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Coenzyme Q10 levels in idiopathic and varicocele-associated asthenozoospermia

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Summary

Levels of coenzyme Q10 (CoQ10) and of its reduced and oxidized forms (ubiquinol, QH_2, and ubiquinone, Q_{ox}) have been determined in sperm cells and seminal plasma of idiopathic (IDA) and varicocele-associated (VARA) asthenozoospermic patients and of controls. The results have shown significantly lower levels of coenzyme Q10 and of its reduced form, QH_2, in semen samples from patients with asthenospermia; furthermore, the coenzyme Q10 content was mainly associated with spermatozoa. Interestingly, sperm cells from IDA patients exhibited significantly lower levels of CoQ10 and QH_2 when compared to VARA ones. The QH_2/Q_{ox} ratio was significantly lower in sperm cells from IDA patients and in seminal plasma from IDA and VARA patients when compared with the control group. The present data suggest that the QH_2/Q_{ox} ratio may be an index of oxidative stress and its reduction, a risk factor for semen quality. Therefore, the present data could suggest that sperm cells, characterized by low motility and abnormal morphology, have low levels of coenzyme Q10. As a consequence, they could be less capable in dealing with oxidative stress which could lead to a reduced QH_2/Q_{ox} ratio. Furthermore, the significantly lower levels of CoQ10 and QH_2 levels in sperm cells from IDA patients, when compared to VARA ones, enable us to hypothesize a pathogenetic role of antioxidant impairment, at least as a cofactor, in idiopathic forms of asthenozoospermia.

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Introduction

The overproduction of reactive oxygen species (ROS) can be deleterious to sperm and has been associated with male subfertility (Aitken & Clarkson, 1987). Owing to their high content of polyunsaturated fatty acids as major building blocks of cellular and intracellular membranes, human spermatozoa are particularly prone to oxygen-induced damage. In addition, the relationship between incidence of spermatozoa with midpiece defects and increased rate of lipoperoxidation has been demonstrated (Rao *et al.*, 1989). Such a relationship assumes an important meaning, especially if associated with other reports demonstrating a marked increase of ROS in oligospermia, in sperm motility defects and/or abnormal sperm morphology, both in animals and in man (Alvarez & Storey, 1982; Aitken *et al.*, 1989; Suleiman *et al.*, 1996).

To counteract the deleterious effects of ROS, spermatozoa possess a number of antioxidant systems, including chain-breaking antioxidants, capable of decreasing oxidant radical levels that would otherwise initiate and propagate free radical chain reactions. Ubiquinol is an important liposoluble chain-breaking molecule associated with membranes and lipoproteins (Frei *et al.*, 1990; Stocker *et al.*, 1991; Kontush *et al.*, 1994), and is present in human sperm (Alleva *et al.*, 1997). Whereas the antioxidant power of ubiquinol in plasma and lipoproteins is well documented (Stocker *et al.*, 1991; Kontush *et al.*,

1994; Alleva *et al.*, 1997), its involvement as a protective molecule against ROS-induced damage in human sperm is still relatively unknown.

Previous studies have shown the presence of higher levels of coenzyme Q10 (CoQ10) in normal semen samples compared to pathological ones (Mancini *et al.*, 1994; Lewis *et al.*, 1995). In the present study, we investigated the levels of total CoQ10 and of its reduced and oxidized forms (ubiquinol and ubiquinone, respectively) in sperm cells and in seminal plasma from subjects affected by idiopathic (IDA) or varicocele-associated (VARA) asthenozoospermia, to identify a possible pathogenetic role of these substances in subfertile men, where reduced sperm motility was the main clinical problem.

Patients and methods

Twenty-four males, affected by asthenozoospermia, were enrolled in the study. The patients were selected at the Andrology Unit of the Division of Endocrinology, Umberto I Hospital, University of Ancona (Italy). All subjects underwent medical screening, including history and clinical examination and presented a clinical history of subfertility of at least 2 years. Testicular volume was evaluated in each patient using Prader's orchidometer. The following investigations were also performed to furnish a complete diagnosis: mixed antiglobulin reaction (MAR) test (SperMar test, Diasint, Florence, Italy) for antispermatozoa antibodies (Ab); sperm culture and urethral specimens collection for the detection of *Chlamydia* and *Mycoplasma U. urealyticum*; FSH, LH, testosterone (T), oestradiol (E2) and prolactin (PRL) assays, using commercial radioimmunoassay kits; testicular, prostatic and seminal vesicles ultrasonography and echo-colour Doppler of venous spermatic plexus, for the detection of anatomical abnormalities and varicocele.

The following criteria were adopted for patient eligibility: (i) sperm count $>20 \times 10^6$ ml^{-1}, sperm motility (class a + b; see below for motility grading) <50% at two distinct sperm analyses; (ii) normal serum levels of gonadotrophins, T, E2 and PRL; (iii) absence of infectious genital diseases, anatomical abnormalities of the genital tract, and antispermatozoa Ab; (iv) absence of systemic diseases.

Two distinct groups of patients were identified on the basis of echo-colour Doppler evidence of reflux in spermatic venous plexus: (i) patients with varicocele-associated asthenozoospermia (VARA, $n=12$; mean age 35 ± 2), and (ii) patients with idiopathic asthenozoospermia (IDA, $n=12$; mean age 33 ± 5 years).

The control group ($n=5$) included 5 normal donors (mean age 31 ± 4 years) with a sperm count $>20 \times 10^6$ ml^{-1}, and normal sperm motility (class a + b > 50%) at two distinct sperm analyses, according to World Health Organization criteria (WHO, 1999). All other conditions described above were respected, including absence of reflux in the spermatic venous plexus.

To assess the source of CoQ10 and its reduced and oxidized forms in seminal plasma, an additional group of azoospermic patients was included in the study. In fact, a relevant aliquot of CoQ10 could theoretically be derived from the fraction of dead spermatozoa present in the ejaculate. Semen samples from azoospermic patients ($n=5$) due to testicular failure were collected; diagnosis of testicular failure was made on the basis of high serum levels of FSH (>15 mUI ml^{-1}) and a reduced testicular volume (<10 ml), as determined by Prader's orchidometer.

Seminal fluid analysis

Semen quality was assessed by the same biologist in terms of sperm concentration, motility and morphology, in accordance with the World Health Organization criteria (WHO, 1999). Briefly, seminal fluid was obtained by masturbation after 3–5 days of sexual abstinence. The samples were kept in the Andrology lab at room temperature and processed within 1 h of ejaculation. Sperm count was determined with the Makler chamber. Motile spermatozoa were assessed by phase contrast microscopy (10 µl of semen was delivered onto a glass slide and covered with a 22×22 mm coverslip) and graded as follows: class a and b, fast and weak forward motility; class c, non-progressive motility; class d, immobile spermatozoa. Sperm morphology was evaluated on smears of seminal fluid, stained with Giemsa method and observed by oil immersion light microscopy.

Percoll fractionation of semen samples

Sperm cells were separated from round cells (sperm cells precursors and leukocytes) by a Percoll gradient. Percoll stock solution (100%) was diluted to 90% by adding (9 : 1 v/v) 1.5 M NaCl 100 mM HEPES, pH 7.4. The liquefied semen samples were layered on top of discontinuous 40–80% Percoll gradients and centrifuged

at room temperature at $500 \times g$ for 18 min. Sperm cells recovered from the top fraction and the pellet were washed with 0.15 M NaCl. Round cells remained in the intermediate layers. The concentration and viability of cell samples were determined once again prior to ubiquinol/ubiquinone measurements.

Ubiquinol/ubiquinone assay

CoQ10, QH_2 and Q_{ox} levels were assayed in sperm cells (asthenozoospermic patients and control group) and in seminal plasma using a Beckman Gold HPLC System (Beckman Instruments, San Ramon, CA, USA) equipped with an electrochemical detector (EC, ESA 5100 model, Bedford, MA, USA), fitted with a 5021 conditioning cell and a 5010 analytical cell. The three electrodes in series were, respectively, set at −0.6; −0.15; +0.6 V.

After adding 250 µl of isopropanol to 10×10^6 sperm cells or 50 µl of seminal plasma, test tubes were vortexed for 1 min, and centrifuged for 3 min at $1500 \times g$. Following centrifugation, 100 µl of the isopropanol upper phase were directly injected into the HPLC apparatus. The mobile phase was prepared by dissolving lithium perchlorate 10 mM (final concentration) in methanol : isopropanol : ethanol (78 : 2 : 20), filtered under vacuum and degassed under argon. Identification of the ubiquinol and ubiquinone peaks was carried out by injection of pure standards at known concentrations, calculated on the basis of the extinction coefficients (Podda *et al.*, 1996).

Statistical analysis

Statistical analysis was performed using the SAS statistical package. Results are reported as mean values ± standard error. Differences among the samples were evaluated by *t*-test and the Kolmogorov-Smirnov and Shapiro-Wilk tests were used to appraise whether the data were random samples from a normal distribution.

Results

Table 1 shows the results of cell count, motility and number of cells with abnormal morphology found in the groups examined. As expected, after patient preselection, the control group had a significantly higher sperm motility and a significantly lower percentage of spermatozoa with abnormal morphology than the IDA and VARA groups. VARA and IDA patients had comparable cell counts and percent of teratozoospermia, whereas the motility of VARA patients' spermatozoa was slightly higher than that observed in IDA patients.

Table 1. Semen features in studied patients

Groups	*n*	Spermatozoa millions ml^{-1}	Motility class a+b	Teratozoospermia (%)
Controls	5	64.0 ± 8.0	56.0 ± 1.9	41.2 ± 1.7
IDA	12	48.3 ± 5.2	26.0 ± 2.5*§	68.4 ± 2.7*
VARA	12	55.8 ± 5.0	33.1 ± 2.1*	63.7 ± 0.9*

*Controls vs. IDA or VARA, $P < 0.001$.
§IDA vs. VARA, $P < 0.05$.

Tables 2 and 3 show the levels of QH_2, Q_{ox}, total CoQ10 and the QH_2/Q_{ox} ratio, in IDA and VARA patients and controls, in sperm cells and seminal plasma, respectively. Sperm cells of the control group had significantly higher levels of QH_2, Q_{ox} and total CoQ10 than those of the IDA and VARA groups. Analogous results were obtained with seminal plasma (Table 3). Moreover, sperm cells of IDA patients had significantly lower levels of QH_2 and of total CoQ10, compared to those of VARA patients (Table 2).

Also QH_2, Q_{ox}, total CoQ10 and QH_2/Q_{ox} ratio in seminal plasma of azoospermic men were similar to those of the control group and significantly higher than those of IDA and VARA patients.

The QH_2/Q_{ox} ratio was significantly higher in the control group than the IDA patients, both in sperm cells (Table 2) and seminal plasma (Table 3). Furthermore, it was significantly higher in the seminal plasma of the control group than in the VARA one (Table 3).

Discussion

Defective sperm function is the most common cause of subfertility and is still difficult to evaluate and treat. This difficulty stems in part from an incomplete understanding of the pathophysiology of sperm function. In this context, excessive production of ROS by abnormal spermatozoa and/or by contaminating leukocytes was recognized as one of the few defined etiologies for male subfertility. In fact, the capacity of human sperm to fertilize mainly depends on sperm motility and membrane integrity, and ROS are known to impair such parameters by inducing lipid peroxidation (Alvarez & Storey, 1982;

Table 2. Coenzyme Q10 levels in sperm cells

Groups	*n*	QH_2 (ng/10^6 sperm cells)	Q_{ox} (ng/10^6 sperm cells)	CoQ10 (ng/10^6 sperm cell)	QH_2/Q_{ox}
Controls	5	$2.29 \pm 0.24^{\circ\circ}$	2.23 ± 0.14	$4.63 \pm 0.26^{\circ\circ}$	$1.02 \pm 0.08^{\circ}$
IDA	12	$0.95 \pm 0.05^{\circ\circ}$	1.70 ± 0.13*	$2.65 \pm 0.14^{\circ\circ}$	$0.60 \pm 0.07^{\circ}$
VARA	12	1.36 ± 0.09*§	1.79 ± 0.14*	$3.08 \pm 0.14^{\circ\circ}$§	0.81 ± 0.07

*Controls vs. IDA or VARA, $P<0.05$.
**Controls vs. IDA or VARA, $P<0.01$.
°Controls vs. IDA or VARA, $P<0.02$.
°°Controls vs. IDA or VARA, $P<0.001$.
§IDA vs. VARA, $P<0.05$.

Table 3. Coenzyme Q10 levels in seminal plasma

Groups	*n*	QH_2 ng ml^{-1}	Q_{ox} ng ml^{-1}	CoQ10 ng ml^{-1}	QH_2/Q_{ox}
Controls	5	44.40 ± 2.22	26.80 ± 1.06	71.80 ± 3.62	1.67 ± 0.13
IDA	12	12.33 ± 0.79**	16.66 ± 0.75**	29.66 ± 1.13**	0.75 ± 0.05*
VARA	12	20.16 ± 0.91**	17.25 ± 0.73**	37.41 ± 1.70**	1.18 ± 0.06*
Azoospermic Patients	5	39.40 ± 1.50	23.60 ± 0.81	63.80 ± 2.85	1.68 ± 0.11

*Controls and azoospermic patients vs. IDA or VARA, $P<0.01$.
**Controls and azoospermic patients vs. IDA or VARA, $P<0.001$.

Aitken & Clarkson, 1987; Aitken *et al.*, 1989; Rao *et al.*, 1989; Suleiman *et al.*, 1996).

Seminal fluid normally contains antioxidants which are likely to quench ROS, thus protecting spermatozoa from oxidative damage. However, during infection and/or inflammation, or in the presence of environmentally toxic substances, or for metabolic reasons still unknown, such antioxidant defences may decrease thus generating a situation of oxidative stress. In this context, low concentrations of chain-breaking antioxidants may either represent a cause, or, in some cases, be an indication of sperm oxidative stress (Lucesoli & Fraga, 1994).

The antioxidant role of QH_2 has been well established in plasma lipoproteins (Stocker *et al.*, 1991; Kontush *et al.*, 1994; Alleva *et al.*, 1995), but its role in semen is at present uncertain. Previous studies have shown higher levels of CoQ10 in normal than in pathological semen fluid (Mancini *et al.*, 1994; Lewis *et al.*, 1995). In patients with varicocele, the correlation between CoQ10 and sperm motility was lacking (Mancini *et al.*, 1998).

In the present investigation, we have clearly shown that all patients with asthenozoospermia (IDA and VARA groups) have lower levels of total CoQ10 and QH_2 when compared to patients with normal sperm motility, and that CoQ10 is mainly concentrated in the cell fraction. It is worthwhile noting that total CoQ10, QH_2, Q_{ox}, as well as QH_2/Q_{ox} ratio in seminal plasma of azoospermic men, were found to be similar to those of the control group. These data clearly indicate that the source of CoQ10 in seminal plasma is the secretory activity of the genital tract, rather than leakage from dead or damaged spermatozoa.

Furthermore, QH_2/Q_{ox} ratio is lower in sperm cells from patients with idiopathic asthenozoospermia and in seminal plasma from patients with idiopathic or varicocele-associated asthenozoospermia. Studies conducted in blood or lipoproteins clearly suggest that the ubiquinol/ubiquinone ratio can be considered an index of the oxidative status (Lagendijck *et al.*, 1997). Furthermore, it was clearly demonstrated in rat testes after acute iron intoxication, that oxidative damage to lipids and DNA is concurrent with decrease of antioxidants and the QH_2/Q_{ox} ratio (Lucesoli & Fraga, 1994). In the light of these recognized data, the QH_2/Q_{ox} ratio could therefore be considered an index of oxidative stress and our results would suggest the presence

of increased oxidative stress in semen from IDA and VARA patients compared to controls.

Whether the reduced levels of antioxidants are a primary cause or the consequence of ROS excess in the semen of IDA and VARA patients, is still an open question. However, the significant reduction of total CoQ10 and of ubiquinol in sperm cells from IDA patients compared to VARA ones enables us to hypothesize that a lack antioxidant reserve could play a pathogenetic role, or at least be a cofactor, in different forms of asthenozoospermia. Non-enzymatic total radical-trapping antioxidant potential of seminal plasma has been recently highlighted (Rhemrev *et al.*, 2000) and we might reasonably suggest that CoQ10 is one of the compounds contributing to its total antioxidant buffer capacity.

In conclusion, data from the present study suggest that total CoQ10, ubiquinol levels and the QH_2/Q_{ox} ratio should be evaluated in subfertile patients, both as a microenvironmental alteration index (oxidative stress) and for a deeper pathogenetic insight.

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