

## Oral carnitine supplementation increases sperm motility in asthenozoospermic men with normal sperm phospholipid hydroperoxide glutathione peroxidase levels

Andrea Garolla, Ph.D.,<sup>a</sup> Matilde Maiorino, Ph.D.,<sup>b</sup> Alberto Roverato, Ph.D.,<sup>c</sup>  
Antonella Roveri, Ph.D.,<sup>b</sup> Fulvio Ursini, Ph.D.,<sup>b</sup> and Carlo Foresta, Ph.D.<sup>a</sup>

<sup>a</sup>Departments of Histology, Microbiology and Medical Biotechnologies, University of Padova, and <sup>b</sup>Biological Chemistry, University of Padova, Padova; and <sup>c</sup>Department of Social, Cognitive and Quantitative Sciences, University of Modena and Reggio Emilia, Modena, Italy

**Objective:** To clarify the role of carnitine supplementation in idiopathic asthenozoospermia and to look for a rationale for its use in asthenozoospermic patients.

**Design:** Blind clinical study.

**Setting:** Academic.

**Patient(s):** Thirty asthenozoospermic patients divided in two groups according to phospholipid hydroperoxide glutathione peroxidase (PHGPx) levels.

**Intervention(s):** Placebo for 3 months, then oral L-carnitine (2 g/day) for 3 months; semen samples were collected at baseline, after placebo, after carnitine administration, and again after 3 months with no drugs.

**Main Outcome Measure(s):** Evaluation of seminal parameters and determination of seminal PHGPx levels, measured as rescued activity.

**Result(s):** When asthenozoospermic subjects were divided in two groups on the basis of PHGPx levels, we observed an improvement of mean sperm motility only in the group of patients with normal PHGPx levels.

**Conclusion(s):** Phospholipid hydroperoxide glutathione peroxidase has an important role in male infertility, and carnitine treatment might improve sperm motility in the presence of normal mitochondrial function. (*Fertil Steril*® 2005;83:355–61. ©2005 by American Society for Reproductive Medicine.)

**Key Words:** Asthenozoospermia, carnitine, mitochondrial function, phospholipid hydroperoxide glutathione peroxidase, sperm motility

A normal motility pattern in ejaculated spermatozoa is a basic requirement for male fertility. Because adenosine triphosphate (ATP) supports the chemical–mechanical coupling catalyzed by dyneins (specific ATPases of the flagellum), it is widely accepted that both glycolysis and oxidative phosphorylation are required for optimal sperm function (1), and thus it can be assumed that impaired motility might follow a bioenergetic shortage, although other mechanisms are still possible.

In mammalian spermatozoa, mitochondria, the major source of ATP, are wrapped in a keratinous structure, called the *capsule*, which derives from the outer membrane. A major component of this structure is the selenoenzyme phos-

pholipid hydroperoxide glutathione peroxidase (PHGPx) (2). This monomeric member of the family of glutathione peroxidases (3, 4) is specifically expressed in testis (5) and has been seen to play a crucial role in spermatogenesis, being involved in building up the sperm mitochondrial capsule (2). During epididymal maturation, a redox switch takes place in spermatozoa, sparked by glutathione depletion, which primes protein thiol oxidation catalyzed by PHGPx. The enzyme, by using protein thiols as an alternative donor substrate in the peroxidatic reaction, produces disulfides involved in the formation of the highly cross-linked protein network of the capsule. While catalyzing protein thiol oxidation, the enzyme is cross-linked with itself and possibly other proteins and becomes catalytically inactive (2).

Phospholipid hydroperoxide glutathione peroxidase activity, lost during this functional switch, can be “rescued” for analytical purposes, by drastic reductive treatment (6), and a lower “rescued” activity has been shown in asthenozoospermic subjects and interpreted as a biomarker of a defective

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Reprint requests: Carlo Foresta, Ph.D., Center for Male Gametes Cryo-preservation, Via Modena 9, Padova I-35128, Italy (FAX: 39-49-8213222; E-mail: carlo.foresta@unipd.it).

structure and function of the capsule and thus of a “weakness” of mitochondria (7). This conclusion was supported by the observation that lower “rescued” activity of PHGPx was associated with faster progressive loss of motility during in vitro incubation.

Taking advantage of the analysis of PHGPx levels in spermatozoa as a marker of correct structure and function of the mitochondrial capsule, in this study we addressed the issue of the efficacy of a carnitine supplementation to ameliorate motility in asthenozoospermic subjects.

Carnitine is required for transport and oxidation of long-chain fatty acids (8–11). A vitamin nature of carnitine is true only in lower species, in which nutritional deficiency induces a bioenergetic gap associated with lipid accumulation in the cytosol (12). Consistently, carnitine nutritional deficiency does not exist in humans, and severe functional deficiency can only take place in rare cases in which the expression of genes coding for endogenous synthesis or metabolism is altered (13, 14). Nevertheless, carnitine supplementation has been seen to ameliorate sperm motility in some cases of idiopathic asthenozoospermia (15), an effect possibly related to the particularly high concentration in the seminal fluid (16–18). In fact, the positive effect of carnitine supplementation could be rationalized in terms of an optimization of the bioenergetic function of spermatozoa when a saturation of all enzymatic systems involved is not reached by endogenous synthesis.

Here, we report evidence of an effect of carnitine supplementation in ameliorating motility of spermatozoa in idiopathic asthenozoospermic patients, when optimal capsule structure and thus seemingly mitochondrial function is conserved, as judged by PHGPx levels above a critical threshold.

## MATERIALS AND METHODS

### Patients

We studied 30 idiopathic asthenozoospermic patients (aged  $34.1 \pm 4$  years [mean  $\pm$  SD]) who consulted our center for infertility. We confirmed the asthenozoospermic pattern on at least three semen analyses carried out at 1-month intervals by the same analyst. Patients were not affected by prior or concomitant endocrine illnesses, cryptorchidism, or clinical or instrumental evidence of varicocele. Consumption of medications during the 3 months before the study was excluded. Presence of seminal infections, mycoplasma, and chlamydia was ruled out by sperm culture, and the presence of sperm antibodies was excluded with a commercial test (Sperm Mar Test; Ortho Diagnostic System, Milan, Italy). Thirty fertile donors (aged  $34.7 \pm 6$  years) who were proved normozoospermic were used as controls for PHGPx content and seminal parameters.

The Ethics Committee of the Medical Faculty of the University of Padova approved the study, and informed consent was obtained from each participant.

**TABLE 1**

**Seminal parameters of normozoospermic fertile donors and of asthenozoospermic patients at different stages of the study.**

	Asthenozoospermic patients (n = 30)				
	Normo-zoospermic donors (n = 30)	T0 (baseline)	T3 (after 3 months of placebo)	T6 (after 3 months of carnitine)	T9 (3 months from the end of any treatment)
Spermatozoa ( $10^6$ /mL)	$105.7 \pm 115.6$	$66.6 \pm 48.7^b$	$69.6 \pm 40.7$	$71.8 \pm 38.1$	$67.2 \pm 35.4$
Motility (% a + b)	$51.6 \pm 11.8$	$27.1 \pm 10.7^c$	$27.4 \pm 9.0$	$34.6 \pm 12.7^a$	$30.1 \pm 10.3$
Normal morphology (%)	$53.4 \pm 12.8$	$32.4 \pm 11.9^c$	$31.1 \pm 7.5$	$31.6 \pm 8.6$	$32.1 \pm 10.1$
Viability (%)	$82.5 \pm 9.1$	$75.8 \pm 10.2^b$	$77.6 \pm 11.5$	$78.1 \pm 10.9$	$74.4 \pm 11.6$

Note: Results are given as mean  $\pm$  SD. P values of  $<.05$  and  $<.01$  were regarded as significant and highly significant, respectively.  
<sup>a</sup>P  $<.05$  vs. T3.  
<sup>b</sup>P  $<.01$  vs. normozoospermic donors.  
<sup>c</sup>P  $<.001$  vs. normozoospermic donors.

Garolla. Carnitine, PHGPx, and sperm motility. Fertil Steril 2005.

**TABLE 2**

**Motility (percent a + b) at different study stages for asthenozoospermic patients, according to PHGPx level.**

	T0 (baseline)	T3 (after 3 months of placebo)	T6 (after 3 months of carnitine)	T9 (3 months from the end of any treatment)
PHGPx ≤105 mU/mg	23.9 ± 10.3	26.7 ± 10.1	24.8 ± 10.1	24.2 ± 9.8
PHGPx >105 mU/mg	29.3 ± 10.7	27.8 ± 8.5	41.1 ± 9.8 <sup>a</sup>	34.1 ± 8.8

Note: Result are given as mean ± SD. *P* values of <.05 and <.01 were regarded as significant and highly significant, respectively.

<sup>a</sup>*P* < .001 vs. T3.

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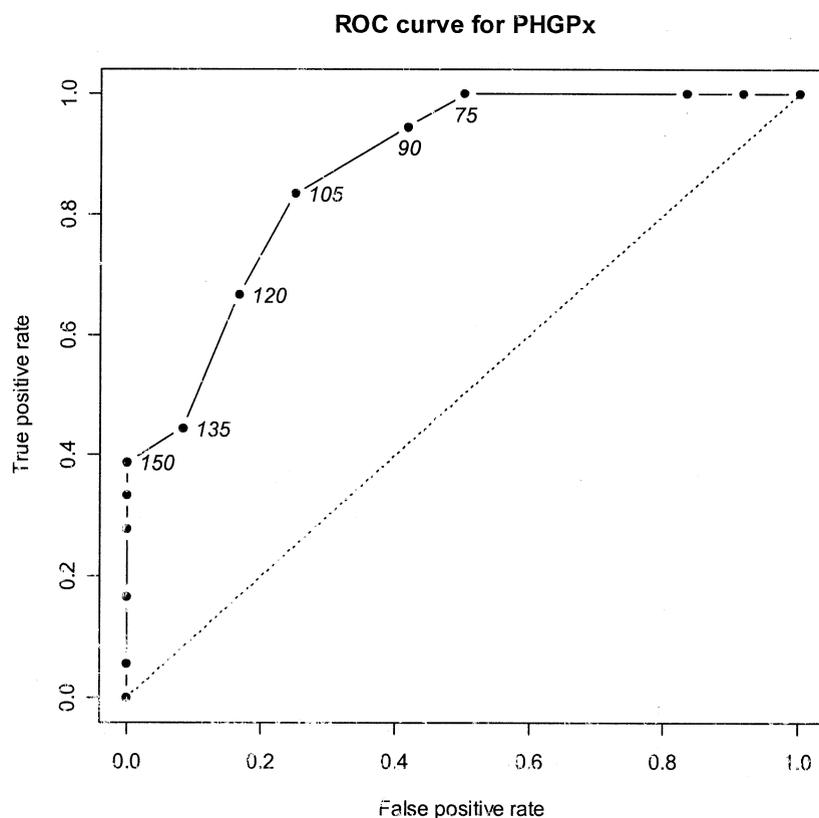
### Semen Processing

Semen samples were obtained by masturbation after 3–5 days of sexual abstinence. After liquefaction at 37°C, seminal volume and pH, sperm concentration, motility, morphol-

ogy, and viability were evaluated according to World Health Organization guidelines (19). To exclude observer bias, sperm motility of the samples was determined by the same person, blind to PHGPx status, microscopically at ×400

**FIGURE 1**

Receiver operating curve for PHGPx content in spermatozoa. The obtained cut-off level of 105 mU/mg produces a true-positive rate of 0.83 and a false-positive rate of 0.25, thus apparently optimizing the balance between sensitivity and specificity.



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magnification after 30 minutes at 37°C. Motility was assigned to the following categories: rapidly progressive (type a), slowly progressive (type b), not progressive (type c), and immotile (type d). Observation of sperm morphology was carried out in at least 200 consecutive Papanicolaou-stained cells for each sample. Spermatozoa were considered normal if there were no defects of the head (length, 4.0–5.5  $\mu\text{m}$ ; breadth, 2.5–3.3  $\mu\text{m}$ ; shape, oval; length/breadth 1.5:1.75; acrosome easily distinguishable), the neck, midpiece, tail, or center part.

The inclusion criterion for a classification of asthenozoospermia was motility (type a + b) <50%. Seminal volume and pH were in the normal range both in control and infertile subjects (semen volume 2.0–4.3 mL; pH 7.6–8.2).

### Measurement of PHGPx Content

Because PHGPx is largely inactive in sperm, the specific activity does not provide any useful information. Therefore, the activity measurement requires a “rescuing” procedure (6), and the results are referred to as “PHGPx content.” A summary of the adopted procedure is reported here. Ejaculates were diluted with phosphate-buffered saline (PBS) and centrifuged at  $600 \times g$  for 10 minutes at 4°C. The pellet containing spermatozoa was washed twice with PBS and stored at  $-20^\circ\text{C}$  for up to 1 week. Pellets were dissolved in 0.1 mol/L Tris-HCl, 6 mol/L guanidine-HCl, pepstatin A (0.5  $\mu\text{g}/\text{mL}$ ), and 0.1 mol/L 2-mercaptoethanol, pH 7.5. Proteins were measured by the Lowry method and the sample diluted to a final concentration of 0.5 mg/mL with the same buffer.

Before activity measurement, mercaptoethanol and guanidine-HCl were removed by passing the sample (0.25/0.5 mL) twice through an NAP-5 column (Pharmacia, Uppsala, Sweden) equilibrated with elution buffer (0.1 mol/L Tris-HCl, pH 7.5, containing 3 mmol/L reduced glutathione (GSH), 5 mmol/L ethylenediaminetetraacetic acid, and 0.1% [vol/vol] Triton X-100). Activity of PHGPx was then measured in 2.2 mL of this buffer, to which glutathione reductase (GSSG reductase, 0.6 IU/mL) and 0.03 mmol/L reduced nicotinamide adenine dinucleotide phosphate (NADPH) were added. The mixture was incubated for 5 minutes at 25°C and, after recording the basal rate of NADPH oxidation, the reaction was started by adding 30  $\mu\text{mol}/\text{L}$  phosphatidylcholine hydroperoxide, prepared as described in the original procedure.

The activity of PHGPx was spectrophotometrically measured at room temperature from the time course of absorbance decrease at 340 nm. The basal rate, although negligible, was subtracted from the enzyme activity rate. Activity of PHGPx was calculated from the regression curve obtained from three different amounts of sample. Activity is given in milliunits (mU), defined as nanomoles of substrate converted per minute.

We measured sperm PHGPx only at the beginning and then considered its value as a constant throughout the study.

During the set-up of the method for PHGPx evaluation, we performed inter- and intra-assay tests that demonstrated both the reproducibility of the measurement and that this parameter remained constant in different samples from the same patient over time. We confirmed PHGPx levels on at least two semen samples evaluated at 1-month intervals in a blind fashion by two different analysts.

### Treatment

Asthenozoospermic patients were orally and daily supplemented with placebo (1 flacon 10 mL/day, containing d-1 malic acid, sodium benzoate, sodium saccarinatum biidrate, pineapple aroma  $1 \times 1,000$ , and purified water) for 3 months (T0 to T3), and then with L-carnitine (2 g) (1 flacon 10 mL/day, containing d-1 malic acid, sodium benzoate, sodium saccarinatum biidrate, pineapple aroma  $1 \times 1,000$ , purified water, and L-carnitine [2 g] intact salt) for a further 3 months (T3 to T6) in a blind fashion. Seminal analyses were performed at the start of the study (T0), after 3 months of placebo (T3), after 3 months of carnitine therapy (T6), and after 3 months from the end of therapy (T9) by the same person, in a blind fashion with respect to treatment type and PHGPx status.

### Statistical Analysis

Statistical comparison between groups was assessed by the Student's *t*-test for either paired or unpaired data. The results are given as mean  $\pm$  SD. *P* values of <.05 and <.01 were regarded as significant and highly significant, respectively. The analysis was carried out with the free statistical software “R.” The association of variables was evaluated by multivariate linear regression.

In Table 1, the empirical distribution of sperm concentration is asymmetric with positive skewness. For this reason, comparison of means has been carried out with respect to log-transformed data, whose distribution looks approximately normal. Nevertheless, to improve readability of Table 1, we give the mean and SD for the original, untransformed data.

### RESULTS

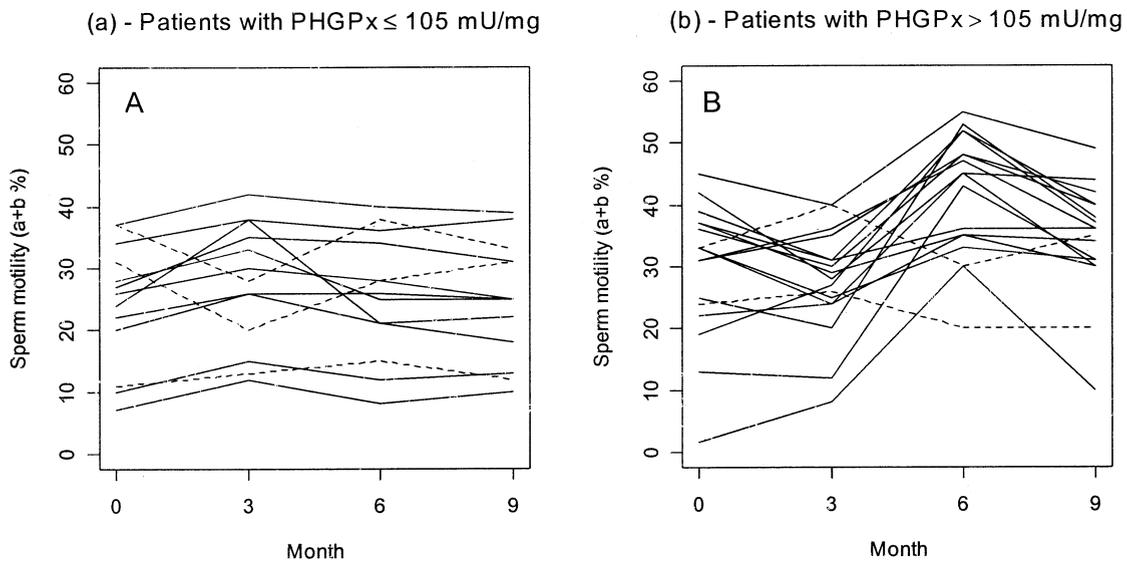
As previously observed (7) in spermatozoa of asthenozoospermic patients, PHGPx levels ( $121.0 \pm 50.2$  mU/mg of protein) were lower than in healthy controls ( $195.1 \pm 53.1$  mU/mg of protein) ( $P < .001$ ).

Seminal parameters of the asthenozoospermic patients, compared with those of fertile subjects, presented the typical pattern of asthenozoospermia: decreased progressive motility (a + b <50%) and a reduced percentage of normal sperm morphology ( $32.4\% \pm 11.9\%$  vs.  $53.4\% \pm 12.8\%$ ). On the other hand, sperm concentration ( $>20 \times 10^6/\text{mL}$ ) and viability (viable cells >50%) were in the normal range.

Seminal parameters of fertile controls and of the asthenozoospermic patients at the different times of the study [base-

## FIGURE 2

Sperm motility of asthenozoospermic patients at different stages of the study. Observed motility values of subjects (A) below the PHGPx threshold and (B) above the threshold are reported.



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line (T0), after 3 months of placebo administration (T3), after 3 months of carnitine supplementation (T6), and 3 months after the end of therapy (T9)] are reported in Table 1. After 3 months of carnitine supplementation (T6), a significant increase in sperm motility was observed ( $P < .05$ ), which disappeared when supplementation was suspended (T9). Other seminal parameters (sperm count, morphology, and viability) did not change during the study.

The receiver operating characteristic curve for PHGPx is shown in Figure 1. This curve shows that the PHGPx level has a good discriminatory power in asthenozoospermic subjects in separating responders from nonresponders to carnitine supplementation. At a PHGPx level of 105 mU/mg, a true-positive rate of 0.83 and a false-positive rate of 0.25 were observed, providing a satisfying balance between sensitivity and specificity. A minor shift of the threshold to values  $< 105$  mU/mg would lead to an improvement of the true-positive rate, smaller than the corresponding increase in the false-positive rate. As an example, with a threshold set at 90 mU/mg, the true-positive rate becomes 94.4% (11.1% more than when the threshold was 105 mU/mg), but the false-positive becomes 41.7% (16.7% more than the value obtained with a threshold of 105 mU/mg). Similarly, a shift of the threshold to a PHGPx value  $> 105$  mU/mg decreases the false-positive rate, but the effect is counteracted by a decrease of the true-positive rate.

We can conclude that, if the sensitivity and the specificity of the procedure are considered on the same footing, then the PHGPx value 105 mU/mg is an optimal threshold value.

Figure 2 reports the motility values at different times of the study on subjects sorted in two groups on the basis of the 105 mU/mg threshold for PHGPx content. Among the 13 patients with sperm PHGPx levels  $\leq 105$  mU/mg (Fig. 2A), carnitine improved sperm motility only in 3 subjects (dashed lines). Among the 17 patients with PHGPx levels  $> 105$  mU/mg (Fig. 2B), the supplement effectively increased motility in 15, whereas motility worsened in 2 patients (dashed lines).

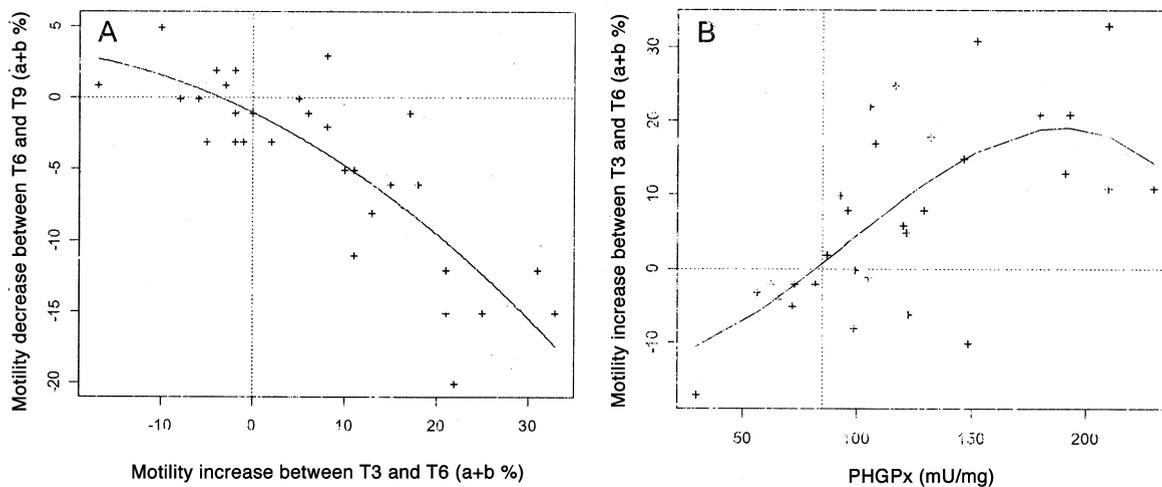
Table 2 reports the motility data at different times of the study on subjects sorted into two groups on the basis of the threshold PHGPx level.

The relation between PHGPx and sperm motility showed no correlation during placebo treatment, whereas a significant positive correlation was observed after carnitine supplementation (data not shown). The fitted linear regression is  $y = 14.2 + 0.17x$  ( $P < .001$ ), with 95% confidence intervals of 4.7–23.8 and 0.10–0.24 for the slope parameter.

The plot of PHGPx content vs. the relative increment of motility in T6 vs. T3 is reported in Figure 3A, indicating that carnitine supplementation is progressively more efficient in affecting motility when PHGPx activity is higher, up to a saturation point when a normal physiologic content is reached. Conversely, the decrease of motility, taking place after the end of supplementation, is also correlated with previous carnitine supplementation (Fig. 3B). The greater the increase at T6, the greater the decrease at T9.

**FIGURE 3**

Relationship between PHGPx content and sperm motility increase after carnitine supplementation. **(A)** Relationship between sperm motility increase after carnitine supplementation (T3 – T6) and sperm motility decrease when supplementation is discontinued. The difference (T6 – T9) of the motility results obtained after carnitine supplementation (T6) and when supplementation is discontinued (T9) is plotted vs. the difference (T3 – T6) obtained after placebo (T3) and after carnitine treatment (T6). Data fit a polynomial regression, and the value of the adjusted  $R^2$  coefficient is 0.67. This figure clearly shows that in all cases, a sperm motility increase after carnitine supplementation corresponds with a decrease at 3 months after the end of treatment. **(B)** The difference (T6 – T3) of the motility results obtained after placebo administration (T3) and after carnitine supplementation (T6) is plotted vs. PHGPx content. Data fit a polynomial regression, and the adjusted  $R^2$  coefficient is 0.40. Phospholipids hydroperoxide glutathione peroxidase content, evaluated as “rescued” specific activity in spermatozoa, did not change throughout the study.



Garolla. Carnitine, PHGPx, and sperm motility. *Fertil Steril* 2005.

**DISCUSSION**

Asthenozoospermia is a relevant issue in male infertility management. The efficiency of sperm motility, required for fertilization capacity, might decrease in the presence of different factors, eventually leading to infertility. A failure in producing metabolic energy is the most reasonable cause of asthenozoospermia when obvious extracellular causes (e.g., infection, varicocele) have been ruled out. Spermatozoa are cells sentenced to death, and it seems reasonable that a reduced sperm motility represents the initial hallmark of depressed mitochondrial function, eventually leading to sperm death.

It is generally accepted that although human sperm produce ATP from glycolysis, mitochondrial oxidative phosphorylation also accounts for an equally important energy supply (19). In normal sperm, mitochondria are embedded in a keratin-like matrix containing, among other proteins, a substantial amount of PHGPx. The capsule formation takes place in late spermatogenesis and during epididymal maturation, through a massive protein thiol oxidation catalyzed by PHGPx and primed by GSH depletion (2).

The actual function of the capsule is not known, and only a nonspecific role in stabilizing the midpiece has been sug-

gested, so that defective architecture of the capsule can be easily seen as an event leading to mitochondrial dysfunction and diminished ATP production. This hypothesis is supported by our observation that a lower PHGPx level in the capsule is associated with asthenozoospermia and faster decline of motility during *in vitro* incubation (7).

The evidence reported here, that carnitine improves sperm motility when asthenozoospermic subjects have PHGPx levels above a critical threshold, fits the above notion. The effect of carnitine is usually seen on the basis of the well-known effect on mitochondrial long-chain fatty acid metabolism and transport (8–11). Moreover, recent studies indicate that the activation of the formation of a long-chain fatty acid carnitine derivative could also be beneficial through a different mechanism. Carnitine supplementation, by pulling the formation of long-chain fatty acid carnitines that are known to have a protective effect on cell biomembranes (20), could prove competent for the prevention of the mitochondrial phase of apoptosis (21, 22).

Although this specific antiapoptotic effect of carnitine has not been specifically addressed in spermatozoa, it is tempting to speculate that the particularly high concentration in seminal plasma (16, 17, 23) could be involved in delaying a pro-

grammed death pathway in spermatozoa. In this respect, carnitine function can be seen as coherent and synergistic with that of capsule, and thus of PHGPx. Apparently, carnitine cannot protect mitochondria when already damaged or weakened by a defective capsule structure. The progressive loss of capacity to convert metabolic energy is a peculiar feature of mammalian spermatozoa (24, 25). The pathway of the decay, however, has to be precisely tuned, and infertility might arise from too fast a decay. In this light, the observed synergism between carnitine and capsule PHGPx content can be seen on the basis of the common issue of delaying the loss of mitochondrial bioenergetic function and eventually sperm motility.

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