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Correlation of sperm motility with mitochondrial enzymatic activities

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Until now, little attention has been paid to the contribution of mitochondrial dysfunction to germinal tissue disorders. The target of this study was to investigate the relationship between sperm motility and mitochondrial respiratory chain enzyme activities. The results obtained showed that semen samples of control individuals (n = 33) have substantially higher activities of complexes I, II, and IV compared with those of asthenozoospermic subjects (n = 86). Moreover, a direct and positive correlation was found in the whole population studied between spermatozoa motility and all the mitochondrial respiratory complex activities assayed (I, II, I+III, II+III, and IV). The ratio of these enzymes to citrate synthase (a reliable enzymatic marker of mitochondrial volume) activities did not correlate with sperm motility. This suggests that motility depends largely on the mitochondrial volume within the sperm midpiece. These observations could be of physiopathological relevance because they suggest that factors affecting the mitochondrial energy production could be then responsible for particular cases of idiopathic asthenozoospermia.

Current understanding of the role of mitochondria in disease is expanding rapidly, and it is now clear that mitochondrial dysfunction is responsible for a wide variety of disorders. The organs involved in these syndromes are those with a high demand for respiratory energy such as the brain, heart, skeletal muscle, kidneys, liver, endocrine system, and other somatic tissues *(1)*. Little attention has been paid to mitochondrial disorders in germinal tissues. However, several recent reports have considered the possibility that mitochondrial dysfunction could be implicated as a factor in infertility *(2–4)*. In particular, some evidence suggests that mitochondria could play a key role in the energy maintenance of spermatozoa motility, one of the major determinants of male fertility *(5)*. Thus, the presence of a shorter midpiece length and fewer mitochondrial gyres in some asthenozoospermic subjects with respect to a reference group *(6)* has been observed; a correlation between mitochondrial volume and sperm length and flagellar beat frequencies have also been shown *(7)*. Moreover, a correlation between mitochondrial activities and motile spermatozoa proportion has been shown using cytochemical techniques *(8)* and flow cytometry methods *(9)*. However, biochemical evidence supporting a relationship between mitochondrial function and spermatozoa motility is still scarce. Until now, this evidence has been limited to decreased spermatozoa motility induced by treatment with inhibitors of the mitochondrial energy metabolism *(10–13)*.

The common final pathway in the mitochondrial energy metabolism is the electron transfer chain, composed of two mobile carriers (coenzyme Q and cytochrome C) and four multimeric enzymatic complexes (complexes I, II, III, and IV), all embedded in the inner mitochodrial membranes *(14)*. Biochemically defined alterations of these respiratory chain activities are usually associated with all kinds of mitochodrial disorders *(15)*. The target of this study was to investigate the relationship between sperm motility and mitochondrial respiratory chain enzyme activities. The results obtained strongly suggest that sperm motility largely depends on the whole energy production originating in the mitochondrial compartment.

Materials and Methods

PATIENTS AND CONTROL SUBJECTS

We prospectively studied 119 subjects attending the andrology unit of our Hospital. Seminograms were performed according to WHO recommendations. After semen liquefaction, 10 μL was placed onto a glass slide and covered with a clean coverslip, and at least four microscope fields were examined per sample. The sperm in each field were classified into one of the four classifica-

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tions of motility (a, b, c, or d) according to WHO recommendations. Percentages of "a" (rapid progressive motility, moving swiftly in a straight line) plus "b" (slow or sluggish progressive motility, less linear in their progression) were considered as progressively motile spermatozoa *(16)*. Samples with observed spermatozoa agglutination, very high viscosity, or moderate or severe oligozoospermia were discarded. No patient with signs of flagellar disturbance (very low motility but healthy vitality) was found.

Vitality was assayed by the eosin-nigrosin method *(16)* (mean ± SD, 47.58 ± 15.56). A positive and direct correlation was found between vitality and motility of the whole population ($r = 0.4135$; $P < 0.001$).

sample preparation

Semen (0.5–2 mL) was centrifuged for 10 min at 600*g* at room temperature. Seminal plasma was eliminated, and the pellet was washed with saline solution. Samples were centrifuged again for 10 min at 600*g*, supernatants were discarded, and the spermatozoa were resuspended in the required volume of 20 mmol/L potassium phosphate buffer, pH 7.0, to give a final concentration of 2×10^{11} spermatozoa/L. Samples were then homogenized by freeze-thawing before analysis. The appropriateness and validity of mitochondrial disruption by freeze-thawing in hypotonic medium to measure respiratory complex activities has been described previously *(17)*.

respiratory chain complexes assay

The activities of NADH dehydrogenase (complex I) *(18)*, succinate dehydrogenase (complex II) *(19)*, NADH cytochrome C reductase (complex I and III) *(18)*, succinate cytochrome C reductase (complex II and III) *(18)*, cytochrome C oxidase (complex IV) *(20)*, and citrate synthase (CS) *(21)* were measured spectrophotometrically using a Beckman DU-650 Spectrophotometer by monitoring the reduction of ferricyanide (complex I), 2,6-dichlorophenolindophenol (complex II), cytochrome C (complex I+III and II+III), the oxidation of cytochrome C (complex IV), and the appearance of free coenzyme A (CS) in spermatozoa homogenates as previously described. Reactions were monitored for 1 min for complex I and 5 min for the remaining enzymatic activities. Intraassay precision (n = 12) of the same homogenate gave CVs <5% for all the enzymatic assays except for complexes II and IV; CVs for complexes II and IV were 10%. Protein was measured by the method of Lowry et al. *(22)*. Specific activities were expressed as nmol · min^{-1} · mg $protein^{-1}$ *(23)*. All chemicals were from Boehringer Mannheim or Sigma Chemicals.

statistical analysis

Results were expressed as mean ± SD. Differences between means were evaluated by a two-tailed Student's *t*-test for impaired samples. Correlations and linear regression analysis were calculated by a SPSSWIN packet (SPSS, Inc.).

Results

Table 1 shows the mitochondrial enzyme-specific activities of spermatozoa of asthenozoospermic and control individuals. According to WHO recommendations, asthenozoospermia was assigned to semen samples with <50% progressively motile spermatozoa. Eighty-six asthenozoospermic patients (range, 0–45%; 26.7% ± 12.9, mean motility ± SD) and 33 control samples (range, 50–70%; 54.1% ± 5.9, mean motility ± SD) were assayed. The mean age of both groups did not differ substantially (31.6 ± 6.3 years in the asthenozoospermic group and 31.1 ± 8.1 years in the control group). The results showed a significant decrease of the specific activities of complex I ($P = 0.015$), complex II ($P = 0.020$), complex IV ($P = 0.019$), and CS ($P = 0.017$) in asthenozoospermic subjects. However, no significant differences were found in the specific activities in which complex III (complex I+III and complex II+III) was assessed.

The large size of the sample allowed us to perform a statistical regression analysis of the whole population studied, correlating sperm motility and the specific activity of each complex. As shown in Fig. 1, a positive and significant correlation was found in all the specific activities with respect to sperm motility. However, the correlation was lower when the specific activity of complex III (I+III and II+III) was measured, with respect to activities of complexes I and II alone. Because complex III requires the endogenous presence of the mobile carrier coenzyme Q, which could be released from membranes along the experimental procedure, the assessments including complex III are the least reliable of those assayed in this work.

Table 1. Mitochondrial enzyme specific activities in spermatozoa from control and asthenozoospermic individuals

Enzymatic activities	Control subjects[a]	Asthenozoospermic subjects[a]
Citrate synthase	261.4 ± 102.2 (33)	209.7 ± 105.2 (86)[b]
Complex I	715.3 ± 340.0 (33)	544.5 ± 335.7 (83)[b]
Complex II	38.3 ± 16.0 (32)	30.0 ± 17.2 (79)[b]
Complex I + III	18.5 ± 8.2 (32)	18.0 ± 10.2 (75)
Complex II + III	10.8 ± 4.9 (31)	9.8 ± 5.9 (73)
Complex IV	41.8 ± 16.3 (33)	34.3 ± 15.0 (85)[b]

[a] Results are expressed as nmol · min^{-1} · mg $protein^{-1}$ (mean value ± SD). Sample sizes are indicated in parentheses.
[b] Significantly different from control subjects: $P \leq 0.020$.

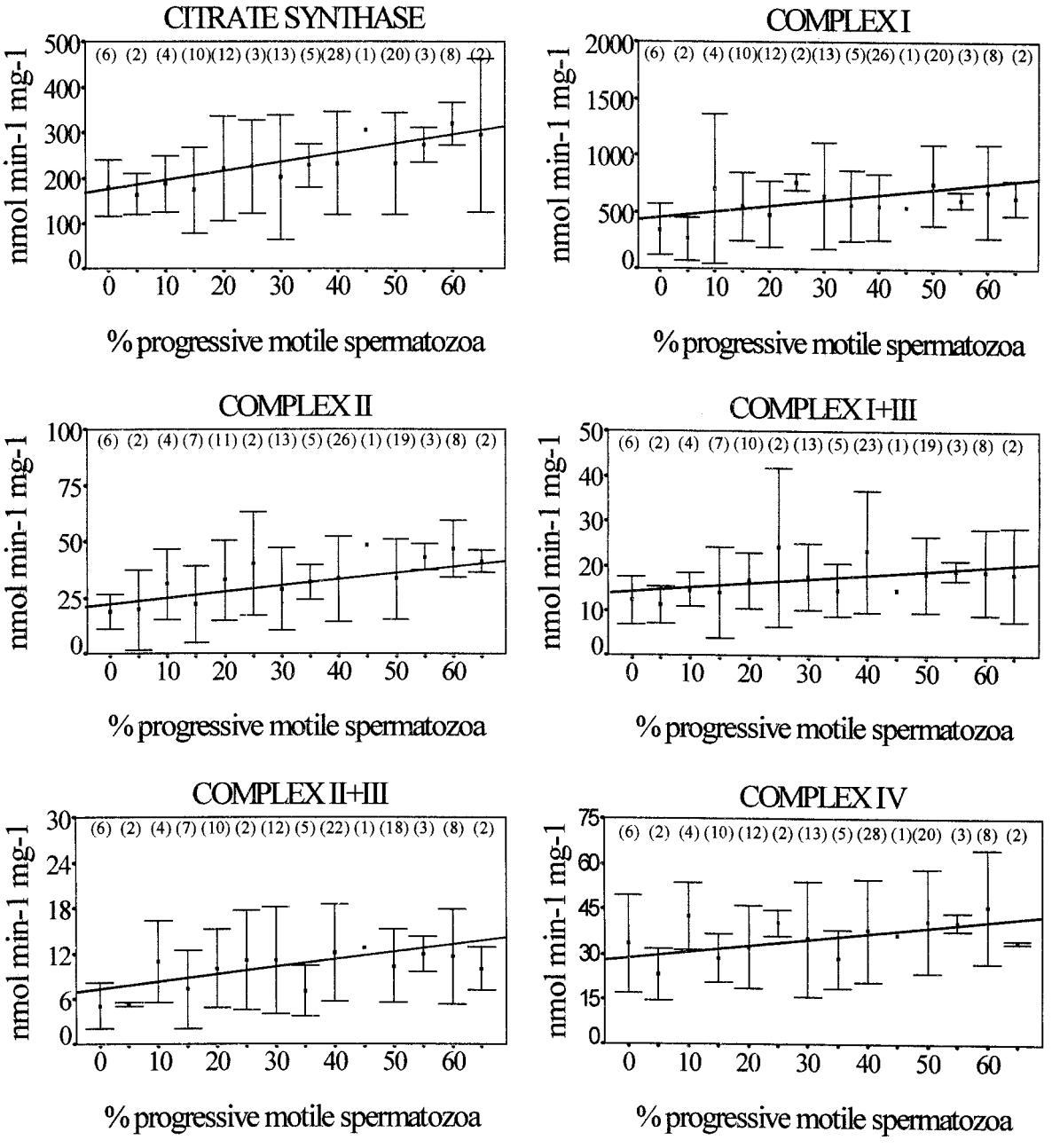

Fig. 1. Correlation between sperm motility and specific activities in spermatozoa.

Linear regression analysis was performed representing sperm motility vs the specific activities of CS ($y = 1.9x + 158.9$, $r = 0.2984$, $P < 0.001$); complex I ($y = 4.5x + 438.7$, $r = 0.2215$, $P = 0.008$); complex II ($y = 0.3x + 21.0$, $r = 0.3220$, $P < 0.001$); complex I+III ($y = 0.1x + 14.2$, $r = 0.2055$, $P = 0.017$); complex II+III ($y = 0.1x + 7.4$, $r = 0.2399$, $P = 0.007$); complex IV ($y = 0.2x + 29.1$, $r = 0.2256$, $P = 0.007$). Values show the mean ± SD of the specific activities found for each motility group. The sample size of these motility groups are indicated in *parentheses* in the *upper part* of every graph.

Therefore, the low correlation obtained for these complexes is less relevant.

The results obtained point out a positive correlation between mitochondrial enzyme-specific activities and sperm motility. However, respiratory complex activities directly reflect the electron transfer capacity, whereas CS is usually considered a reliable marker of the number and/or volume of mitochondria *(24)*. Therefore, the ratio of respiratory complex/CS activities would indicate whether the correlation with sperm motility was because of a partial enrichment in electron transfer chain activities or an overall increase in the mitochondrial metabolic function. The results obtained showed that respiratory chain complexes I, II, I+III, II+III, and IV, when compared with CS activities, did not correlate with sperm motility (complex I, $r = 0.0310$, $P = 0.370$, n = 116; complex II, $r = 0.1491$, $P = 0.059$, n = 111; complex I+III, $r = -0.0036$, $P = 0.485$, n = 107; complex II+III, $r =$

0.0893, $P = 0.184$, n = 104; complex IV, $r = -0.0881$, $P = 0.171$, n = 118).

Discussion

The results obtained clearly show a direct and positive correlation in the whole population between spermatozoa motility and mitochondrial enzyme-specific activities. The observation that the activities of enzyme complexes when compared with that of CS did not correlate significantly with sperm motility suggests that this motility depends largely on mitochondrial volume within the sperm midpiece rather than on specific enrichment of the complexes themselves within each mitochondrion. These results provide strong biochemical evidence to previous ultrastructural observations supporting the correlation between mitochondrial volume and sperm motility *(7)* and strongly suggest that sperm motility largely depends on the whole energy production originating in the mitochondrial compartment.

On the other hand, a previous report noted that patients with primary mitochondrial disorders had reduced sperm motility, with their spermatozoa mitochondria showing the characteristic ultrastructural features of mitochondrial disorders *(25)*. In addition PCR amplification of mtDNA has shown a substantially higher incidence of a mtDNA deletion in patients with asthenozoospermia as compared with unaffected individuals *(26)*. mtDNA deletions are invariably associated with defects in mtDNA-encoded complexes I, III, and IV. However, we have observed a correlation with motility in both mtDNA-encoded complexes (e.g., complexes I and IV) and nuclear DNA-encoded complexes (e.g., complex II), which could not be explained entirely by the presence of different contents in the deleted mtDNA in our samples.

Despite the relatively large degree of scatter found in the mitochondrial enzyme-specific activities, we have found a linear relationship between these specific activities and sperm motility of the population studied in this work, clearly showing a relationship between mitochondrial energy production and cell motility. The pathological relevance of this finding rests in concluding that factors affecting mitochondrial energy production in a given individual could give rise to motility impairment. Such factors could include physiological conditions *(27)* or the well-known sensitivity of sperm to reactive oxygen species *(28)*, xenobiotics toxicity *(29)*, or mtDNA mutations *(26)*. These factors could then be responsible for particular cases of asthenozoospermia and could perhaps contribute to the large variability found in our results.

In conclusion, we have found a close and positive relationship between sperm motility and mitochondrial enzyme-specific activities, suggesting that more specific mitochondrial dysfunctions could be the underlying cause of idiopathic asthenozoospermia. The biochemical assessment of mitochondrial enzymes in sperm samples of idiopathic asthenozoospermic individuals could help to identify cases of mitochondrial-based asthenozoospermia.

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