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UVB irradiation as a tool to assess ROS-induced damage in human spermatozoa

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SUMMARY

One of the consequences of oxygen metabolism is the production of reactive oxygen species (ROS) which in a situation of imbalance with antioxidants can damage several biomolecules, compromise cell function and even lead to cellular death. The particularities of the sperm cell make it particularly vulnerable to ROS attack compromising its functionality, mirrored in terms of fertility outcome and making the study of the origin of sperm ROS, as well as the alterations they cause very important. In the present work, we used UVB irradiation, an easy experimental approach known as a potent inducer of ROS formation, to better understand the origin of ROS damage without any confounding effects that usually exist in disease models in which ROS are reported to play a role. To address these issues we evaluated sperm mitochondrial ROS production using the Mitosox Red Probe, mitochondrial membrane potential using the JC-1 probe, lipid peroxidation through BODIPY probe and vitality using PI. We observed that UVB irradiation leads to an increase in sperm mitochondrial ROS production and lipid peroxidation that occur previously to an observable mitochondrial dysfunction. We concluded that sperm UVB irradiation appears to be a good and easily manipulated *in vitro* model system to study mitochondria-induced oxidative stress in spermatozoa and its consequences, which may be relevant in terms of dissecting the action pathways of many other pathologies, drugs and contaminants, including endocrine disruptors.

INTRODUCTION

Reactive oxygen species (ROS) are well-recognized by-products of oxygen metabolism and normally exist in all cells in an equilibrium with antioxidants. Oxidative stress takes place when this fine balance is disturbed because of ROS excess, antioxidant depletion, or both (Orrenius *et al.*, 2007; Limón-Pacheco & Gonsebatt, 2009) and is dependent on the metabolic activities of a given tissue and its particular redox status (Limón-Pacheco & Gonsebatt, 2009). While at moderate levels, ROS can exert some positive effects, participating in relevant signalling pathways such as the defence against pathogens or the preparation for fertilization in spermatozoa, excessively high levels are known for their damaging effects, affecting all biomolecules and ultimately leading to a cellular stress or even death (Matés & Sánchez-Jiménez, 1999; Agarwal *et al.*, 2003; Orrenius *et al.*, 2007; Tremellen, 2008). Although ROS production can originate from oxydases, oxygenases and non-enzymatic reactions, mitochondria are both the major producer and target of ROS in mammalian cells (Orrenius *et al.*, 2007). Moreover, mitochondria are known to be involved in a panoply of other processes such as energy production, steroidogenesis, calcium signalling, insulin production, lipid and xenobiotic metabolism and apoptosis, among others, all of which implicated in several signalling pathways (Wallace & Starkov, 2000; Brookes *et al.*, 2004; Dimmer & Scorrano, 2006; Orrenius *et al.*, 2007; Papadopoulos & Miller, 2012).

The importance of mitochondria is also reflected through all the spermatogenic process and ultimately in sperm function (Ruiz-Pesini *et al.*, 1998; Meinhardt *et al.*, 1999; Marchetti *et al.*, 2002, 2012; O'Connel 2002, 2003; Gallon *et al.*, 2006; Nakada *et al.*, 2006; Ramalho-Santos *et al.*, 2009; Sousa *et al.*, 2011; Aitken *et al.*, 2012). Dysfunction of this organelle is thus capable of affecting cellular function, with effects ranging from small alterations to disease and loss of gamete function.

In fact, ROS and mitochondrial dysfunction are involved in several pathologies and/or conditions, such as diabetes, ageing and infertility (Wallace, 1999; Brownlee, 2001; Agarwal *et al.*, 2003; Baker & Aitken, 2005; Newsholme *et al.*, 2007; Tremellen, 2008; Pitocco *et al.*, 2010; Terman *et al.*, 2010; Sivitz & Yorek, 2010; Amaral *et al.*, 2013). The fact that the majority of these processes affect human sperm function makes the study of the origin of sperm ROS, as well as the alterations they cause, particularly important. Furthermore, the particularities of

spermatozoa make them more vulnerable to ROS attack (Agarwal *et al.*, 2003; Baker & Aitken, 2005; Tremellen, 2008). These characteristics include a clearly localized mitochondrial distribution pattern, cell membranes high in polyunsaturated fatty acids (prone to ROS attack) and minimal cytoplasm (thus low antioxidant content), rendering the extrapolation of data from other cell types very difficult.

In the present work, we used UVB irradiation-induced ROS formation properties as an easy experimental approach, to better understand the origin of such damage and to access induced ROS-mediated alterations in human spermatozoa, without the confounding effects that usually exist in animal models for diseases or the addition of harsh chemical agents (e.g. H_2O_2) (Misro *et al.*, 2004; Dietrich *et al.*, 2005; Sousa *et al.*, 2009; Jang *et al.*, 2010; Sánchez *et al.*, 2012).

In fact along with the confirmed involvement of UVB irradiation in DNA damage (Kielbassa *et al.*, 1997; Pfeifer *et al.*, 2005; Xu *et al.*, 2010), apoptosis triggering (Kulms & Schwarz, 2002; Naik *et al.*, 2007) and damage of other cellular structures such as membranes and cytoplasmic components (Schwarz 1998), UVB irradiation is also known for being a potent inducer of ROS formation (Black, 1987; Wlaschek *et al.*, 2001; Heck *et al.*, 2003). This and the related oxidative stress seem to be at least partly mediated by mitochondria (Paz *et al.*, 2008; Birch-Machin & Swalwell, 2010; Gonzales Maglio *et al.*, 2011) and may be responsible for the previously referred alterations.

The UVB irradiation has previously been used in experiments mainly on spermatozoa from marine species which are naturally exposed to such putative damage, with effects being reported on sperm motility and fertilization ability (Au *et al.*, 2002; Lu & Wu, 2005a; Zan-Bar *et al.*, 2005; Nahon *et al.*, 2009), membrane integrity (Lu and Wu, 2005b) and DNA integrity (Pruski *et al.*, 2009). Lu and Wu (2005a) showed that some of these alterations involved increased levels of ROS and higher lipid peroxidation after spermatozoa were exposed to UVB and UVA. Nonetheless the origin of such ROS and the cascade of events that result in the decreased motility and lower fertilization rates remain to be determined. The same is true regarding UVB-induced alterations in the DNA of human spermatozoa detected and localized recently by Raman Confocal spectroscopy (Mallidis *et al.*, 2011).In fact, the description of the effects of UVB irradiation on human spermatozoa are scarce although there are some reports regarding the effects of longer and shorter wavelengths (UVA and UVC irradiation) (Makler *et al.*, 1980; König *et al.*, 1996; Torres *et al.*, 2010).

In this study, we assessed human sperm ROS production and its possible involvement in mitochondrial (dys)function as well as sperm lipid peroxidation and viability after UVB irradiation during different time periods and confirmed that sperm UVB irradiation represents a suitable and easily manipulated *in vitro* model system that may therefore help clarify ROS-induced alterations particular to the sperm cell.

MATERIALS AND METHODS

SUBJECTS

This project was conducted according to the Helsinki declaration with the approval of the ethics committee of the University Clinic of Münster and all recruited subjects signed informed consents. Fresh human sperm samples were obtained from six healthy normozoospermic donors after a 2–5 days period of sexual abstinence. All semen analyses and related procedures were performed in accordance with the World Health Organization guidelines (WHO, 2010). After a 30-min period to allow for semen liquefaction, sperm concentration, motility and viability (eosin/nigrosin) were determined. No leucocytes or any other round cells were observed in any of the ejaculates. Following analysis, samples were washed twice in fresh sperm preparation medium (Origio medicult medium) by centrifugation at 600 ***g***, for 5 min.

Chemicals

The fluorescent probes JC-1 (T3168), BODIPY 581/591 C_{11} (D3861) and MitoSox Red (M36008) were purchased from Invitrogen-Molecular probes (Eugene, OR, USA). Antymicin (A8674), superoxide dismutase (SOD; S7571) and Carbonyl cyanide 3-chlorophenylhydrazone (CCCP; C2759) were purchased from Sigma (St. Louis, MO, USA). Sperm preparation medium (Origio medicult medium; 10700060A), was purchased from Origio (Måløv, Denmark).

Sperm ROS (superoxide) production

Mitochondrial superoxide generation was determined using the Mitosox red probe (Msox), according to the previously described method (Koppers *et al.*, 2008). Msox is a cationic fluorogenic dye that has a tetraphenylphosphonium group which allows it to selectively target the mitochondria. Once oxidized, Msox displays a red fluorescence. Spermatozoa (2 million) were incubated with Msox to a final concentration of 3 μM for 15 min at 37 °C. Accompanying controls were prepared namely: incubation with Antimycin (80 μM, positive control) and SOD (500 U/mL, negative control). Afterwards the sperm suspension was washed with PBS-supplemented medium [PBS supplemented with 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.3% (w/v) bovine serum albumin (BSA), 5 mM glucose, 10 mM lactate, 1 mM pyruvate and 1% (v/v) penicillin/streptomycin, pH 7.2–7.4] (600 ***g***, 5 min) and resuspended in fresh PBS. Msox red fluorescence was measured by flow cytometry (Beckman Coulter Cytomics, Krefeld, Germany).

Sperm mitochondrial membrane potential

Sperm mitochondrial membrane potential (MMP) was assessed using the JC-1 fluorescence probe (Amaral & Ramalho-Santos, 2010). JC-1 is a cationic lipophilic probe that can selectively enter the mitochondria and is able to shift its fluorescent emission from green to red according to MMP. When the MMP is high, the probe accumulates in the matrix forming the so called 'J-aggregates' that emit red fluorescence. In contrast, with low MMP JC-1 remains in its monomeric form displaying green fluorescence. A decrease in the ratio of red/green fluorescence in a population of cells indicates the extent of mitochondrial depolarization.

Sperm samples (2 million) were incubated with JC-1 at a final concentration of 2 μM for 15 min at 37 °C. An appropriate control was prepared using CCCP (50 μM), an uncoupler that disrupts MMP, resulting in a shift from red to green fluorescence. After incubation, samples were washed (600 ***g***, 5 min) with PBS-supplemented medium. The green/red fluorescence was then measured using flow cytometry.

Sperm lipid peroxidation

Lipid peroxidation was monitored using BODIPY 581/591 C_{11} (Aitken *et al.*, 2007). This probe is incorporated into the sperm membrane and when oxidized responds with a fluorescence spectral emission change from red to green. BODIPY was added to spermatozoa (2 million) at a final concentration of 5 μM. Sperm samples were incubated for 45 min at 37 °C. The sperm suspension was then washed (600 ***g***, 5 min) and resuspended in fresh PBS-supplemented medium (with 1 mg/mL polyvinyl alcohol-PVA instead of BSA). An appropriate positive control was also prepared by incubating the samples with ferrous sulphate (80 μM), a peroxidation promoter. BODIPY fluorescence was then measured with a flow cytometer.

Sperm viability

Able to enter only compromised spermatozoa, propidium iodide at a final concentration of 10 μg/mL, was used to evaluate sperm viability using flow cytometry (Aitken *et al.*, 2007).

UVB irradiation protocol

After incubation of the human spermatozoa with the above-mentioned fluorescent probes, sperm samples were exposed to 312-nm UV irradiation (UVB) (Saur GmbH, Reutlingen, Germany) for 0, 30, 60, 120 and 180 sec (Mallidis *et al.*, 2011). Samples were then analysed by flow cytometry as described below.

Flow cytometry analysis

Analyses were conducted using a Beckman Coulter Cytomics FC 500 analyser with an argon laser that operates with an excitation wavelength of 488 nm coupled with the following emission filters: 530/30 band pass (FL-1 channel/green), 585/42 band pass (FL-2 channel/red) and >620 nm long pass filter (FL-3 channel/far red) according to the manufacturer's instructions (Beckman Coulter). Data were collected and analysed using CXP Software from Beckman Coulter. Non-spermatozoa-specific events were gated out and 50 000 cells were examined per experiment.

Statistics

All statistical analyses were carried out using the SPSS (statistical package for the social sciences program), version 20.00, software for windows (SPSS Inc., Chicago, IL, USA). All variables were checked for normal distribution, using the Shapiro–Wilk test. Results are presented as mean ± SEM of the number of experiments indicated and statistical significance assessed using the independent samples *t*-test. Multiple comparisons were performed using one-way ANOVA, and $p \leq 0.05$ was considered significant. Bivariate correlation (r) was evaluated by calculating the Pearson correlation coefficient with a two-tailed significance (p).

RESULTS

UVB treatment stimulates ROS production in sperm mitochondria

Mitochondria are the main cellular producers (and also targets) of ROS. In this study, we assessed the production of superoxide, the major form of ROS produced by the mitochondria, in human spermatozoa after UVB treatment. We observed that the number of spermatozoa labelled by Msox, which correspond to spermatozoa with higher ROS production, increased after 60 sec of exposure, reaching a maximum after 180 sec (Fig. 1).

UVB treatment affects sperm MMP

The mitochondrial proton gradient originates an electrochemical potential (Δp) resulting in a pH (ΔpH) and in a voltage ($\Delta\psi$m) gradient across the mitochondrial inner membrane (Newmeyer & Ferguson-Miller, 2003). The transmembrane electrical potential ($\Delta\psi$) is the main component of the electrochemical gradient, accounting for more than 90% of the total proton motive force (Moreira *et al.*, 2005), and is thus crucial for good mitochondrial performance. Therefore, changes associated with $\Delta\psi$ are critically important in studies regarding mitochondrial function.

We observed that UVB irradiation caused a decrease in sperm MMP, that is, sperm population with high MMP mitochondria

Figure 1 Mitochondrial superoxide production in spermatozoa exposed to UVB radiation. *Left part* shows flow cytometry results obtained in spermatozoa without UVB irradiation (A) and after 60″ (B), 120″ (C) and 180″ (D) of UVB irradiation. Red fluorescence was detected in the FL2 channel. The population producing superoxide corresponds to *B* regions of the histograms. On the graph on the *right* (E), increased superoxide production (*B* region) is observable after 60″ of UVB exposure. Superoxide production was determined as described in the material and methods section. Data (n = 6) are expressed as mean ± SEM, *$p \leq 0.05$; ***$p \leq 0.001$, compared with the control (no UVB irradiation).

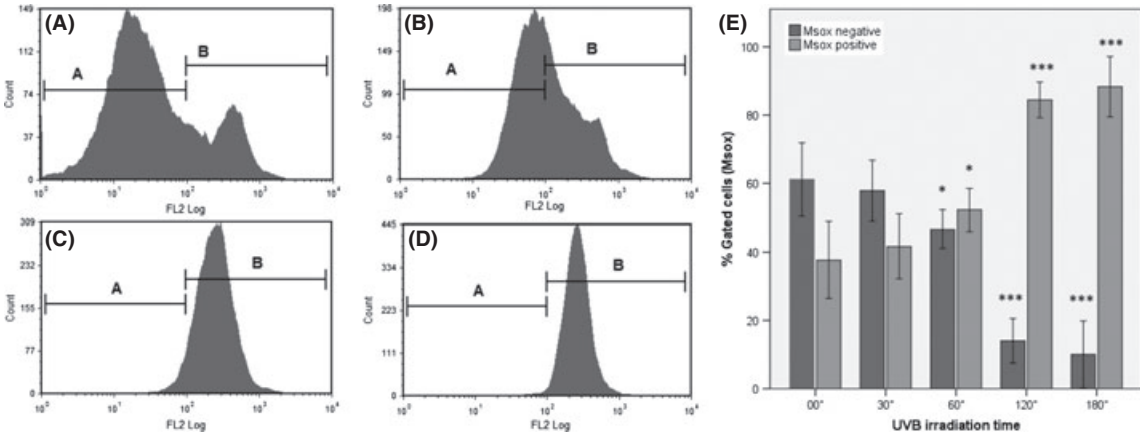
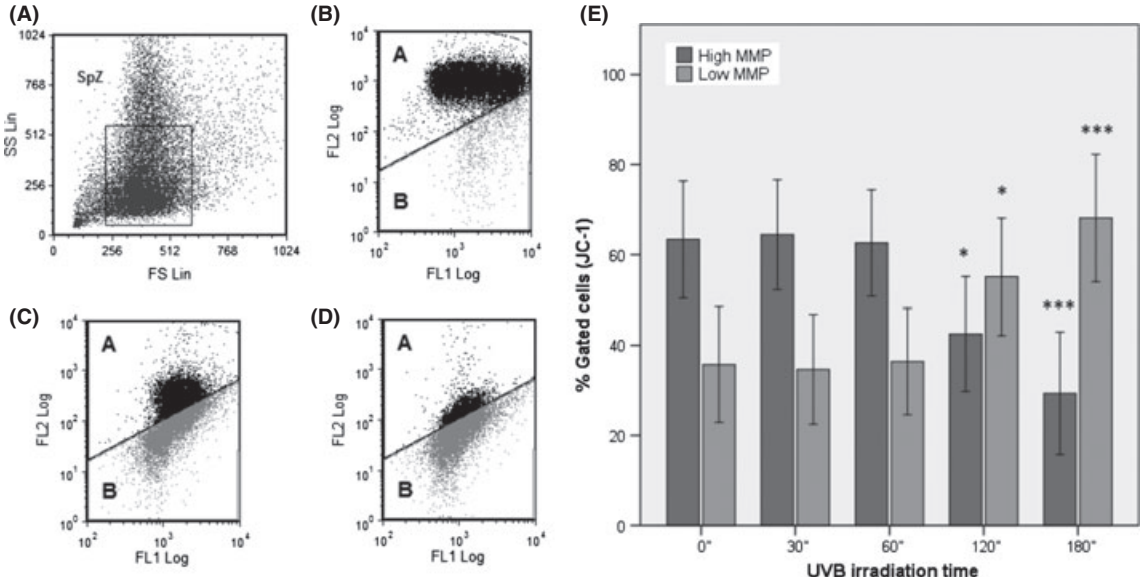


Figure 2 Mitochondrial membrane potential (MMP) in sperm exposed to UVB radiation. *Left part* shows MMP flow cytometry results for selected sperm population (A), sperm without UVB irradiation (B) and after 120″(C) and 180″(D) of UVB irradiation. The red fluorescence is detected in the FL2 channel and the green in the FL1 channel. The *A* region on the dot plots (B–D) represent the area with sperm with high MMP and the *B* region sperm with low MMP. In the *right part* (E), the MMP decrease is observable after 120″ of UVB exposure. MMP was determined as described in the materials and methods section. Data ($n = 6$) is expressed as mean ± SEM, *$p \leq 0.05$; *** $p \leq 0.001$, compared to the control (no UVB irradiation).



(red) decreased whereas sperm population with low MMP (green) increased. This effect was dependent on the time of exposure (Fig. 2), being significant at 120 sec and peaking at 180 sec. At this time (180 sec), the observed effect was similar to the one using the mitochondrial uncoupler CCCP, a substance that collapses MMP.

UVB treatment results in lipid peroxidation

DNA and sperm membranes are the most likely targets of oxidative stress-induced injury (Agarwal *et al.*, 2003; Baker & Aitken, 2005; Tremellen, 2008). In fact, owing to the high proportion of unsaturated fatty acids and low levels of antioxidant defences, the sperm membrane is highly susceptible to peroxidation. As noted for ROS formation, we observed that lipid peroxidation increased significantly after 60 sec of UVB exposure peaking at 180 sec of exposure (Fig. 3), mirroring the effect described for UVB-ROS production.

Sperm viability

A decrease in sperm viability was evident after 120 sec of UVB exposure, reaching a minimum after 180 sec of UVB irradiation (Fig. 4). These results appear to be in agreement with those obtained for mitochondria functionality (using JC-1, Fig. 1) indicating that sperm viability and mitochondrial functionality are connected, a relationship further confirmed by the existence of a strong correlation between these two parameters (Table 1). In the same vein, sperm viability was shown to be negatively correlated with sperm ROS production and lipid peroxidation (Table 1).

DISCUSSION AND CONCLUSIONS

Although ROS can exert some positive effects on spermatozoa at low levels, participating in crucial steps such as capacitation and the acrosome reaction, it is now suggested that at higher levels ROS mediate sperm damage and as such may be important contributors to infertility in men (Tremellen, 2008). The peculiar characteristics of spermatozoa make them extremely prone to oxidative alterations (Agarwal *et al.*, 2003; Baker & Aitken, 2005) and thus stress the importance of studying the origin of such ROS, and the resulting changes in sperm function they mediate. This can only be done using a controlled ROS-generating system.

In the present work, we used UVB irradiation to study the ROS riddle in human spermatozoa, as this method had already been proven to increase ROS production (Black, 1987; Wlaschek *et al.*, 2001; Heck *et al.*, 2003; Paz *et al.*, 2008). In addition, the feasibility of this approach in spermatozoa is guaranteed by previous studies conducted in spermatozoa from marine animals, which are naturally exposed to this kind of irradiation (Au *et al.*, 2002; Lu & Wu, 2005a,b; Zan-Bar *et al.*, 2005; Nahon *et al.*, 2009; Pruski *et al.*, 2009).

Keeping in mind that mitochondria are the main producers of ROS, and also given their importance in sperm function (Ruiz-Pesini *et al.*, 1998; Meinhardt *et al.*, 1999; Marchetti *et al.*, 2002, 2012; Gallon *et al.*, 2006; Nakada *et al.*, 2006; Sousa *et al.*, 2011; Aitken *et al.*, 2012) we focused on this organelle. To confirm whether an increase in ROS production occurred after UVB irradiation in human spermatozoa and if the origin of such ROS production was mitochondrial, we specifically assessed mitochondrial superoxide production and found increases after 60 sec of UVB exposure and a maximum after 180 sec. Results which are in accordance with a previous study of Lu and collaborators (Lu and Wu, 2005a) conducted in sea urchin spermatozoa, although the probe used in this case was less specific. To investigate whether this increase was associated with mitochondrial dysfunction, we assessed MMP and found that human spermatozoa exposed to UVB irradiation presented a decrease in this important indicator of

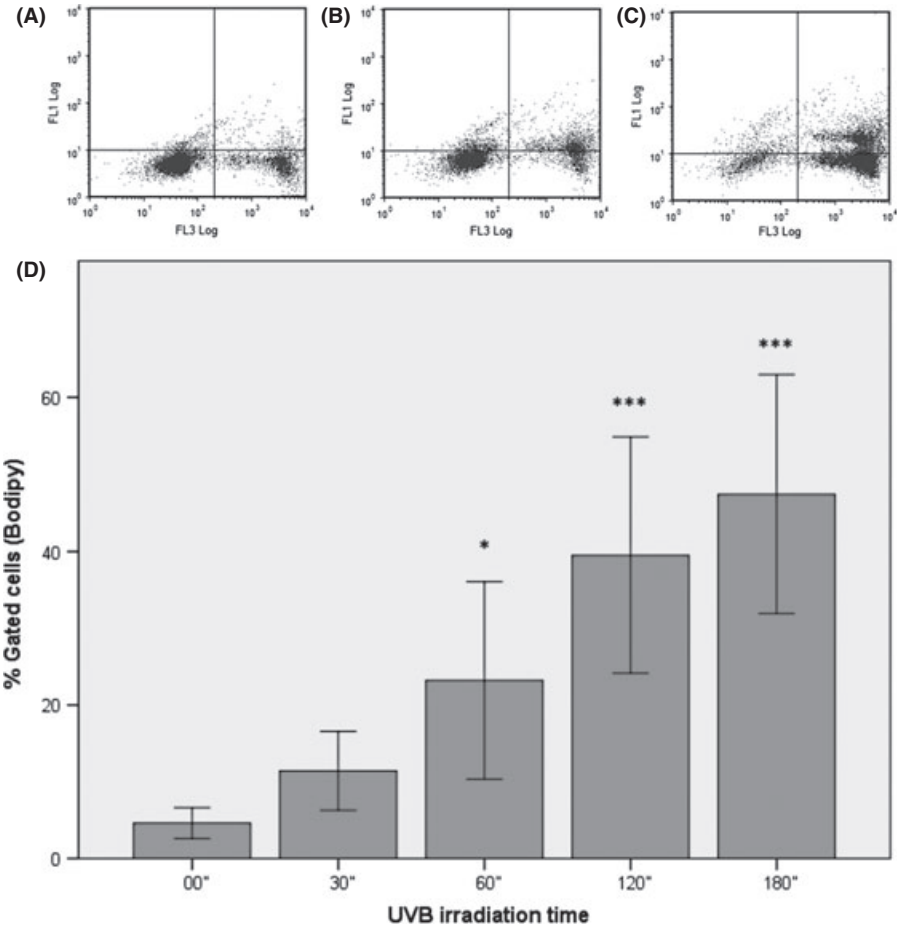


Figure 3 Sperm membrane lipid peroxidation after UVB exposure. *Upper part* show flow cytometry results for sperm labeled with BODIPY (FL1-green) and PI (FL3-red) without UVB irradiation (A) and after 60″ (B) and 180″(C) of UVB irradiation. In the *lower part* (D), increased lipid peroxidation is observable after 60″ of UVB exposure. Lipid peroxidation was determined as described in the material and methods section. Data ($n = 6$) is expressed as mean ± SEM, $*p \leq 0.05$; $***p \leq 0.001$, compared to the control (no UVB irradiation).

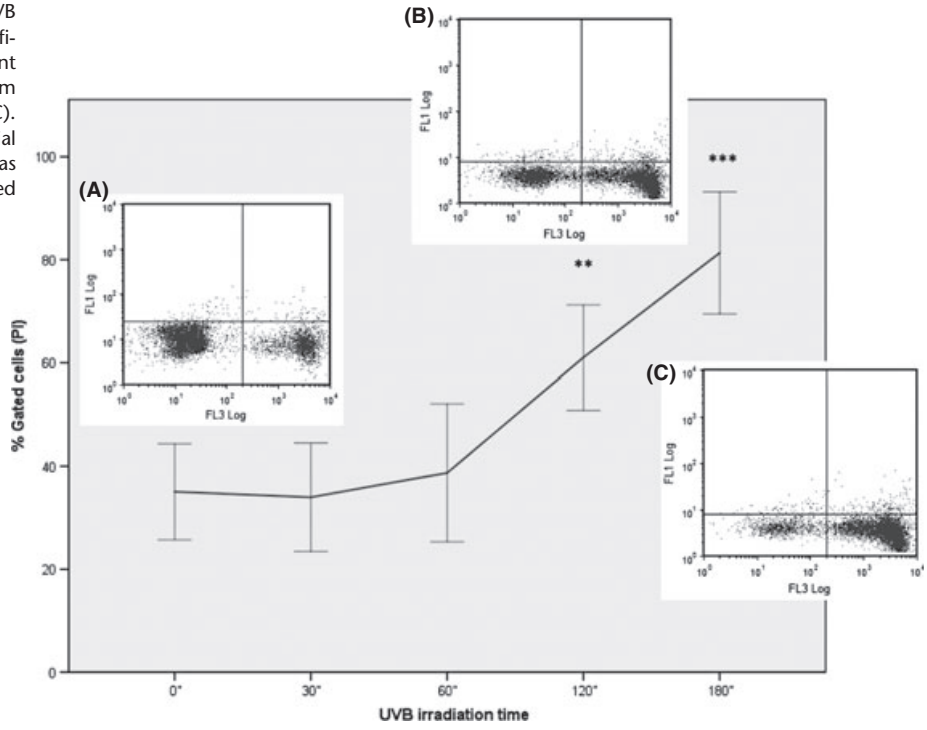


Figure 4 Sperm PI positive (dead) cells after UVB radiation exposure. A decrease in viability is significant after 120″ of UVB exposure. The gates represent flow cytometry data regarding non irradiated sperm (A) and sperm irradiated for 120″ (B) and 180″ (C). Viability was determined as described in the material and methods section. Data ($n = 6$) is expressed as mean ± SEM, $**p \leq 0.01$; $***p \leq 0.001$, compared to the control (no UVB irradiation).

Table 1 Correlations between evaluated parameters following UVB irradiation

	Low MMP (JC-1 green)	High superoxide production (MSox positive)	Lipid peroxidation (BODIPY positive)	Disrupted membrane (PI positive)
High MMP (JC-1 red)	−0. 999***	−0.740***	−0.646***	−0.954***
Low MMP (JC-1 green)		0.555***	0.641***	0.948***
High superoxide production (MSox positive)			0.640***	0.844***
Lipid peroxidation (BODIPY positive)				0.604***

Pearson correlation coefficients (r) obtained between all variables studied are presented, ***$p \leq 0.001$.

mitochondrial function after 120 sec of irradiation reaching minimum values after 180 sec, an observation which again corroborates the findings of Lu and Wu (2005b) in sea urchin spermatozoa. Both results clearly indicate that sperm mitochondrial function is affected by UVB exposure in a time-dependent manner, an effect closely correlated with changes in sperm viability. Interestingly, the increase in ROS production we observed occurred before any decrease in MMP became apparent, an effect that has been previously observed in human spermatozoa (Koppers *et al.*, 2008; Aitken *et al.*, 2012). Nonetheless, what is unequivocal is that the combined results of JC-1 and Msox experiments confirm the oxidative nature of UVB damage, and focus these events to the mitochondria. Results that are supported by recent findings in which the great majority of ROS production in human spermatozoa had been traced back to the mitochondria and which production is directly related with sperm quality (Koppers *et al.*, 2008, 2010; Aitken *et al.*, 2012). However, we cannot preclude the existence of other ROS-generating systems that might contribute to increased ROS levels and associated damage in a cumulative fashion, mainly because human spermatozoa is endowed with some oxidases capable of ROS production, such as NOX5 (Agarwal *et al.*, 2003; Baker & Aitken, 2005; Tremellen, 2008; Musset *et al.*, 2012), an issue that needs to be addressed in future studies. Nonetheless, several strong correlations were found in this study supporting our results. In fact, it was clearly demonstrated that the MMP was negatively correlated with ROS levels, as expected.

Next, we hypothesized that if UVB damage to spermatozoa is oxidative, the sperm membrane will be highly responsive to such damage because of its high content in PUFAS, and thus an increase in lipid peroxidation should be expected (Koppers *et al.*, 2008; Aitken *et al.*, 2012). In fact, we observed that lipid peroxidation increases following the same pattern as ROS production. The UVB-induced lipid peroxidation is not exclusive to human sperm cells or to UVB-induced effects, as an increase in lipid peroxidation has also been observed in sea urchin spermatozoa exposed to UVB and UVA (Wu and Lu, 2005a), in human spermatozoa exposed to UVC (Torres *et al.*, 2010) and in keratinocytes, cornea and lens cells exposed to UVA and UVB (Punnonen *et al.*, 1991; Satori *et al.*, 2011). Moreover, in the present work, lipid peroxidation was positively correlated with mitochondrial ROS production and negatively correlated with MMP, in accordance with previous work conducted in human spermatozoa (Koppers *et al.*, 2008; Aitken *et al.*, 2012) thus strengthening this connection. Overall, the increase in ROS production and increase in lipid peroxidation previous to any apparent mitochondrial dysfunction suggest that the ROS produced in the mitochondria may also affect mitochondrial membranes, complexes, mtDNA and cause a decrease in MMP (and ultimately cell death). Furthermore, the ROS production is not induced by a depolarization of the mitochondria, but rather precedes it, and might actually induce it. Dysfunctional mitochondria contribute to the establishment of a vicious cycle (Harman, 1972) in that they produce even more ROS which in turn lead to a progressive increase in damage to other cellular components which ultimately results in a general bioenergetic failure. Moreover, mitochondrial dysfunction may also trigger the apoptotic process, already described in several cell types exposed to UVB irradiation (Kulms & Schwarz, 2002; Naik *et al.*, 2007). This may explain why sperm viability decreased only at 120 sec, mimicking the pattern of mitochondrial dysfunction. In fact, the correlation obtained between viability and MMP was even higher than with the increase in ROS production, indicating once more the importance of this organelle for sperm functionality, in accordance with previous reports (Ruiz-Pesini *et al.*, 1998; Marchetti *et al.*, 2002, 2012; Gallon *et al.*, 2006; Sousa *et al.*, 2011).

Overall, the mechanisms operating in the spermatozoa seem to be similar to the ones described in human skin in which it was demonstrated that, after UVB irradiation, cells progressed to apoptotic cell death displaying increased levels of ROS production and mitochondrial depolarization (Paz *et al.*, 2008; Gonzales Maglio *et al.*, 2011).

The cellular consequences of the increase in mitochondrial ROS production, decreased mitochondrial functionality and membrane lipid peroxidation might therefore explain the decrease in sperm motility, fertilization capacity (Au *et al.*, 2002; Lu & Wu, 2005a; Zan-Bar *et al.*, 2005; Nahon *et al.*, 2009) and DNA damage observed in spermatozoa from marine species after UVB exposure (Pruski *et al.*, 2009) as well as UVB-induced alterations detected in the DNA of human spermatozoa by Raman Spectroscopy (Mallidis *et al.*, 2011) all of which corroborating the already known importance of mitochondria in sperm function.

To our knowledge, this is the first study that presents results clearly pointing to mitochondria-mediated UVB-induced sperm damage. However, the implications of this mitochondrial dysfunction and increased production of ROS on other sperm functional parameters should be addressed in the future. Regardless, sperm UVB irradiation appears to be a good and easily manipulated *in vitro* model system to study mitochondria-induced oxidative stress in spermatozoa and its consequences, which may be relevant in terms of dissecting the action pathways of many other pathologies, drugs and contaminants, including endocrine disruptors.

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DISCLOSURES

The authors declare no conflict of interests.

AUTHOR CONTRIBUTIONS

S.A., V.S. and C.M. conceived and designed the research study. S.A. and K.R. performed the experiments. S.A., K.R., J.R.-S., C.M. and S.S. analysed and discussed the data. S.A. wrote the manuscript.

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