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Enolase Isoforms Activities in Spermatozoa From Men With Normospermia and Abnormospermia

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ABSTRACT: Total enolase as well as enolase-αα (ENO-αα, ubiquitous) and enolase-S (ENO-S, sperm-specific) activities were measured in total and Percoll-selected sperm from 30 normospermic fertile men and 20 abnormospermic infertile patients. The total enolase activity was significantly higher in total sperm from patients with abnormospermia compared with normospermic patients (11.1 ± 1.9 vs. 4.8 ± 0.5 mIU/10^7 sperm $P < .05$). ENO-αα activity was significantly higher in total sperm from abnormospermic men than from normospermic men ($P < .05$). ENO-αα activity in Percoll-selected sperm was significantly lower compared with total sperm in both group of patients; however, for the same sperm fraction ENO-αα activity did not differ between normospermic and abnormospermic men. ENO-αα activity was related to the cell contamination ratio and to the percentage of spermatozoa with abnormal morphology. Furthermore, ENO-αα was positively correlated with the percentage of immature sperm showing an excess of residual cytoplasm. ENO-S activity was significantly higher in total sperm from normospermic patients than from abnormospermic patients ($P < .05$). ENO-S activity in Percoll-selected sperm was not significantly different compared with total sperm in both group of patients. However, this activity was significantly lower in Percoll-selected sperm from abnormospermic men compared with normospermic men ($P < .05$). ENO-S activity was not related to the cell contamination ratio but was significantly correlated with the percentage of spermatozoa with normal morphology. The 2 enolase isoforms seem to reflect 2 opposite aspects of sperm cells quality: ENO-αα is associated with abnormal spermatozoa, immature spermatozoa, or both; and ENO-S is associated with normal spermatozoa. As an additional index to distinguish normal from abnormal semen, the ENO-S:ENO-αα ratio was evaluated for total and Percoll-selected sperm in both groups. This ratio seems to be a new, valuable marker of the global sperm quality in a given semen sample, and may represent a predictive index of sperm fertilizing potential.

Key words: Human sperm, glycolytic enzyme, Percoll gradient, enzyme marker, sperm immaturity, sperm morphology.

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Among the additional sperm parameters studied to improve the classification and management of men with infertility, is enzymatic determination. Indeed, independent studies have indicated that defective sperm function is frequently associated with elevated activities of certain enzymes, including creatine kinase (Huszar et al, 1988; Sidhu et al, 1998), lactic acid dehydrogenase (LDH; Casano et al, 1991; Orlando et al, 1994; Lalwani et al, 1996; Aydin et al, 1997), diaphorase (Gavella and Lipovac, 1992), adenylate kinase (Dolcetta et al, 1986), and glucose-6-phosphate dehydrogenase (Aitken et al, 1994). The variations of these metabolic proteins were not directly involved in sperm dysfunction but could be considered as markers of the normality of sperm differentiation. Enolase is a glycolytic enzyme that catalyzes the conversion of 2-phosphoglycerate in a dehydration step to yield phosphoenolpyruvate. The presence of a sperm-specific enolase isoform, demonstrated by Edwards and Grootegoed in 1983, led us to consider this enzyme as a possible new marker. Indeed, in human spermatozoa, 2 enolase isoforms were found, an unusual sperm-specific isoform, designated ENO-S, and a classical isoenzyme found in broad tissue, ENO-αα. Since the preliminary work of Edwards and Grootegoed (1983), no other data were found in the literature on the levels of this enzyme associated with sperm quality.

The objective of this study was to measure total enolase and the activities of its isoforms in spermatozoa of men with normospermia and men with abnormospermia in order to investigate a possible relationship between this enzyme and sperm function.

Materials and Methods

Reagents

The ATP Monitoring Kit 5080-200 was provided by Labsystems (Helsinki, Finland). Percoll was purchased from Nidacon (Gothenburg, Sweden). All other chemicals used were of purest analytical grade and obtained from Sigma Chemical Co (St Louis, Mo).

Patients

The study was conducted between April and December 2000. A total of 38 semen samples were classified into 2 groups: 1) nor-

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mospermic fertile patients (panel 1) according to criteria of the World Health Organization (WHO, 1992): sperm concentration $>20 \times 10^6$ sperm, sperm total motility ≥50%, sperm normal morphology ≥30%, and who had already conceived children through natural means (n = 18); 2) abnormospermic infertile patients (sperm concentration $<20 \times 10^6$ sperm, sperm total motility <50%, sperm normal morphology <30%) (n = 20). The distribution of clinical diagnoses of abnormospermic patients was idiopathic infertility (n = 11), varicocele (n = 3), and unilateral cryptorchidism (n = 6). Specimens from these patients were used in the analysis of enolase isoform activities in total sperm or in Percoll-selected sperm (P95 fraction), and in assessing the relationships between the presence of these isoforms and the functional competence of the spermatozoa.

In order to address additional questions concerning the relationship between enzyme isoforms and sperm maturity, samples were collected from an additional panel of normospermic patients (n = 12, panel 2) who had conceived children through natural means.

The mean age of the normospermic patients was 34.7 ± 0.9 (range 24–46) years and 33.6 ± 0.8 (range 27–39) years for abnormospermic patients.

Total Sperm Preparation for Enolase Determination

Semen collection and analysis were performed according to the WHO (1992) recommended procedure. Semen samples were collected by masturbation in our laboratory after 3–4 days of sexual abstinence. After complete liquefaction, ejaculate volume, sperm count, leukocytes count, and motility were determined by routine procedure. Viability was evaluated using the eosin-nigrosin test. When round cells were observed in the ejaculate, the differentiation between white blood cells and spermatogenic cells was performed using the benzidin-cyanosin coloration technique, which results in brown-colored polymorphonuclear granulocytes that represent more than 80% of leukocytes in semen. Sperm morphology was assessed after staining with Shorr dye, according to the David criteria (David et al, 1975). The original semen sample (1–2 mL) was centrifuged at 500 × *g* for 10 minutes at room temperature. The pellet was washed with 10–15 volumes of ice-cold 20 mM tris(hydroxymethyl)aminomethane (Tris), 150 mM NaCl, 5 mM $MgCl_2$ pH 7.4, and centrifuged at 1200 × *g* for 10 minutes at 4°C. The sperm pellet containing the whole sperm population of the ejaculate was resuspended in 500 μL of washing buffer and the sperm count was determined (total sperm).

To characterize the cell contamination of the preparation we calculated the ratio (expressed as %) of the number of leukocytes plus round cells to the number of spermatozoa. This ratio will be designated as the cell contamination ratio. A maximum of 10×10^6 spermatozoa were transferred to an Ependorff microtube and centrifuged as above. The latter pellet was then vortexed for 1 hour at 4°C in 500 μL of 20 mM Tris, 5 mM $MgCl_2$ devoid of NaCl, and supplemented with 0.5% octyl-β-D-glucopyranoside. Finally, each extract was frozen and stored at −80°C. Before analysis each sample was thawed at room temperature, then centrifuged at 10 000 × *g* for 10 minutes at 4°C. The enolase measurement was performed on the supernatant. The results were expressed as IU/L and calculated for each sample in mIU/10^7 spermatozoa.

Percoll Fractionation of Semen Samples

Two-Step Percoll—Migrated sperm fractions were obtained by centrifuging on a Percoll gradient (Yates et al, 1989) semen samples from 14 normospermic and 13 abnormospermic men. After complete liquefaction an aliquot of 1–2 mL was centrifuged at 500 × *g* for 10 minutes at room temperature. The seminal fluid was removed and the sperm pellet was suspended in 1 mL of Earle medium and layered on top of a two-step discontinuous gradient obtained with 1 mL of both 47.5% and 95% Percoll in a 15-mL conical tube. The tubes were then centrifuged at 500 × *g* for 20 minutes at room temperature. After centrifugation the P95 fraction was collected and the sample was processed as indicated above for total sperm to obtain the lysis of spermatozoa.

Four-Step Percoll—The semen samples from 12 normospermic men (panel 2) were fractionated on a discontinuous four-step Percoll gradient. After centrifugation at 500 × *g* for 10 minutes at room temperature, the pelleted sperm was resuspended in 1 mL of Earle medium and layered onto a discontinuous four-step Percoll gradient. Percoll density gradients were formed by layering 1 mL of each different concentration of Percoll solution (47.5%, 60%, 80%, 95% Percoll) into a 15-mL conical tube. After centrifugation at 500 × *g* for 20 minutes at room temperature, each fraction was collected and processed as indicated above for total sperm prior to the enolase assay.

Sperm Residual Cytoplasm Assessment by Diaphorase Assay

A cytochemical staining procedure was used, involving NADH-NADPH and nitroblue tetrazolium (NBT; Caldwell et al, 1976). An aliquot from each Percoll-selected fraction (4-step Percoll gradient, panel 2) was incubated with 1 mM NADH-NADPH, 0.5 mM NBT in phosphate-buffered saline (pH 7.4) at 37°C and 5% CO_2 for 3 hours in a closed tube. Then the sperm were plated onto slides and examined microscopically (1000×) for the presence of midpiece staining (blue-black precipitate). A midpiece cytoplasmic droplet greater than double the width of the normal midpiece was considered significant. A minimum of 200 spermatozoa per slide were examined.

Bioluminescence Determination of Enolase Activity

We determined total enolase activity according to a method described by Viallard et al (1985). In this procedure the phosphoenolpyruvate formed from 2-phosphoglycerate (G2P) by enolase is transformed into adenosine triphosphate (ATP) in the presence of pyruvate kinase and adenosine diphosphate. We measured the rate of production of ATP with a bioluminometer (Biocounter M 2010; Lumac, Basel, Switzerland) using a luciferin-luciferase system. Enzyme activity was expressed as mIU, defined as the amount of enzyme that catalyzes the conversion of 1 nmol of substrate per minute. As previously described (Viallard et al, 1985), the lowest activity measured by this method is 0.4×10^{-6} IU of enolase. The assay is linear from 0.4×10^{-6} to 50×10^{-6} IU with a variation coefficient of 6.7% and 2.2% for the low and high values, respectively.

Electrophoresis Determination of Enolase Isoforms Activity

Relative ENO-αα and ENO-S activities were determined according to the method described by Viallard et al (1986). Briefly the isoforms were separated by electrophoresis on a cellulose acetate plate (Titan III Iso-Flur 3905; Helena, Beaumont, Tex). The separated enolase isoforms were detected by overlaying the plate with a reactional mixture, which via a multistep reaction, produces NADPH.

The fluorescent bands corresponding to the enolase isoforms were quantified under UV light with a scanning fluorometer (Cliniscan II Astron Densitometer, Helena). The enolase nature of the fluorescent bands was verified by omitting the enolase substrate (G2P) of the revelation medium.

The relative activity of each isoform was calculated from the total activity of the sample, and the relative proportion was measured after electrophoresis.

Statistical Analysis

The Wilcoxon-signed rank test (nonparametric test) for paired data was used to compare the parameters between Percoll-selected and total sperm, or between the 4 sperm subpopulations selected by the 4-step Percoll method. Differences between normospermic (panel 1) and abnormospermic groups were examined with the Mann-Whitney *U*-test. Linear regression analysis was performed to examine the relationship between enolase parameters and cell contamination ratio, the percentage of spermatozoa with residual cytoplasm, and the percentage of spermatozoa with normal or abnormal morphology. A P value $< .05$ was considered statistically significant.

Results

Enolase Isoforms Electrophoretic Separation

The pattern of sperm enolase isoforms is presented in Figure 1. It is characterized by a band with the same migration that the αα isoenzyme of brain extract has; and another band, ENO-S, with an unusual electrophoretic migration. The latter band was located between the αα and αγ isoenzyme of brain extract. This brain extract is used as a reference to characterize enolase isoforms present in other tissues.

Enolase Levels in Total Sperm

Table 1 presents the spermiologic characteristics of the 2 normospermic panels and the abnormospermic group. Sperm numeration, vitality, total motility, and normal morphology were significantly lower in abnormospermic patients compared with those in panel 1. The spermiologic parameters of the abnormospermic group correspond to oligoasthenoteratospermic patients.

As shown in Table 2 the 2 sperm categories were well differentiated by their enolase parameters. Total and ENO-αα activities were significantly higher in sperm extracts from the abnormal group ($P < .05$), whereas ENO-S activity was significantly lower in the abnormal group ($P < .05$).

ENO-αα Levels in Total and Percoll-Selected Sperm

In order to explain ENO-αα activity level differences between the 2 groups, we studied the correlation between ENO-αα activity and the round cell contamination ratio of the sample, and between ENO-αα activity and the percentage of sperm with abnormal morphology (Figure 2A and B). For these 2 parameters a positive correlation was found when the data from the 2 groups of patients were gathered (panel 1 of the normospermic group and the abnormospermic group; $r = 0.686$, $P < .01$ and $r = 0.450$, $P < .05$). However the data corresponding to the abnormal specimens showed a high dispersion.

In a further study, we measured the enolase isoform activities in Percoll-selected sperm (P95 fraction). In both groups, ENO-αα activity was significantly lowered in the P95 sperm fraction compared with that of total sperm. The values obtained for the P95 fraction of the abnormal group were not significantly different from those of the normal group (Table 3).

ENO-S Levels in Total and Percoll-Selected Sperm

ENO-S activity was significantly higher in total sperm from normospermic patients. The ENO-S level remained constant compared with total sperm after Percoll selection in both groups (Table 3). A positive correlation between ENO-S and the percentage of spermatozoa with normal morphology was found (Figure 2D; $r = 0.428$, $P < .05$). There is no correlation between ENO-S activity and the cell contamination ratio (Figure 2C). The percentage of morphologically normal spermatozoa before and after Percoll selection was not significantly different (49% ± 4% vs 38% ± 4%, respectively, in normospermic patients and 17% ± 2% vs 15% ± 2%, respectively, in abnormospermic patients). In the P95 sperm fraction the ENO-S activity remained significantly lower in the abnormal group compared with the normal group (Table 3), as shown for total sperm.

Relationship Between Sperm With Residual Cytoplasm and Enolase Isoforms Activity

The relationship between the percentage of sperm exhibiting retention of residual cytoplasm and enolase measurement was assessed in 4 sperm subpopulations separated by Percoll gradient centrifugation from normal semen (panel 2). As shown in Figure 3A, the ENO-αα activity was inversely proportional to the Percoll density of the sperm fraction. The high ENO-αα activity in the P47.5 fraction can be explained by the cell contamination of this fraction (15% ± 2%). For the P80 fraction the cell contamination ratio was lower (2.5%), and it was even lower in other fractions.

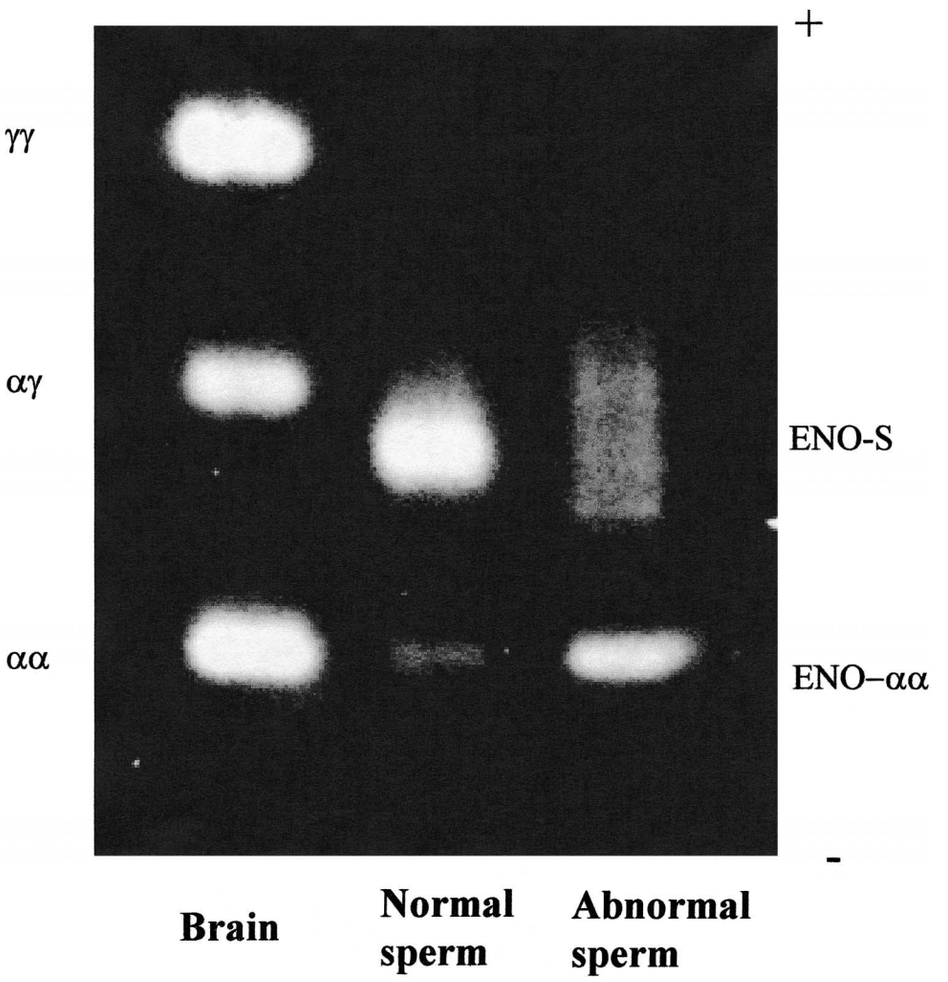

Figure 1. Electrophoretic separation of enolase isoforms of total sperm extracts from normospermic and abnormospermic men. A human brain sample that contains the 3 soluble isoenzymes of enolase (αα, γγ, and αγ) is included for comparison. The bands were revealed under fluorescence after migration on cellulose acetate.

The ENO-S activity was unchanged in the different Percoll-selected subpopulations (3.66 ± 0.34, 3.84 ± 0.41, 3.81 ± 0.32, and 3.80 ± 0.62 mIU/10^7 sperm for P95, P80, P60, and P47.5 fractions, respectively).

Using NADH/NADPH diaphorase staining to monitor sperm residual cytoplasm retention, we observed that the different sperm subpopulations (P95, P80, and P60 fractions) had significantly different percentages of spermatozoa exhibiting cytoplasm retention (8.9% ± 1.2%, 14.2% ± 2.1%, and 22.8%± 2.4%, respectively; $P < .05$; Figure 3B).

Figure 3C shows that ENO-αα activity correlated with the percentage of sperm with residual cytoplasm ($r = 0.563$; $P < .05$).

Estimation of the ENO-S:ENO-αα Ratio

The inverse variation of enolase isoforms led us to try to increase the specificity of determination of our enzyme by calculating the ENO-S:ENO-αα ratio (S:αα). The mean S:αα ratio was significantly higher both in total and P95-selected sperm from normospermic men compared with abnormospermic men (3.3 ± 0.4, range 1.1–5.7 vs 0.5 ± 0.1, range 0.1–1.0, $P < .001$ in total sperm; 7.6 ± 0.8, range 4.5–15.2 vs 2.4 ± 0.5, range 0.2–5.7, $P < .001$ in the 95% Percoll fraction). Figure 4 presents the different values of the S:αα ratio in total sperm of the 2 groups and, for comparison, the corresponding values after Percoll selection. The S:αα ratio allows a marked separation of the 2 groups in Percoll-selected sperm as well as in total sperm, with minor overlap of the values.

Discussion

In 1983, Edwards and Grootegoed were the first to describe the enolase pattern of human spermatozoa com-

Table 1. *Semen characteristics of the 50 studied patients**

Semen Parameters	Normospermic Samples		Abnormospermic Samples (n = 20)
	Panel 1 (n = 18)	Panel 2 (n = 12)	
Volume (mL)	3.4 ± 0.2 (1.7–5.2)	4.4 ± 0.4 (3.0–7.9)	3.8 ± 0.3 (1.9–7.4)
Numeration (10^6 sperm/mL)	129 ± 22 (40–380)	127 ± 21 (58–285)	6 ± 1† (0.5–16)
Numeration (10^6 sperm per ejaculate)	472 ± 108 (100–1976)	589 ± 138 (210–1817)	24 ± 5† (2–76)
Vitality (%)	83 ± 2 (70–91)	85 ± 2 (75–91)	70 ± 4† (24–92)
Total motility (%)	61 ± 1 (50–70)	59 ± 2 (45–72)	36 ± 2† (14–49)
Normal morphology (%)	53 ± 3 (30–70)	44 ± 3† (30–60)	13 ± 2† (2–26)
Round cells (10^6/mL)	3.5 ± 0.8 (1–13)	2.9 ± 0.6 (1–6)	1.6 ± 0.4 (0.1–9)
Leukocytes (10^6/mL)	0.07 ± 0.05 (0–0.8)	0.05 ± 0.03 (0–0.24)	0.12 ± 0.06 (0–1.0)

* Results are mean ± SEM. Extreme values appear in parentheses.
† $P < .05$ compared with panel 1 of normospermic samples.

posed of the ubiquitous αα isoenzyme and by an unusual isoform (ENO-S), characterized by its particular electrophoretic migration and its sperm specificity. Our results using electrophoresis on cellulose acetate confirm those of Edwards and Grootegoed. In this study, we associated the quantitative measurement of total enolase with the qualitative data of electrophoresis to determine the activity of each isoform. In order to evaluate whether these parameters could help to differentiate normal and abnormal semen, we performed the analysis on 2 well-characterized semen groups showing quite different spermiologic parameters. The first group consisted of fertile normospermic men who had conceived children through natural means, and the second group of infertile abnormospermic men who had never conceived children through natural means.

We measured a higher total enolase activity in spermatozoa from abnormospermic men than in normospermic men. The presence of ENO-αα was demonstrated in the 2 groups, with a higher level in abnormospermic men. Two hypotheses can be proposed to explain this finding. First, ENO-αα is associated with a high level of cell contamination in abnormal semen—indeed, a positive correlation exists between ENO-αα levels and cell contamination ratio. Furthermore, ENO-αα activity is sevenfold higher in total sperm from the abnormal group and was only twofold higher after Percoll selection. Second, ENO-αα could arise from the spermatozoa themselves. In favor of this hypothesis, ENO-αα activity is present in the Percoll-selected sperm extracts that were free of contaminating cells (P95 fraction, panel 1), and a positive correlation exists between ENO-αα and spermatozoa with abnormal morphology (total semen). Thus, the presence of ENO-αα could also be associated with abnormal sperm. That there is no difference in ENO-αα activity in sperm from the 95% Percoll group from normospermic and abnormospermic men is consistent with the notion that ENO-αα is mostly present in the residual cytoplasm surrounding the postacrosomal and midpiece regions of immature sperm and not in mature sperm isolated from the 95% Percoll pellet, which for the most part, are devoid of or have reduced residual cytoplasm. A high proportion of abnormal spermatozoa in the semen of abnormospermic men reflects abnormal spermatogenesis. Spermatogenesis is an orderly process in which male germ cells pass through sequential phases of differentiation to subsequently develop into mature spermatozoa. Before the release of sperm from germinal epithelium of the testis or during early epididymal transit, residual cytoplasm is normally removed from spermatozoa. Failure to release excess cytoplasm results in retention of a cytoplasmic mass

Table 2. *Total enolase, enolase-αα, and enolase-S activities expressed as mIU/10^7 sperm in total sperm from normospermic (panel 1) and abnormospermic men**

Group	Total Enolase Activity (mIU/10^7 sperm)	ENO-αα Activity (mIU/10^7 sperm)	ENO-S Activity (mIU/10^7 sperm)
Normospermic (n = 18)	4.8 ± 0.5 (1.6–10.7)	1.2 ± 0.2 (0.2–3.4)	3.8 ± 0.5 (1.4–9)
Abnormospermic (n = 20)	11.1 ± 1.9† (1.3–31.2)	8.8 ± 1.7† (0.9–27)	2.3 ± 0.2† (0.3–4.0)

* Results are mean ± SEM. Extreme values appear in parentheses.
† $P < .05$ compared with normospermic patients.

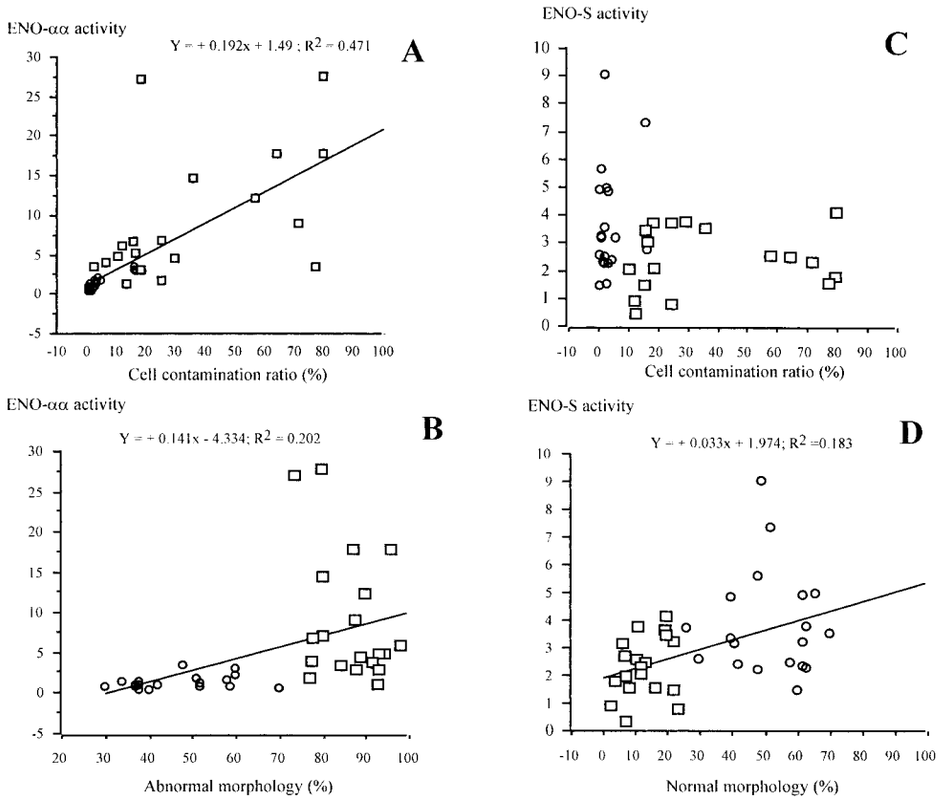

Figure 2. Analysis of the relationships between enolase isoform activities (mIU/10^7 sperm) and either cell contamination ratio (ratio value of precursor germ cells plus leukocytes per milliliter to sperm count per milliliter) or the morphological status of the analyzed spermatozoa in total semen. **(A)** Correlation between ENO-αα activity and cell contamination ratio. **(B)** Correlation between ENO-αα activity and percentage of sperm with abnormal morphology. **(C)** Correlation between ENO-S activity and cell contamination ratio. **(D)** Correlation between ENO-S and percentage of sperm with normal morphology. Normospermic samples (panel 1) appear as open circles (○), abnormospermic samples appear as open squares (□).

in the midpiece region of the spermatozoon. A number of studies have reported that retention of residual cytoplasm is associated with defective sperm function (Gavella et al, 1995; Gomez et al, 1996; Gergely et al, 1999).

Table 3. *Enolase-αα and enolase-S activity (mIU/10^7 sperm) in spermatozoa from total sperm and from 95% Percoll-selected sperm fraction from normospermic (panel 1) and abnormospermic men**

	Normospermic (n = 14)	Abnormospermic (n = 13)
ENO-αα activity		
Total sperm	1.4 ± 0.3 (0.5–3.4)	9.3 ± 2.5‡ (1.7–27.5)
Percoll-selected sperm	0.6 ± 0.1† (0.2–1.3)	2.1 ± 0.8† (0.2–10.7)
ENO-S activity		
Total sperm	4.0 ± 0.5 (2.2–10.0)	2.6 ± 0.3‡ (0.7–4.0)
Percoll-selected sperm	4.4 ± 0.5 (1.4–7.8)	2.6 ± 0.5‡ (0.4–7.0)

* Results are mean ± SEM. Extreme values appear in parentheses.
† $P < .05$ compared with total sperm.
‡ $P < .05$ compared with normospermic men.

In this study we have evaluated, in normospermic men (panel 2), the relationship between sperm with residual cytoplasm and sperm ENO-αα activity in 4 subpopulations isolated from the same semen by discontinuous Percoll density gradient. The high ENO-αα activity found in the P47.5 fraction can be attributed to the high amount of contaminating cells. The other fractions (P60, P80, and P95), which had negligible amounts of contaminating cells, were well discriminated by ENO-αα activity (lower activity in the P95 fraction). In addition, these subpopulations had significant differences in the percentage of sperm with residual cytoplasm (a lower percentage in the 95% Percoll sperm fraction). A cytochemistry approach (Zini et al, 1998) or lipid analysis (Ollero et al, 2000) obtained the same results, confirming a different maturity status of these sperm subpopulations. We demonstrated that ENO-αα was positively correlated with the percentage of sperm exhibiting residual cytoplasm in sperm fractions. These results showed that ENO-αα could be used as a marker of sperm immaturity, as it is for creatine kinase activity (Huszar et al, 1993), NADPH oxidase-like activity (Aitken et al, 1997), superoxide dismutase activ-

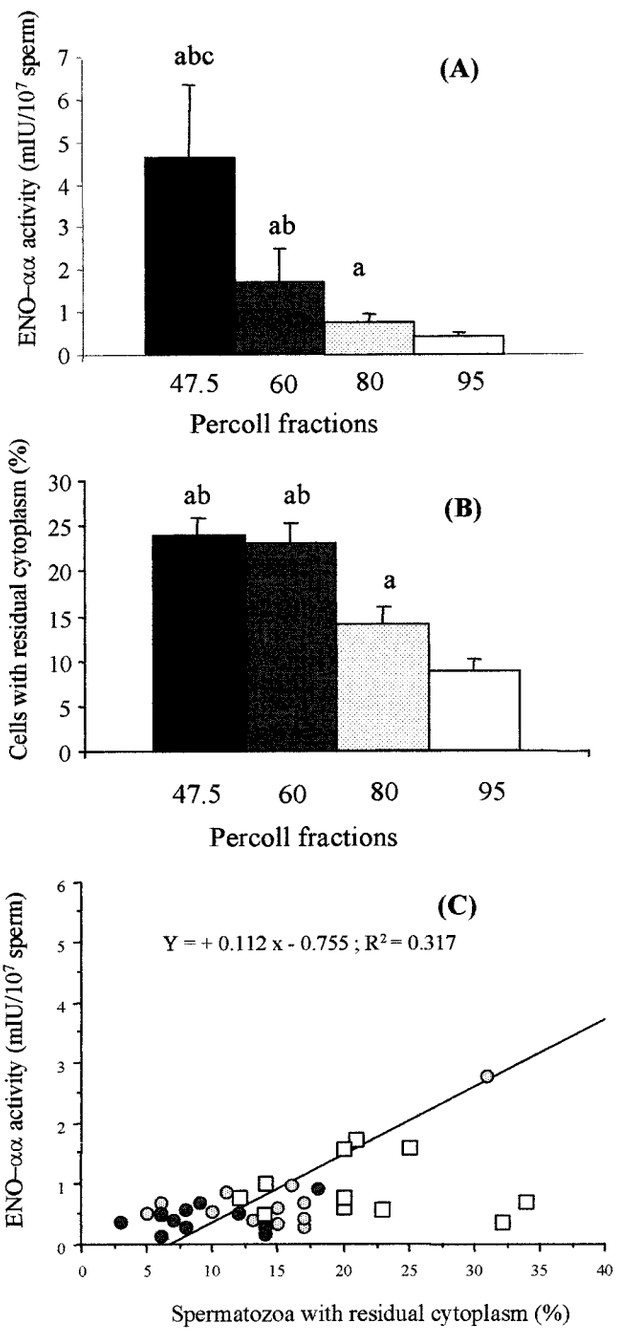

Figure 3. Analysis of the relationship between ENO-αα isoform activity (mIU/10^7 sperm) and the percentage of spermatozoa with residual cytoplasm in sperm populations isolated from the 47.5%, 60%, 80%, and 95% Percoll fractions, respectively. The semen samples were normospermic (n = 12, panel 2). **(A)** ENO-αα activity in sperm subpopulations isolated from 47.5%, 60%, 80%, and 95% Percoll fractions. **(B)** Percentage of spermatozoa with residual cytoplasm in sperm subpopulations isolated from 47.5%, 60%, 80%, and 95% Percoll fractions. **(C)** Correlation between ENO-αα activity and the percentage of sperm with residual cytoplasm. The different Percoll fractions are represented as ● for 60%, ☺ for 80%, and □ for 95%. [a]Compared with P95, $P < .05$; [b]compared with P80, $P < .05$; [c]compared with P60, $P < .05$.

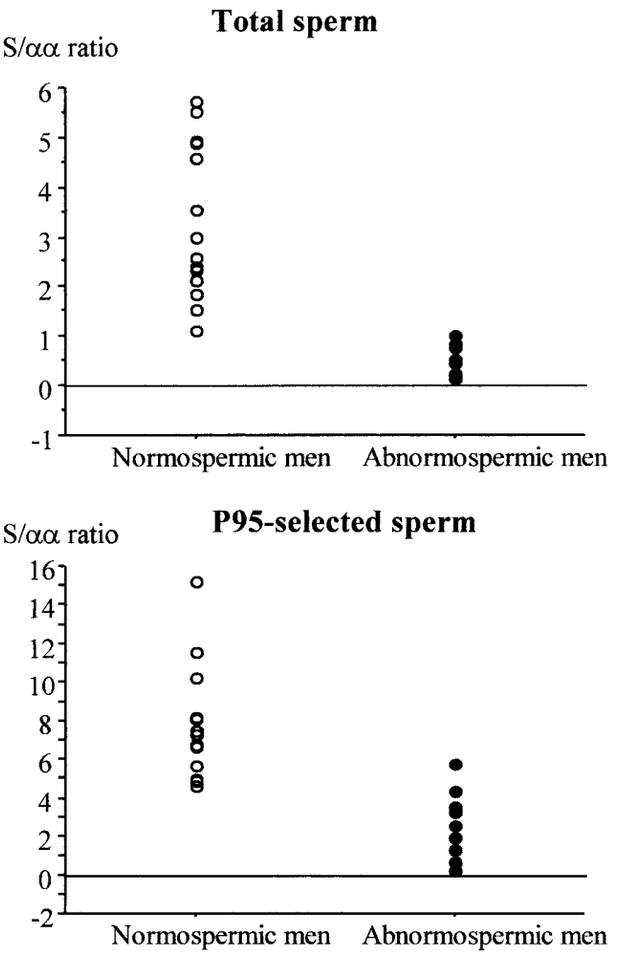

Figure 4. Distribution of ratio values of ENO-S activity to ENO-αα activity (S:αα) in total and P95-selected sperm from normospermic men (panel 1) or men with abnormospermia. The normospermic samples appear as open circles (○); abnormospermic samples appear as solid circles (●).

ity (Aitken et al, 1996), and the activity of several glycolytic enzymes including glucose-6-phosphate dehydrogenase and lactate dehydrogenase (Casano et al, 1991; Aitken et al, 1994).

The presence of ENO-S was demonstrated in both groups, with a higher level in normospermic men. There was no difference in ENO-S activity between total sperm and the Percoll-selected fraction for a result expressed per 10^7 spermatozoa. Thus, Percoll selection does not show a difference in the distribution of populations containing ENO-S. These results were confirmed by the 4-step Percoll selection method in which each sperm fraction presented a similar level of ENO-S. It could be noticed that the morphological data obtained in the different sperm fractions of the 2 Percoll procedures did not show any significant difference in the percentage of spermatozoa with normal morphology. The twice higher ENO-S levels found in normal semen samples compared with abnormal

samples could thus be explained by the difference in the percentage of spermatozoa with normal morphology (49% vs 17%). Finally, in total semen, a positive correlation was found between ENO-S and the percentage of spermatozoa with normal morphology. Altogether, these results suggested ENO-S as a possible marker of sperm with normal morphology.

The study of enolase isoforms provides 2 parameters for describing different aspects of spermatozoa: ENO-αα characterizes abnormal immature spermatozoa, and ENO-S characterizes normally developed spermatozoa.

The inverse variation of enolase isoforms brought us to try to increase the specificity of determining our enzyme by calculating the ENO-S:ENO-αα ratio. A high S:αα value characterized normal semen and a low S:αα value characterized abnormal semen. Huszar et al (1992) developed a similar approach for creatine kinase (CK) by using the isoenzyme CK:MM ratio rather than the CK activity for increasing the diagnostic value of its enzyme marker. It appears that the S:αα ratio would be a more specific index than the ENO-S or ENO-αα activity for differentiating normal vs abnormal semen both in 95% Percoll-selected sperm and in total sperm. The cutoff value for total sperm could be set to 1 and was lower than the P95 fraction, which was 4. However the sample discriminating power of the S:αα ratio was not superior in Percoll-selected sperm compared with total sperm. The diagnostic application of the S:αα ratio in total sperm is complicated by the existence of leukocytes and precursor germ cells, which contain ENO-αα activity; however, this index has the advantage of taking into account the whole cells of the ejaculate and can be easily used in routine practice. Furthermore, after Percoll selection, this index can be used to assess the quality of spermatozoa that are intended for in vitro fertilization.

These preliminary results, obtained in semen with well-defined and opposite spermiologic characteristics, must be completed by a study that includes more samples with unselected spermiologic charateristics. The enolase parameters could allow a better classification and management of infertile men in relation to advanced techniques of in vitro fertilization, and we suggest that the S:αα ratio may be a predictive marker of a man's fertility and may be used to elucidate some cases of male infertility.

In conclusion, the development of defined biochemical criteria for the assessment of human sperm function is an important objective. Enolase constitutes a new candidate for such a diagnostic role. We have demonstrated quantitative and qualitative differences among the spermatozoa of abnormospermic and normospermic men with enolase enzymatic marker. The S:αα ratio seems to be a new, valuable marker of the global quality of spermatozoa in a given semen sample and could represent a predictive index of sperm fertilizing potential.

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