa, in normospermic and oligozospermic Hungarian men.*
Methods: *A study of 109 randomly selected men in a university-based andrology laboratory was done.*
Results: *CK activity differed between normospermic and oligozospermic men (0.21 ± 0.02 vs. 1.19 ± 0.15 CK IU/10^8 sperm; n = 56 and n = 53; mean ± standard error of the mean, respectively). There was an inverse correlation between sperm concentration and CK activity (r = −0.70; n = 109). However, 28% of men in the range with less than 10 million sperm/ml had normal sperm CK activity (below the mean + 2 standard deviations of the group with greater than 30 × 10^6 sperm/ml), whereas 36% of men in the group with 20–30 million sperm/ml and 5% in the group with greater than 30 million sperm/ml had elevated CK activities, indicating that the incidence of mature and immature spermatozoa in specimens is independent from the sperm concentrations.*
Conclusions: *The improved facility of sperm CK activity measurements, compared with sperm concentrations, in the assessment of sperm maturity was confirmed in a Hungarian population. The CK measurements aid the selection of the most efficient treatment for couples with male-factor or unexplained infertility, particularly when considering the options of intrauterine insemination, varicocelectomy followed by a waiting period, or ovulation workup/induction in wives of men who are oligozospermic but may have fertile sperm.*

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INTRODUCTION

In the past few years, it has been established that creatine phosphokinase (CK) activities and the ratios of the CK-M (mature sperm-type CK) to the CK-B (brain-type CK) isoforms in sperm are objective biochemical markers of sperm cellular maturity and fertilizing potential (1–5). The CK activities of sperm represent the retained cytoplasm in immature spermatozoa that have not completed cytoplasmic extrusion during spermiogenesis (6). Retained cytoplasm has also been found to be present in individual spermatozoa by means of CK immunocytochemistry. In the same studies, furthermore, computer-assisted morphometry showed an association between higher CK activity and the increased incidence of morphologically abnormal spermatozoa: due to increased cytoplasmic retention, sperm showed an increased head size, head roundness, and a higher rate of amorphous sperm forms (6). Hemizona assays, in which sperm samples and sperm–hemizona complexes were immunostained with an anti-CK antibody, have demonstrated that immature sperm with cytoplasmic retention do not bind to the zona pellucida (7). The biological rationale underlying this finding has recently become clear. Immature spermatozoa have diminished potential for zona binding and fertilizing because, during spermiogenesis and simultaneously with cytoplasmic extrusion and expression of the mature sperm-specific CK-M isoform, the sperm plasma membrane undergoes a remodeling process which apparently leads to the formation of the zona-binding site(s) (8).

The biochemical parameters of CK activity and CK-M isoform ratio were predictive of male fertility in several studies (9–11). In couples with oligozoosper-

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mic husbands treated with intrauterine insemination, sperm CK activities differed between fertile and infertile men, although the sperm concentration and motility values between the two groups were similar or identical. A logistic regression analysis in fertile and infertile oligozospermic men further indicated that, while the occurrence of pregnancies correlated with CK activities, the sperm concentration values did not contribute to this correlation (4). With respect to in vitro fertilization (IVF), in a blinded study of 84 couples, the CK-M isoform ratios predicted the occurrence of pregnancies in the CK-M fertile group at a rate of 30.4% per cycle, which corresponded to the best IVF pregnancy rates at the time of the study (5).

In the present work, we have initiated sperm CK measurements and examined the utility of objective CK markers in the evaluation of Hungarian men presenting for semen analysis.

MATERIALS AND METHODS

Patient Population

The study population was composed of normospermic and oligozospermic men who presented for semen analysis at the andrology laboratory of the Department of Obstetrics and Gynecology, Albert Szent-Györgyi Medical University, Szeged, Hungary, during a 3-year period beginning in October 1993.

Sperm Protein Extraction and CK Measurements

Specimens were evaluated for sperm concentration and motility by manual techniques. Samples with concentrations of less than 20×10^6 sperm/ml were considered oligozospermic, and those of greater than 20×10^6 sperm/ml were considered normospermic. For CK measurements, each semen sample was washed with 15 to 20 vol of ice-cold 0.15 *M* NaCl, 0.03 *M* imidazole, pH 7.0, at $5000 \times g$ for 15 min to remove all seminal fluid contaminations. The resulting sperm pellet was resuspended in ice-cold 0.03 *M* imidazole, pH 7.0, 10% glycerol, 5 m*M* dithiothreitol, 0.1% Triton X-100. The sperm were disrupted by vortexing vigorously for 30 sec. The sperm extract was clarified by centrifugation at $5000 \times g$, and aliquots were subjected to CK activity determinations by spectrophotometry using a CK kit (Sigma Chemical Co., St. Louis, MO) as described previously (4).

Data Analysis

Statistical analyses, including the mean ± standard error of the mean of each data group, Student's *t* test, and linear regression analyses, were carried out by the SigmaStat program. The normalcy of distributions was established by the Kolmogorov–Smirnov test. Abnormally distributed data were analyzed by the Spearman rank correlation test. Comparisons with *P* values of less than 0.05 were considered significant.

RESULTS

Sperm CK Activity in the Normospermic and Oligozospermic Samples

We determined the sperm CK activities in 56 normospermic (concentration: $54.3 \pm 3.5 \times 10^6$ sperm/ml) and 53 oligozoospermic (concentration: $7.4 \pm 0.6 \times 10^6$ sperm/ml) semen samples. The CK activities were six times higher in the oligozospermic than in the normospermic group (1.19 ± 0.15 vs. 021 ± 0.02 IU/10^8 sperm; $P < 0.001$). An inverse correlation between CK activity and sperm concentration was also evident in the 109 samples ($r = -0.70$, $P < 0.001$; $n = 109$) (Fig. 1).

Distribution of High- and Low-CK Activity Samples Among Various Sperm Concentration Groups

In general, men with oligozospermic specimens are thought to have diminished fertility. However, previous

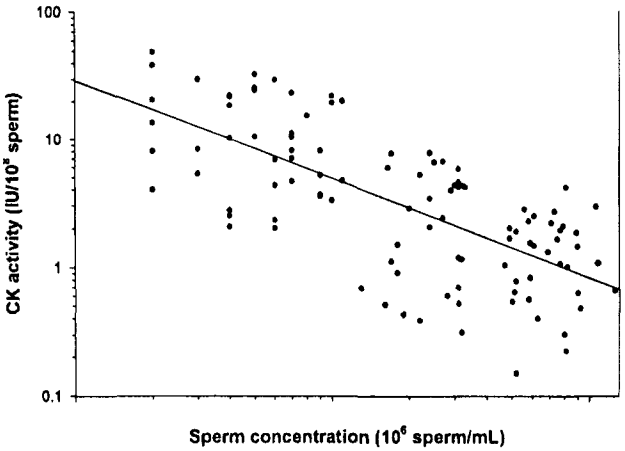

Fig. 1. Correlation between CK activity and sperm concentration in the 109 samples. The data were subjected to logarithmic transformation.

studies have indicated that sperm concentrations are not predictive of fertilizing potential (1–5,9–12). We examined the relationship between CK activity and sperm concentrations by dividing the 109 patients into four groups: severely oligozospermic men (less than 10×10^6 sperm/ml; $n = 40$), oligozospermic men ($10–20 \times 10^6$ sperm/ml; $n = 13$), men with a low normal sperm concentration ($20–30 \times 10^6$ sperm/ml; $n = 11$), and normospermic men with sperm concentrations greater than 30×10^6 sperm/ml ($n = 45$). In accordance with the inverse correlation between sperm concentrations and CK activity in the 109 samples, the mean CK activities were highest in the severely oligozospermic men and lowest in the group with greater than 30×10^6 sperm/ml (Fig. 2). There was also an incremental decrease in CK activity among sample groups with increasing sperm concentrations, and the mean CK activities differed significantly among the four groups (<10 vs. 10–20, $P < 0.05$; <10 vs. 20–30 and >30, $P < 0.001$; 10–20 vs. 20–30 and >30, $P < 0.05$; 20–30 vs. >30, $P < 0.05$). This indicates that there is a increased incidence within the low sperm concentration groups of men showing elevated CK activities due to immature sperm with retained cytoplasm.

Sperm Concentration vs. Sperm CK Activity in the Various Groups

To study the relationship between sperm concentrations and sperm maturities in the four patient groups (Fig. 3), we established the upper level of normal sperm CK activity as 0.43 CK IU/10^8 sperm, based

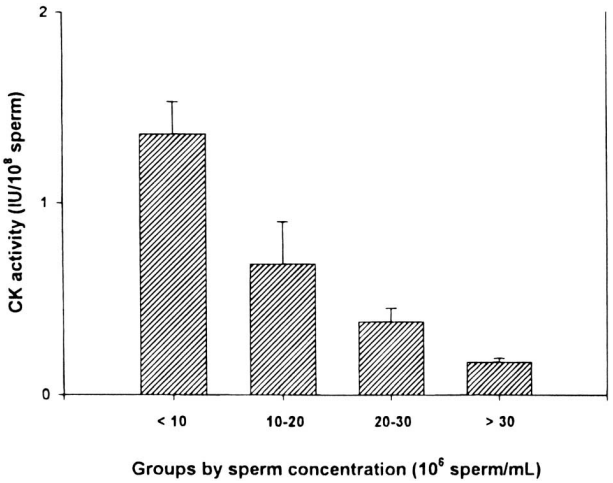

Fig. 2. Distribution of CK activities in the four sperm concentration groups.

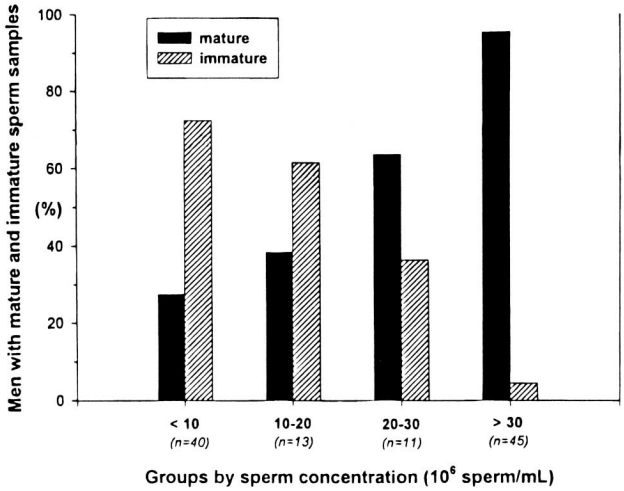

Fig. 3. Proportions of samples with mature and immature spermatozoa in the four sperm concentration groups.

on the mean + 2 standard deviations of the CK activity in the samples with more than 30 million sperm/ml normospermic (0.17 ± 0.13 CK IU/10^8). The incidences of samples above and below this value provide information about the proportions of men who are oligozospermic but have sperm with normal maturity/fertilization potential or who are normospermic but show diminished sperm maturity. As in previous studies, the data indicated a substantial discrepancy between sperm concentration and sperm maturity (1–5, 9–12). In the severely oligozospermic and oligozospermic groups, respectively, 27.5% and 39% of the men had CK values in the normal range. Conversely, in the low-normal and normal sperm concentration groups, 38% and 5% of the men had CK values in the abnormal sperm maturity range, indicating unexplained diminished male fertility. These data clearly demonstrate that sperm maturities are independent of sperm concentrations in these specimens. The most striking finding was the similarity between the incidences of men with mature sperm in the group with 10–20 million/ml sperm concentration and of men with immature sperm in the normospermic group, according to World Health Organization criteria, with 20–30 million sperm/ml, which were 38% and 39%, respectively. Also worthy of note is the presence of men in the patient group with greater than 30 million sperm/ml who had diminished sperm maturity, approximately 5%.

DISCUSSION

The parameters of conventional semen analysis (sperm concentration, motility, and morphology) have

failed to show a consistent correlation with pregnancy rates (1,11,12). In fact, the relationship between semen analysis parameters and male fertility continues to be an unresolved issue (13–15). Other available tests for assessing specific sperm functions, such as acrosomal integrity and response (15–20), the sperm heat stress test (21), and the zona-free hamster ova penetration assay [which is now thought to reflect not only acrosomal function but also the ability of sperm to participate in sperm–oolemma fusion (22,23)], have all failed to reliably predict male fertility and, particularly, infertility. The results improved somewhat when multiple sperm function tests were used, but consistent prediction of diminished fertility still was not possible (13,15,19,22). The sperm function tests are inefficient primarily because they test only one of the several steps involved in sperm–oocyte interaction and fertilization, which may not provide a true assessment of sperm functional integrity. Accordingly, the best predictive value among the sperm function tests was achieved by combining the human sperm–hemizona binding assay and sperm motility, both of which are actual real measures of function necessary for fertilization *in vivo* or in conventional IVF (23).

In a new approach, the Huszar laboratory has pursued the clinical and cell biological aspects of human sperm CK activity and CK-M isoform ratio, which are objective biochemical markers of sperm maturation in spermiogenesis (1–8). The assessment of male fertility by these means provides very reliable data, as demonstrated in clinical studies of couples with oligozospermic husbands who were treated with intrauterine insemination and also in a blinded study of 84 couples treated with IVF (4,5). In the latter study of 62 prospectively fertile and 22 prospectively infertile men, classified by their CK-M ratios as being either above or below the 10% CK-M cutoff value, the pregnancy rates were 30.4 and 0%, respectively. None of the men with sperm samples of diminished fertility caused pregnancies, even though 9 of the 22 men had semen analysis parameters in the normospermic concentration and motility range (5).

Further study of the cell biology of human spermatozoa clarified the relationship between the CK characteristics and the occurrence of pregnancies. Mature spermatozoa, which shed their extra cytoplasm as residual bodies in the adluminal area before being released into the seminiferous tubuli, characteristically have very low levels of cytoplasm (low CK activity). Mature sperm also show a higher concentration of the CK-M isoform because it is expressed only during the last phase of spermiogenesis in elongated spermatids and in mature sperm (1–3). Moreover, the decline of sperm CK activity and the increased expression of the CK-M isoform are closely related (3,24). Another close relationship has been demonstrated between CK and the hemizona assay of sperm binding, based on the study of CK-immunostained sperm fractions and their sperm–hemizona complexes: immature spermatozoa, with high CK activity and cytoplasmic retention, were unable to bind to the zona (3). This suggested that plasma membrane changes take place in spermiogenesis simultaneously with cytoplasmic extrusion and the expression of the new sperm-specific CK-M. The occurrence of this developmental remodeling, which is likely to contribute to the formation of the zona-binding site in sperm, was recently demonstrated by the expression pattern of β-1,4-galactosyltransferase (GalTase), a protein that is present only on the surface of the sperm plasma membrane (8). These results further emphasize that sperm cellular maturity, which is reflected by the CK measurements, is a key underlying element of sperm functional integrity.

In the present study of a Hungarian patient population, we followed sperm CK activity and confirmed the previous findings with respect to both the CK activity differences and the patterns among the groups of men with various CK activities, supporting the lack of a relationship between sperm concentration and sperm maturity (1–6,9). In the normospermic semen samples with a higher percentage of mature spermatozoa, the sperm CK activities were six times lower than in oligozospermic specimens, and an overall inverse correlation was present between sperm concentrations and CK activities. CK activity values differed significantly among the four concentration groups into which the study population was divided (Fig. 2). Most important, semen samples with sperm of normal and diminished biochemical maturity were variously distributed within the four groups (Fig. 3). There was a general predominance of men with immature sperm within the severely oligozospermic group and of men with mature sperm in the group with greater than 30 million sperm/ml. However, we detected a substantial representation of oligozospermic men with mature sperm in the groups with a lower sperm concentration range, groups with less than 10 million and 10–20 million sperm/ml, and of diminished maturity sperm among normospermic men within the group with greater than 30 million sperm/ml (Fig. 3). These findings further demonstrate that sperm concentrations alone are inadequate for assessing a man's fertility. Indeed, the groups with concentrations of 10–20 and 20–30 million sperm/ml contain almost-identical pro-

portions of mature and diminished maturity sperm, although the men in these groups are considered "oligozospermic" and "normospermic" according to the World Health Organization criteria. Also, the subpopulation of normospermic men with diminished sperm maturity indicates that the CK markers are able to detect unexplained male infertility, i.e., men with normospermic semen who show diminished sperm maturity and fertilizing potential.

The present work, while mostly confirmatory, is important for two main reasons. First, although the data were developed from a patient population in Hungary, the CK activity data are essentially identical to those of the original studies in the United States. There are similar significant differences in the mean CK activity values between the various oligozospermic and normospermic groups, and the distribution pattern in the proportions of men with mature and immature spermatozoa in the four sperm concentration groups is likewise similar. Moreover, the Hungarian population also displays the characteristic dissociation between sperm concentrations and sperm maturities in a substantial proportion of the specimens. Second, this work shows how the management of couples with male-factor infertility or unexplained infertility may be made more efficient and specific by means of the objective sperm CK activity tests, which require only a careful estimation of sperm concentration, a CK assay kit, and a simple spectrophotometer.

Identifying men with adequate fertilization potential and, equally important, men with diminished fertilizing potential and infertility, irrespective of sperm concentrations, is the most important aspect of a workup in couples with male-factor or unexplained infertility. Sperm CK activity measurements can contribute to at least three major therapeutic decisions: (i) whether surgery is necessary and what the duration of the subsequent waiting period should be for oligozospermic men with varicocele, which may be problematic if the wife is older than 35 years of age; (ii) whether intrauterine insemination is indicated for a couple with an oligozospermic husband; and (iii) the question of workup and ovulation monitoring in a couple with unexplained infertility, particularly in cases where the wife's infertility factor may be overlooked in the light of the husband's oligozospermia, although his sperm maturity and fertility may be normal. We have recently started an IVF program in Szeged in which the sperm CK measurements will be utilized. The clinical data emerging from the current and future studies will be presented in a follow-up report.

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