Oxidative Stress Negatively Affects Human Sperm Mitochondrial Respiration

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OBJECTIVE To correlate the level of oxidative stress in serum and seminal fluid and the level of sperm deoxyribonucleic acid (DNA) fragmentation with sperm mitochondrial respiratory efficiency.

METHODS Sperm mitochondrial respiratory activity was evaluated with a polarographic assay of oxygen consumption carried out in hypotonically treated sperm cells. A possible relationship between sperm mitochondrial respiratory efficiency, the level of oxidative stress, and the level of sperm DNA fragmentation was investigated.

RESULTS Sperm motility was positively correlated with mitochondrial respiration but negatively correlated with oxidative stress and DNA fragmentation. Interestingly, sperm mitochondrial respiratory activity was negatively affected by oxidative stress and DNA fragmentation.

CONCLUSION Our data indicate that sperm mitochondrial respiration is decreased in patients with high levels of reactive oxygen species by an uncoupling between electron transport and adenosine triphosphate synthesis. This reduction in mitochondrial functionality might be 1 of the reasons responsible for the decrease in spermatozoa motility. UROLOGY 82: 78–83, 2013. © 2013 Elsevier Inc.

Mitochondria of human spermatozoa are involved in the production of adenosine triphosphate (ATP), which is necessary for a variety of cellular processes, thereby assuring the quality and the fertilizing ability of these germinal cells. Mitochondrial ATP is particularly important for sperm cell motility,[1] although an active debate currently exists on the role of cytosolic ATP, produced by glycolysis, in this respect.[2,3] Mitochondria, which are more efficient in terms of ATP yield when compared with cytosolic glycolysis, produce energy by the complex mechanism of oxidative phosphorylation (OXPHOS). In the OXPHOS, the electron transport catalyzed by the respiratory chain is strictly coupled to the adenosine diphosphate (ADP) phosphorylation promoted by ATP synthase.

For proper functionality of spermatozoa, an adequate level of reactive oxygen species (ROS) is required.[4] At low concentrations, ROS are involved in sperm hyperactivation and capacitation, acrosome reaction, spermatozoa-oocyte fusion, and other molecular events implicated in human fertility.[5-8] High levels of ROS have been associated with low sperm motility and infertility.[9-12] Indeed, ROS at elevated concentrations attack and damage almost all biomolecules, including deoxyribonucleic acid (DNA), protein, lipid, and carbohydrate. To contrast this harmful role of ROS, living organisms have developed several antioxidant mechanisms, which are enzymatic and nonenzymatic.[4,8] It is worth mentioning that high levels of ROS in seminal fluid might derive from morphologically or functionally abnormal spermatozoa[11,13] or by an excess of leukocytes, or both.[12] In addition, high levels of ROS might be the consequence of defective antioxidant mechanisms.

What is the relationship between ROS and mitochondria inside spermatozoa? At least in principle, a double link could exist because mitochondria, on the one hand, might represent 1 of the ROS generators[14]; whereas, on the other hand, it might represent 1 of the ROS targets.[8,15] Particularly intriguing is the "mitochondrial theory of aging" which assigns to mitochondria, as ROS generator and as ROS target, a fundamental role in aging.[16] When mitochondria become a target of elevated levels of ROS, the process of OXPHOS might be severely affected as a consequence of a possible damage of proteins and membrane lipids.

In this study, we investigated the level of oxidative stress in serum and seminal fluid of normozoospermic and asthenozoospermic patients, along with the level of sperm DNA fragmentation. A study on the correlation of these parameters with sperm mitochondrial respiratory efficiency has then been carried out.

MATERIALS AND METHODS

Patients

We studied 40 semen samples from the same number of patients (between 26 and 49 years of age) from infertile couples or from

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couples wanting a child. Patients did not have any conditions that might interfere with the semen analysis. This study was conducted according to guidelines established for research on human subjects.

Semen Analysis

Semen samples were collected after 3-5 days of sexual abstinence. Samples were collected by masturbation and examined directly after liquefaction in a period <30 minutes. Semen analysis was performed according to World Health Organization (WHO) guidelines.[17]

The following variables were taken into consideration: ejaculate volume (mL), sperm concentration ($\times 10^6$/mL), total sperm number ($\times 10^6$/ejaculate), and motility (%). Automated computer analysis of sperm motility (Computer-aided sperm analysis − Sperm Class Analyzer, LabIVF Asia Pte Ltd, Singapore) was carried out on all semen samples. In addition, according to WHO guidelines, semen samples were classified as leukocytospermic (leukocyte concentration $>1 \times 10^6$/mL, n = 31) and nonleukocytospermic (n = 9).

Mitochondrial Respiration Studies

Spermatozoa were collected by centrifugation at 800 *g* for 10 minutes at room temperature and washed by resuspension in isotonic salt medium (2 g/L bovine serum albumin, 113 mM KCl, 12.5 mM KH_2PO_4, 2.5 mM K_2HPO_4, 3 mM $MgCl_2$, 0.4 mM ethylenediaminetetraacetic acid, and 20 mM tris adjusted to pH 7.4 with HCl). They were then subjected to hypotonic treatment essentially as previously described.[18,19]

Oxygen uptake by hypotonically treated spermatozoa was measured at 36°C by using a Clark-type oxygen probe (Oxygraph, Hansatech Instruments, King's Lynn, UK), in the presence of mitochondrial respiratory substrates (10 mM pyruvate and 10 mM malate) and 0.76 μM ADP. The rate of oxygen uptake by sperm mitochondria (V) was expressed as nmol $O_2 \times mL^{-1} \times minute^{-1}$. The respiratory control ratio (RCR) was calculated by dividing V_3 (rate of oxygen uptake measured in the presence of respiratory substrates + ADP) by V_4 (rate of oxygen uptake measured with respiratory substrates alone).

Serum Reactive Oxygen Metabolites

Serum levels of reactive oxygen metabolites (ROMs) were measured using a commercially available assay kit (d-ROMs Test, Diacron International, Grosseto, Italy). This test, which accurately detects serum hydroperoxides as their derivatives,[20] is a marker of oxidative stress in the organism. The d-ROMs test is based on the reaction of serum with transition metal ions (ferrous sulfate) to form alkoxy and peroxy radicals. The in vitro-formed radicals react with an alkyl-substituted aromatic amine (N,N-diethylparaphenylendiamine) generating a pink-colored derivative, detectable spectrophotometrically at 505 nm. The results are expressed in Carratelli units according to the following formula: 1 Carratelli = (Abs/minute) × F (a correction factor with an assigned value, according to the results obtained with the calibrator) = 0.08 mg H_2O_2/dL.

Analysis of Sperm DNA Fragmentation

Semen samples were diluted to a concentration of $5\text{-}10 \times 10^6$ cells/mL. Sperm chromatin dispersion test was carried out according to Fernández et al's procedure.[21] Slides were stained with Wright for light field microscopy and the percent of DNA fragmentation was determined for each semen sample.

Lipid Peroxidation Measurement in Semen Samples

The measurement of lipoperoxides (LPO) in whole sperm was performed using a commercially available assay kit (LP Sperm Test, Diacron International, Grosseto, Italy). The assay is based on the ability of peroxides to promote the oxidation of Fe^{2+} to Fe^{3+}. The product of peroxidation (Fe^{3+}) binds to the thiocyanate, developing a colored complex measured photometrically at 505 nm. LPO were expressed as millimoles LPO/10^6 spermatozoa.

Statistical Analysis

Data are presented as mean ± standard deviation. Nonparametric Spearman Rank correlation analysis was performed to identify significant correlations between sperm progressive motility, mitochondrial respiratory efficiency, ROS levels, and DNA fragmentation. A value of $P < .05$ was considered statistically significant. Linear regression analysis was performed to examine the observed relationship between different parameters.

RESULTS

Semen Samples

In this study, the semen samples provided by 40 patients were analyzed as reported previously (data not shown). They were divided into 5 motility classes with 10% increments between classes (5%-15%, n = 12; 16%-26%, n = 8; 27%-37%, n = 8; 38%-48%, n = 7; 49%-59%, n = 5). Sperm motility was measured by computer-aided sperm analysis and ranged from 5% to 55% among semen samples, thereby allowing us to separate the patients into 2 main categories, the normozoospermic (n = 16) and the asthenozoospermic (n = 24) ones. This classification was made according to the criteria of the WHO guidelines, which indicated a value of 32% as the lowest progressive motility for a normozoospermic patient.

Sperm Motility Is Positively Correlated With Mitochondrial Respiration but Is Negatively Correlated With Oxidative Stress and DNA Fragmentation

Sperm motility is 1 of the main elements influencing the quality of semen and consequently its fertilizing ability. We therefore investigated the dependence of progressive motility of spermatozoa on several factors, such as mitochondrial respiratory efficiency, ROMs levels, and DNA fragmentation. Figure 1A shows a strong positive correlation between sperm mitochondrial respiratory efficiency expressed as RCR and progressive motility ($r_s = 0.651$, $P < .0001$). RCR is a commonly used parameter in mitochondrial respiration studies calculated dividing V_3 (rate of oxygen uptake by mitochondria in the presence of substrates + ADP, also known as active state, or state 3, of respiration) by V_4 (rate of oxygen uptake by mitochondria with substrates alone, also known as resting state, or state 4, of respiration). These findings fully confirm that sperm motility requires an active mitochondrial respiration.[1,19,22] On the contrary, Figure 1B shows a strong negative correlation between the levels of

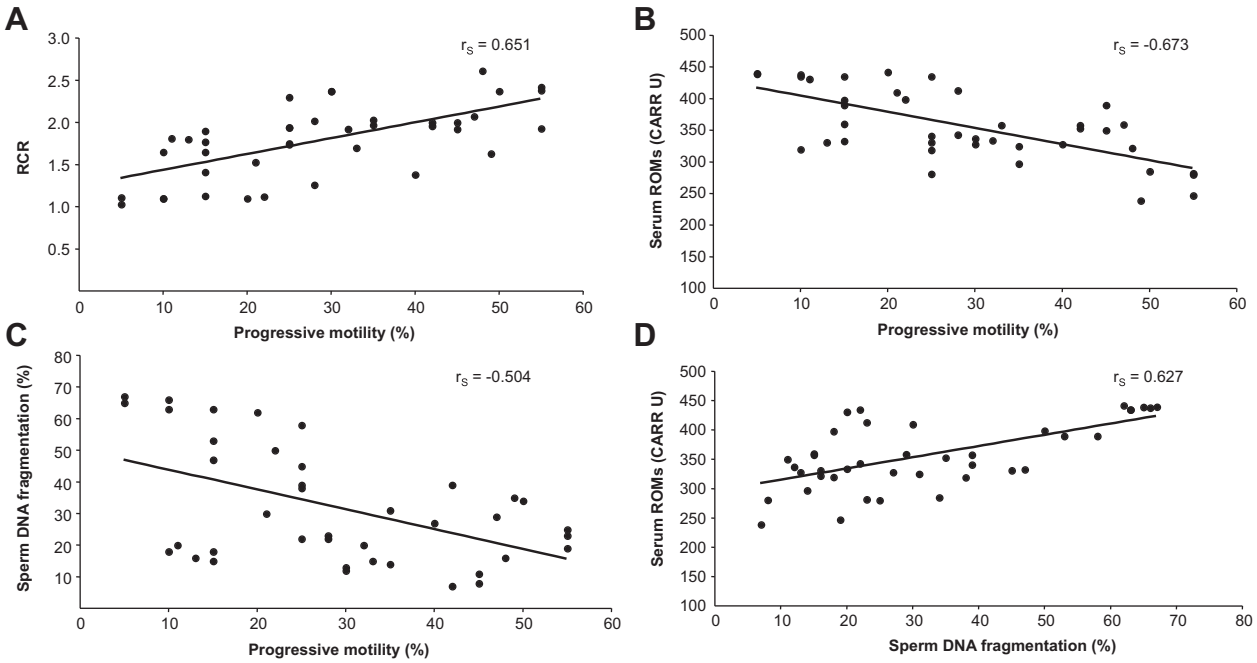

Figure 1. (A) Progressive motility vs respiratory control ratio (RCR) values (P <.0001). **(B)** Progressive motility vs serum reactive oxygen metabolites (ROMs) levels (P <.0001). **(C)** Progressive motility vs sperm deoxyribonucleic acid (DNA) fragmentation (P <.001). **(D)** ROMs levels vs sperm DNA fragmentation (P <.0001).

serum ROMs in the patients analyzed in this study and the percentage of sperm progressive motility (r_s = −0.673, P <.0001). A significant negative correlation, even if less pronounced when compared with that shown in Figure 1B, was also found between sperm DNA fragmentation and sperm progressive motility (Fig. 1C, r_s = −0.504, P <.001). Finally, a significant positive correlation was identified between the levels of serum ROMs and the degree of sperm DNA fragmentation (Fig. 1D, r_s = 0.627, P <.0001).

The Mitochondrial Respiratory Activity Is Negatively Affected by Oxidative Stress and DNA Fragmentation

We then analyzed the dependence of V_3 and V_4, representing active and resting state of sperm mitochondrial respiration, respectively, on the serum ROMs concentration. Interestingly, although V_3 exhibited a low positive correlation with ROMs (Fig. 2A, r_s = 0.284, P <.05), V_4 showed a significantly higher correlation with radical species (Fig. 2B, r_s = 0.526, P <.0005). As a result, a strong negative correlation was found between the levels of serum ROMs and the sperm mitochondrial respiratory efficiency (Fig. 2C, r_s = −0.660, P <.0001).

A similar behavior was found when analyzing the relationship between seminal LPO and sperm mitochondrial respiratory activity. Figure 3A reports the moderate dependence of V_3 on the level of LPO (r_s = 0.336, P <.05); whereas, in Figure 3B the strong correlation between V_4 and LPO (r_s = 0.626, P <.0001) is clearly evident. This different correlation of V_4 and V_3 with LPO levels implied a strong negative correlation of RCR with seminal LPO as shown in Figure 3C (r_s = −0.602, P <.0001).

We eventually investigated the dependence of sperm mitochondrial respiratory efficiency on sperm DNA fragmentation. Although V_3 was virtually independent of DNA fragmentation (Fig. 4A, r_s = 0.011, P >.05), V_4 showed a significant dependence on sperm DNA fragmentation (Fig. 4B, r_s = 0.396, P <.005). As expected, the sperm mitochondrial respiratory efficiency, expressed as RCR, was negatively correlated with sperm DNA fragmentation (Fig. 4C, r_s = −0.603, P <.0001).

COMMENT

ROS production might be beneficial or deleterious for living organisms. This concept applies also in the case of spermatozoa, which require low levels of ROS to manifest their full fertilizing ability. On the contrary, oxidative stress is deleterious for spermatozoa and many other cellular types. Indeed, an excess of ROS has been implicated in several diseases such as diabetes, cancer, atherosclerosis, Parkinson disease, and so forth.[8] Oxidative stress might also be a consequence of unhealthy lifestyles such as smoking, alcohol abuse, or exposition to chemical or electromagnetic pollution. In this study, we therefore investigated the level of serum ROMs, a systemic indicator of oxidative stress, and that of sperm LPO, a specific indicator of oxidative stress in seminal fluid, in a group of patients under andrological control. Indeed, the analysis of blood oxidative status carried out in parallel with seminal profile and sperm oxidative stress might be useful for an overall evaluation of sperm

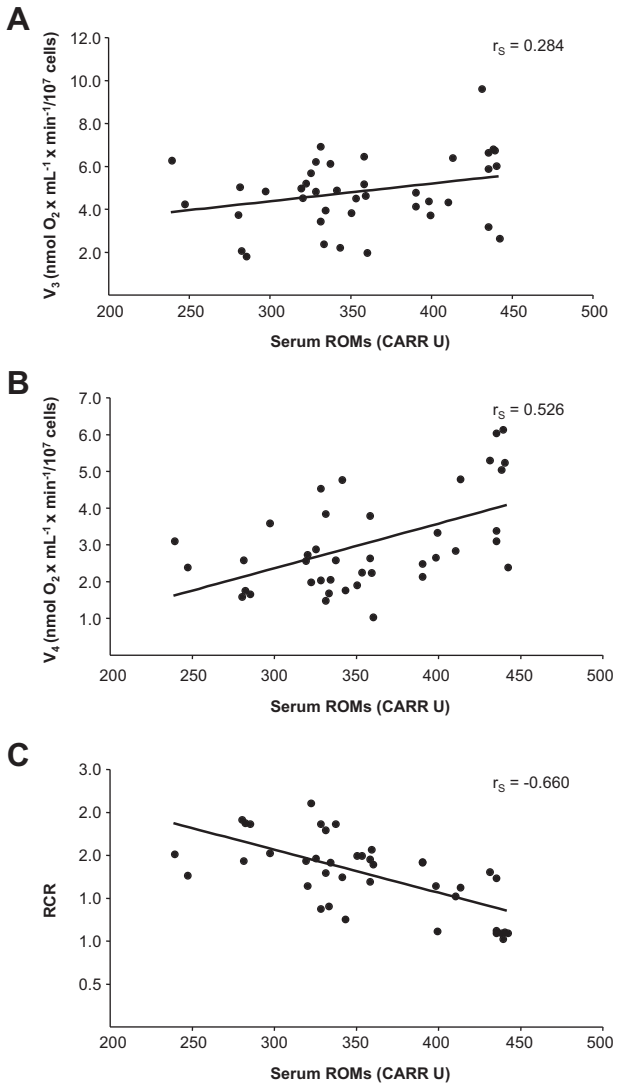

Figure 2. (A) Serum reactive oxygen metabolites (ROMs) levels vs V_3 values ($P < .05$). **(B)** Serum ROMs levels vs V_4 values ($P < .0005$). **(C)** Serum ROMs levels vs respiratory control ratio (RCR) values ($P < .0001$).

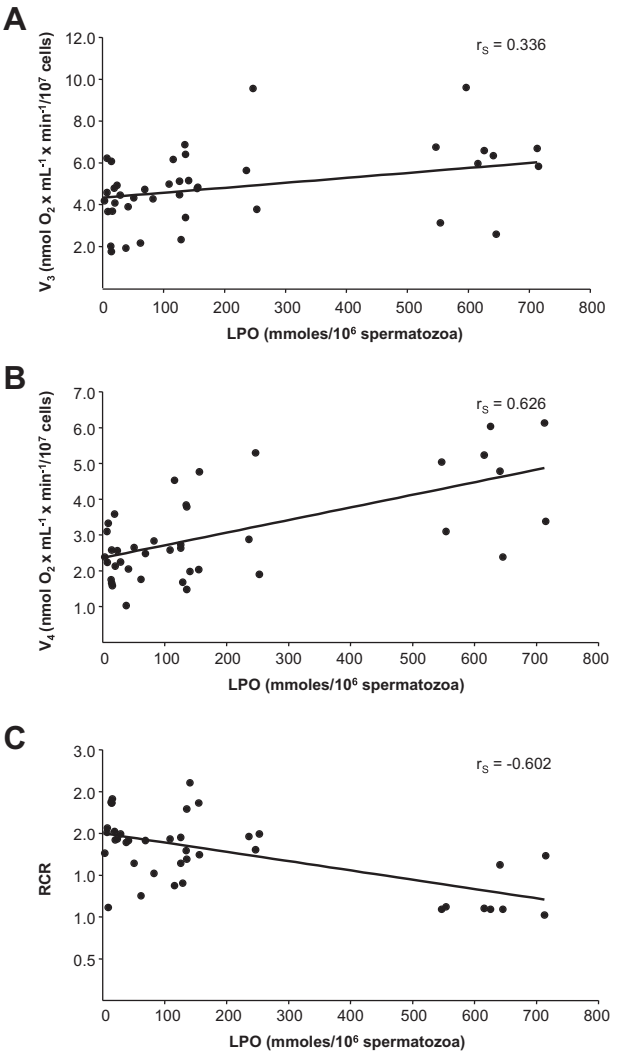

Figure 3. (A) Lipoperoxides (LPO) levels vs V_3 values ($P < .05$). **(B)** LPO levels vs V_4 values ($P < .0001$). **(C)** LPO levels vs respiratory control ratio (RCR) values ($P < .0001$).

quality.[23,24] The degree of sperm DNA fragmentation has also been evaluated.

Interestingly, the negative correlation between oxidative stress, along with DNA fragmentation, and progressive motility of spermatozoa found in this study agrees well with previous results reported in other studies.[8,13] Nevertheless, the molecular mechanisms implicated in this phenomenon are still poorly understood. To shed some light on this intricate field, we analyzed the possible influence of oxidative stress on sperm mitochondrial respiration, 1 of the main determinant of spermatozoa motility.[1,2,19,22] To achieve this, we measured the RCR, which expresses the efficiency of mitochondrial respiration and its coupling with the mitochondrial synthesis of ATP. Interestingly, sperm mitochondrial RCR is negatively correlated with serum ROMs and seminal LPO. The 2 values of oxygen rates (V_3 and V_4) used for RCR calculation, however, showed a different dependence on ROS and LPO. Indeed, although V_3 moderately increased with ROMs and LPO, V_4 showed a significantly higher dependence on both these reactive species. As a result, RCR, which is calculated by dividing V_3 by V_4, negatively correlated with oxidative stress. To the best of our knowledge, this is the first report on a strong negative correlation between oxidative stress and mitochondrial respiratory efficiency in human sperm mitochondria. Notably, a negative correlation between mitochondrial membrane potential, another, although indirect, indicator of sperm mitochondria function, and ROS abundance in seminal fluid has previously been reported.[25,26]

In this study, a positive correlation between oxidative stress and sperm DNA fragmentation has also been found, thereby confirming previous results.[8,27,28] In addition, we found an inverse relationship between sperm DNA fragmentation and sperm mitochondrial respiration. Indeed, RCR negatively correlated with DNA fragmentation as a result of an almost complete independence of V_3 and a positive dependence of V_4 on DNA fragmentation. In

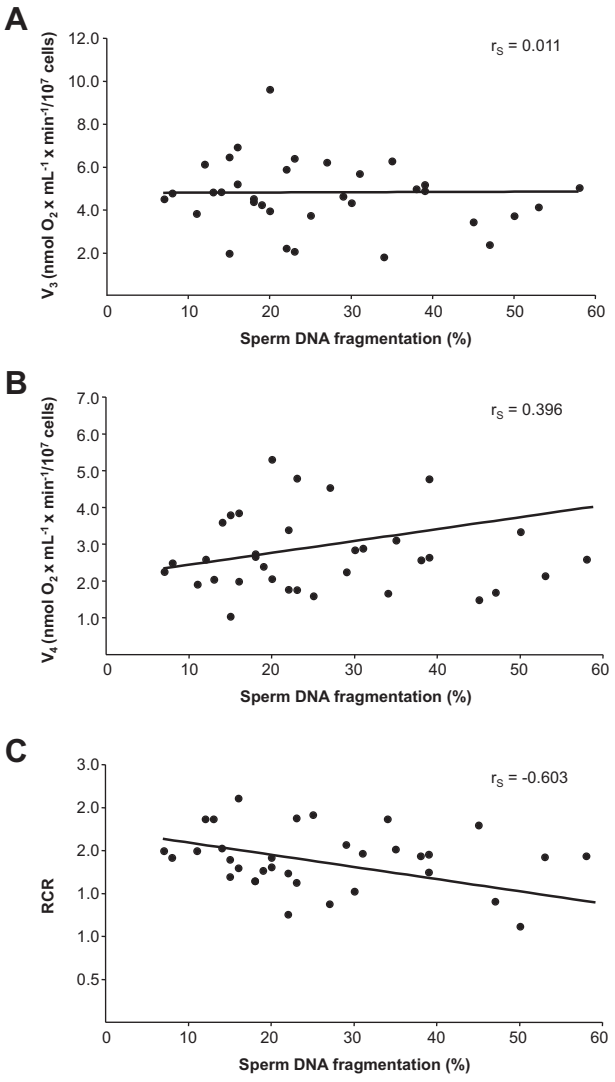

Figure 4. (A) Sperm deoxyribonucleic acid (DNA) fragmentation vs V_3 values ($P > .05$). **(B)** Sperm DNA fragmentation vs V_4 values ($P < .0005$). **(C)** Sperm DNA fragmentation vs respiratory control ratio (RCR) values ($P < .0001$).

this study, the fragmentation of total sperm DNA has been analyzed, but it is likely that sperm mitochondrial DNA was also damaged because the latter is more sensitive to oxidative stress than nuclear DNA.[15,29]

Why does V_4 show such a high dependence on oxidative stress and DNA fragmentation? At first, it must be clarified that an increase in V_4 suggests a stimulus of mitochondrial respiration independent of ADP phosphorylation. In other terms, it seems that during oxidative stress, a lower coupling between mitochondrial respiration and ATP synthesis occurs. It is possible that high levels of ROS might damage the inner mitochondrial membrane in which the respiratory complexes are deeply embedded. The rise in the level of seminal LPO observed in this study might corroborate this hypothesis by taking into account that they might derive from the polyunsaturated fatty acids of the mitochondrial membrane phospholipids. Cardiolipin, a marker phospholipid of the inner mitochondrial membrane, contains considerable amounts of polyunsaturated fatty acids and might therefore represent a suitable target during oxidative stress.[30] The coupling between respiration and ADP phosphorylation requires a strict impermeability of the inner mitochondrial membrane to create, and to productively use, a transmembrane electrochemical gradient, which is the driving force for the synthesis of ATP. Any factor disturbing the electrochemical gradient across the inner mitochondrial membrane uncouples respiration from ATP synthesis.

Nevertheless, we cannot exclude an inverse flux of "cause-effect" for the observed correlations reported in this study that an uncoupling of mitochondria, induced by a currently unknown mechanism, might represent the primary event that, in turn, causes the oxidative stress detected in our semen samples. In fact, a partially uncoupled mitochondrial respiration might promote ROS production, thereby creating a vicious circle in which mitochondria might be the creator and the victim of the oxidative damage.

Eventually, several studies[1] clearly demonstrated a correlation between an impairment of sperm mitochondrial OXPHOS and reproductive ability, most probably caused by low sperm motility. Interestingly, in the present study, a low mitochondrial respiratory efficiency (RCR = 1.5 ± 0.2) and a low sperm progressive motility (28.8 ± 15.7%) were associated to patients (n = 20) who have difficulty in conceiving.

CONCLUSION

The results reported in this study indicate that oxidative stress, along with the concomitant phenomenon of sperm DNA fragmentation, negatively affects sperm mitochondrial respiration by an uncoupling between electron transport and ATP synthesis. This reduced mitochondrial respiratory efficiency might be 1 of the causes responsible for the decrease in progressive motility of human spermatozoa. However, oxidative stress might have further targets beyond mitochondria and therefore other molecular mechanisms might be implicated in the observed reduction of sperm motility.

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