

Melatonin diminishes oxidative damage in sperm cells, improving assisted reproductive techniques

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Abstract: Sperm preparation procedures are a potential generator of oxidative stress-induced DNA damage, which leads to a dramatic drop in fertility. An increasing number of studies suggest that melatonin reduces the oxidative stress induced by manipulation. However, very little is known about the preservative role of melatonin in sperm preparation medium during assisted reproduction procedures. For this aim to be achieved, semen was divided into two fractions and preincubated with and without 1 mM melatonin. Afterwards, both fractions were divided into two subfractions to perform swim-up in the presence and absence of 1 mM melatonin. Labeling with anti-CD46 and antiactive caspase-3 allowed the monitoring of acrosome reaction and apoptosis by flow cytometry. Sperm DNA fragmentation and compaction were analyzed through propidium iodide staining. The normozoospermic and oligozoospermic samples that were preincubated with melatonin underwent a significant increase in the ratio of adequate spermatozoa and a reduction of caspase-3 activation. Additionally, preincubation with melatonin enhanced the migration of sperm cells with compacted DNA in oligozoospermic samples ($P < 0.05$) and prevented DNA fragmentation in normozoospermic samples ($P < 0.05$). In light of the current results, the cytoprotective capacity and innocuousness of melatonin make it a great candidate to be applied in assisted reproduction techniques in order to prevent iatrogenic oxidative damage.

Key words: Melatonin, antioxidants, sperm cells, in vitro fertilization, oxidative damage, assisted reproduction technology, sperm manipulation

1. Introduction

Surveys indicate that 9%–15% of couples endure a prevalence of infertility after 12 months of unprotected intercourse, and 56% of them seek medical care to conceive (Boivin et al., 2007; Agarwal et al., 2014b). Several studies have shown a significant increase in reactive oxygen species (ROS) activity in various types of infertility (Aitken and Clarkson, 1987; Zini et al., 1993; Kodama et al., 1997; Aitken et al., 2009). The spermatozoon is a very sensitive cell to ROS-induced oxidative damage. This is partly due to the fact that the sperm plasma membrane contains a high content of polyunsaturated fatty acid, which confers to sperm cells the needed fluidity for membrane fusion during fertilization (Makker et al., 2009).

The presence of ROS in the seminal fluid can originate from several sources, both endogenous and exogenous. In this sense, cellular components (mature and immature sperm cells, leukocytes, and urogenital epithelial cells) of human semen are considered the major source of ROS, especially leukocytes and immature sperm cells (Agarwal

et al., 2014b). Moreover, sperm cells are characterized by remarkable metabolic activity due to their flagellar movement, which demands a high level of intracellular ATP during the motility phase. Sperm preparation procedures in assisted reproduction technology (ART) are potential generators of oxidative stress causing DNA damage (Balasuriya et al., 2014). The seminal plasma provides antioxidant protection to germ cells against excessive ROS production. Sperm cells become especially vulnerable to the oxidative damage when seminal plasma is removed during sperm preparation for ART (Eid Hammadeh et al., 2009). Therefore, to avoid such an oxidative threat that could cause irreversible fatal injuries, culture media have been supplemented with antioxidants (e.g., ascorbic acid or α -tocopherol) for mitigating the noxious effects of ROS on sperm cells (Keshtgar et al., 2012). Seminal plasma removal is not the only origin of ROS, since the centrifugation itself has a remarkable influence on the generation of endogenous ROS in a g-force and time-dependent manner and it causes greater detriment to

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sperm parameters (Shekarriz et al., 1995). In this regard, apoptosis has been described to be stimulated by ROS in sperm cells (Bejarano et al., 2008). The experimental evidence suggests the preincubation of semen samples with antioxidants as a protection against centrifugation-induced ROS production and damage to processed sperm cells (Agarwal et al., 2014a).

Melatonin is a powerful antioxidant hormone secreted mainly by the pineal gland to regulate the sleep/wake cycle. Melatonin, which activates the primary enzymatic antioxidant machinery in body tissue (Mayo et al., 2002), has also been reported to be present in human semen (Awad et al., 2008). Using rats as a model, the oxidative stress induced by hyperthyroidism in testes is shown to be increased by pinealectomies (Mogulkoc et al., 2006). Experimental studies demonstrate that sperm cells from healthy men incubated with melatonin gain motility and viability (du Plessis et al., 2010; Ortiz et al., 2011), and it causes the ratio of sperm cells with normal morphology to increase (Ortiz et al., 2011). Our previous findings showed that the daily supplementation of 6 mg of melatonin produced some reduction in the oxidative damage caused in sperm DNA (Bejarano et al., 2014). These findings are also linked to reductions in oxidative and/or nitrosative stress due to the antioxidant ability of melatonin (du Plessis et al., 2010; Espino et al., 2010). Therefore, it is not surprising that melatonin has been proposed for supplementation of semen extenders against oxidative stress induced by freezing-thawing processes in human sperm cells, increasing their viability and motility (Karimfar et al., 2015). A number of studies suggest that melatonin reduces the oxidative stress induced by manipulation that generates iatrogenic damage (Cruz et al., 2014), like staining or flow cytometer sorting (Li et al., 2012). All these results are consistent with our previous findings, which described how the exposure of sperm cells to melatonin improves motility, thus increasing the ratio of rapid cells (Ortiz et al., 2011). In light of the aforementioned literature, this study aims to describe the role of the presence of melatonin on manipulation-induced damage during the preparation of sperm cells for ART.

2. Materials and methods

2.1. Reagents

Paraformaldehyde (PFA), Triton X-100, and RPMI 1640 medium were obtained from Sigma (Madrid, Spain). An in situ cell death detection kit, POD, was purchased from Roche (Madrid, Spain). Melatonin was obtained from Fagron Iberica (Barcelona, Spain). All other reagents were of analytical grade.

2.2. Subjects

Human semen was obtained from infertile volunteers at the Extremadura Centre of Human Assisted Reproduction

(Badajoz, Spain), as approved by the local committee, the institutional review board of the University of Extremadura, and the ethics committee of the Mother and Child Hospital (Badajoz, Spain), in accordance with the Declaration of Helsinki. Semen was obtained from 40 men (36.4 ± 2.2 years old), from couples suffering from primary infertility after at least 1 year of regular sexual intercourse, who were undergoing evaluation at our andrology laboratory. Conventional semen evaluation was carried out following the criteria of the World Health Organization (WHO, 2010), allowing the classification of the patients as normozoospermic or oligozoospermic. Twenty of them were classified as normozoospermic and twenty as oligozoospermic. Their partners had not become pregnant after at least 1 year of unprotected intercourse. Each subject was ascertained to be in good health by means of their medical history and a clinical examination including a routine laboratory test and screening. The subjects were all nonsmokers, they were not taking any medication, and they abstained from alcohol. Written consent was obtained from all the participants.

2.3. Sperm preparation

Samples were collected by masturbation after 3–4 days of sexual abstinence and were allowed to liquefy for 30 min at 37 °C. The semen was then divided into two fractions and preincubated with and without 1 mM melatonin (namely Pr[−] or Pr^m samples, respectively) for 30 min at 37 °C, based on earlier research (Espino et al., 2010; Ortiz et al., 2011). Sperm cells were washed in RPMI medium (250 × g, 10 min) and, at the same time, both samples (Pr^m and Pr[−]) were divided into two fractions to perform the swim-up procedure in the presence and absence of 1 mM melatonin (namely Pr[−]Sw[−], Pr[−]Sw^m, Pr^mSw[−], and Pr^mSw^m samples) as shown in Figure 1. After swim-up migration, sperm cells were washed in PBS and fixed with 1% PFA for 15 min. Then samples were washed in PBS-BSA (0.1% bovine serum albumin) and stored in the same medium at 4 °C until their evaluation by flow cytometry.

2.4. Caspase-3 activation

Samples stored in PBS-BSA were washed and resuspended in PBS-Triton (0.1%) to be permeabilized. The samples were incubated with Alexa Fluor 647 rabbit antiactive caspase-3 clone C92-695 (BD Pharmingen, Warsaw, Poland (1:250)) for 30 min at RT. The active caspase-3 was quantified by flow cytometry (Cytomyx FC-500; Beckman-Coulter, Hialeah, FL, USA).

2.5. Sperm acrosome reaction

CD46 is a membrane protein localized in the inner acrosomal membrane. When acrosome reaction occurs, the fusion between the sperm cell membrane and the acrosomal membrane takes place. After acrosome reaction, CD46 is exposed and can be labeled by specific antibodies, thus being a marker for acrosome reaction. To

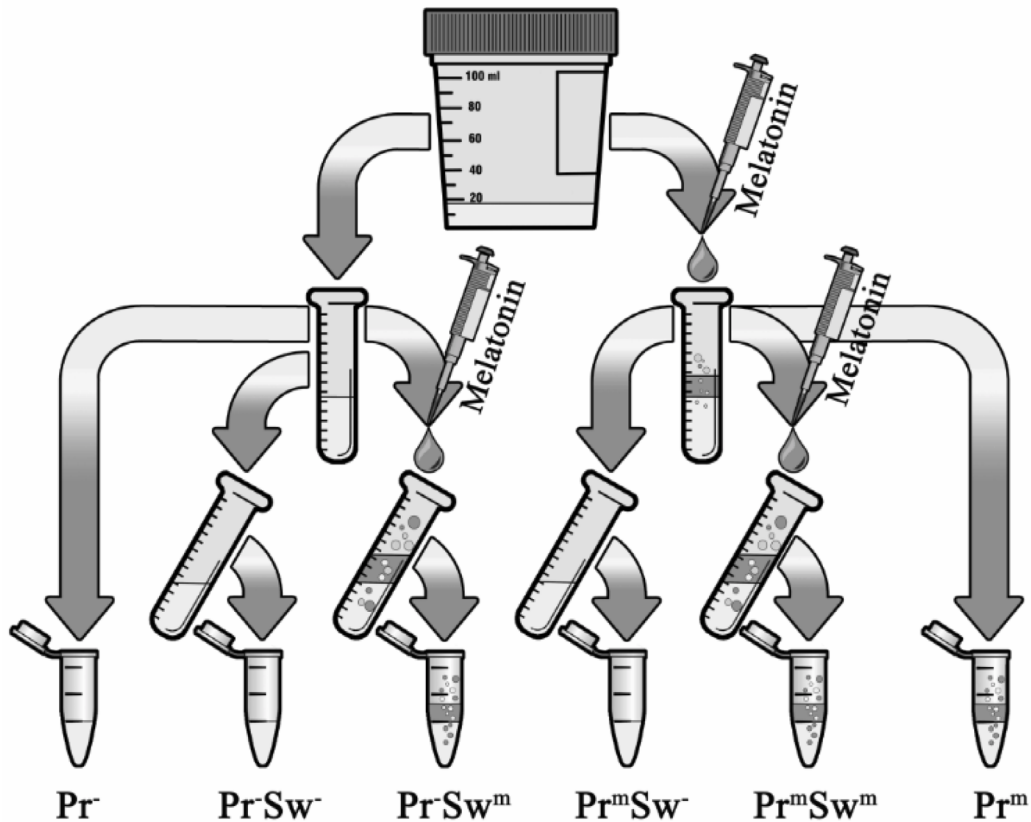


Figure 1. Design of the treatments during the sperm preparation for assisted reproduction technology (ART). The ejaculations were divided into two fractions and preincubated with and without 1 mM melatonin (Pr^- or Pr^m , respectively) for 30 min at 37 °C. Both samples (Pr^m or Pr^-) were divided into two subfractions to be swum-up in the presence or absence of 1 mM melatonin ($PrSw^-$, $PrSw^m$, Pr^mSw^- , and Pr^mSw^m).

study the acrosome reaction, stored samples in PBS-BSA were washed and then incubated with the monoclonal antibody (mAb) FITC (FL1) antihuman CD46 (clone MEM-258 Biolegend, London, UK) for 30 min at room temperature. The CD46 protein was quantified by flow cytometry (Cytomics FC-500).

2.6. Chromatin compaction and fragmentation

The sperm cells were incubated with propidium iodide (PI) and then sperm labeling was quantified by flow cytometry (Cytomics FC-500). The difference in fluorescence intensity of each spermatozoon was due to different DNA compaction. The higher the fluorescence intensity, the greater the chromatin decompaction (Figure 2). Given that apoptotic sperm cells lose their already fragmented DNA, sperm cells with fragmented DNA are located below cells with compacted DNA in the FL3 histogram profile, as shown in Figure 2.

2.7. Statistical analysis

Data were expressed as means \pm SEM of the number of determinations. For multiple comparisons, one-way analysis of variance followed by Tukey's test was used. $P < 0.05$ was considered a statistically significant difference.

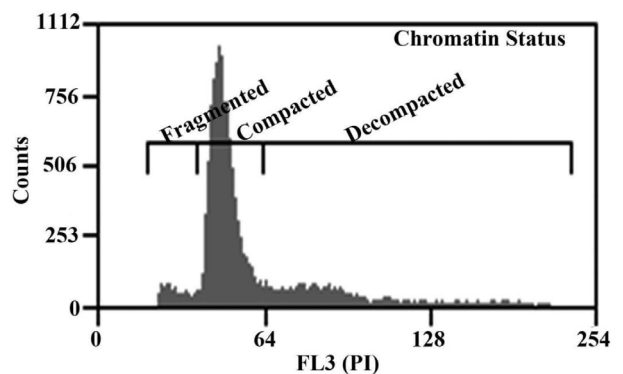


Figure 2. Profile of propidium iodide (PI) fluorescence of stained sperm cells versus number of sperm cells. The histogram shows sperm labeled with PI and quantified by flow cytometry. The difference in fluorescence intensity is due to the DNA compaction of each spermatozoon. The higher the fluorescence intensity, the greater the chromatin decompaction. Sperm cells with fragmented DNA are located below cells with compacted DNA.

3. Results

In both normozoospermic and oligozoospermic samples, flow cytometry indicated a decrease in the ratio of active caspase-3 labeling after preincubation with melatonin (Pr^m) (Figures 3A and 3B). However, only oligozoospermic samples exhibited a significant improvement when the swim-up was performed with the presence of melatonin, independently of the fact that they were preincubated with and without the hormone (Pr^mSw^m , Pr^mSw^m) (Figures 3A and 3B). Hence, melatonin reduced caspase-3 activation in unprocessed sperm cells and prevented it from the damage induced by manipulation in oligozoospermic sperm cells (Figure 3B). Therefore, in light of these results, melatonin could be used to protect sperm cells in those cases in which the swim-up assortment is not carried out.

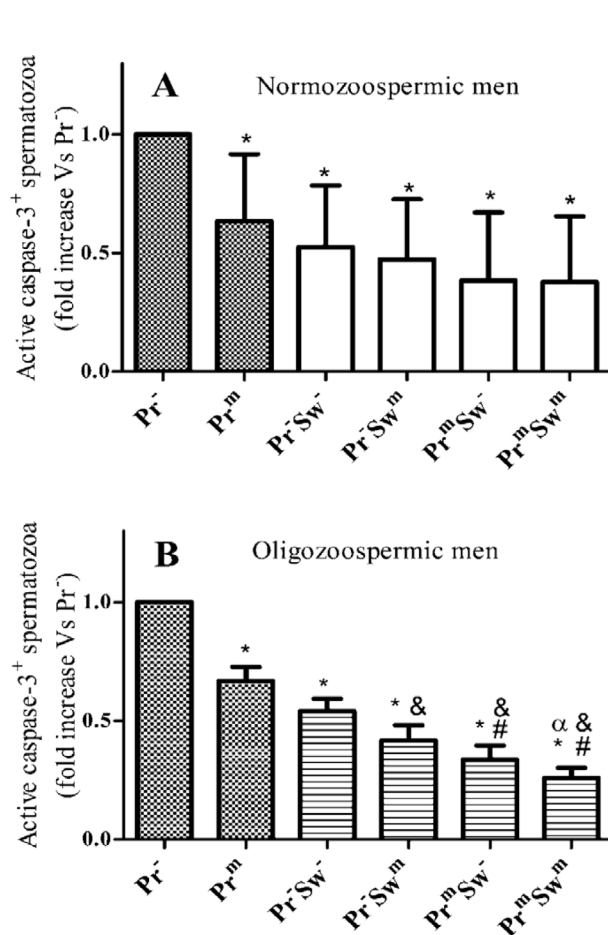


Figure 3. Quantification of sperm cells containing active caspase-3. Caspase-3 activation was estimated as described in Section 2. Values are presented as mean \pm SEM of 20 separate experiments and expressed as fold increase over the non-pretreatment level (Pr^-). *: $P < 0.05$ compared to Pr^- values. &: $P < 0.05$ compared to Pr^mSw^- . #: $P < 0.05$ compared to Pr^m . α : $P < 0.05$ compared to Pr^mSw^- .

Both in normozoospermic and oligozoospermic, anti-CD46 labeling revealed an increase in the ratio of adequate sperm cells when fresh samples were preincubated with 1 mM melatonin (Pr^m) (Figure 4). Moreover, after swim-up selection both in the presence and absence of 1 mM melatonin, the samples that were preincubated with 1 mM melatonin (Pr^mSw^- , Pr^mSw^m) underwent a rise in the ratio of CD46-labeled sperm cells compared to Pr^mSw^- and Pr^m ($P < 0.05$, Figure 4). Slight increases of CD46 externalization were shown when swim-up was performed in the presence of melatonin (Pr^mSw^- , Pr^mSw^m) (Figure 4).

Figure 5A shows that sperm cells from normozoospermic samples that were preincubated with 1 mM melatonin (Pr^m) did not undergo any changes in the ratio of compacted chromatin sperm cells. The

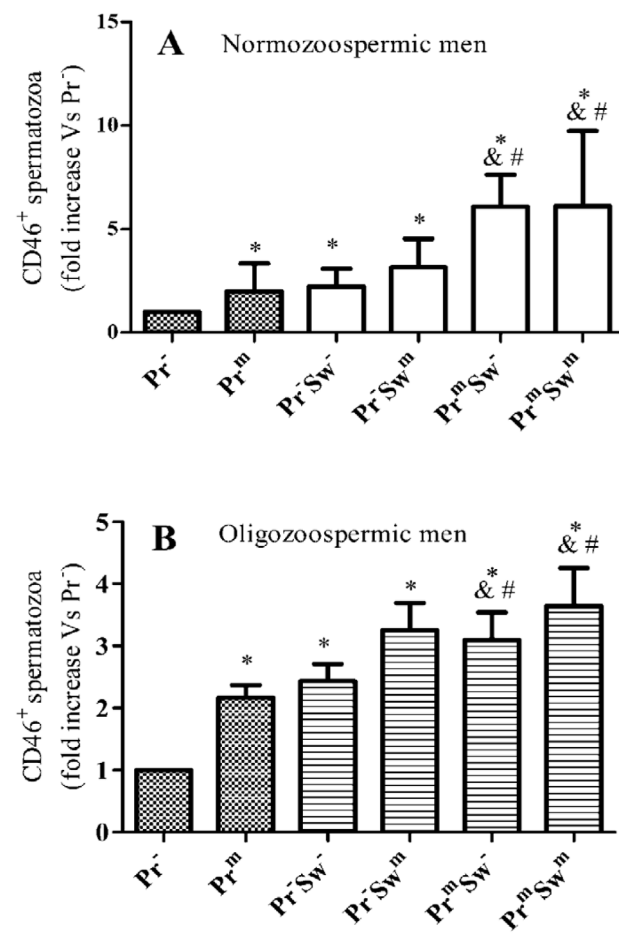


Figure 4. Quantification of sperm cells containing externalized CD46. CD46 externalization was determined as described in Section 2. Values are presented as mean \pm SEM of 20 separate experiments and expressed as fold increase over the non-pretreatment level (Pr^-). *: $P < 0.05$ compared to Pr^- values. &: $P < 0.05$ compared to Pr^mSw^- . #: $P < 0.05$ compared to Pr^m .

presence of melatonin during the swim-up procedure did not induce any improvement in the aforesaid ratio in samples from normozoospermic patients (Figure 5A). Unlike normozoospermic samples, oligozoospermic samples underwent a significant increase ($P < 0.05$) in the compaction ratio when they were preincubated with 1 mM melatonin and selected by swim-up ($\text{Pr}^{\text{m}}\text{Sw}^-$, $\text{Pr}^{\text{m}}\text{Sw}^{\text{m}}$) (Figure 5B). This could mean that the pretreatment with melatonin enhanced the migration of the healthiest sperm cells. On the other hand, no changes could be observed in samples in normozoospermic patients, showing high levels of compaction before migration and/or incubation. As expected, the fresh samples either incubated or not with melatonin showed the same compaction ratios. Given that the compaction occurs during spermiogenesis and also during the epididymal transit, there were no changes in the compaction of sperm cells during our preincubation,

although there was a selection of cells after the swim-up. It is worth highlighting that samples without melatonin pretreatment did not show any increase in the ratio of cells with compacted chromatin, despite the fact that they were treated during the swim-up.

In normozoospermic patients the results revealed a decrease in the ratio of fragmented DNA when fresh samples were preincubated with melatonin (Pr^{m}) regarding the control values (Figure 6). This decrease was emphasized when the swim-up was performed in pretreated cells ($\text{Pr}^{\text{m}}\text{Sw}^-$, $\text{Pr}^{\text{m}}\text{Sw}^{\text{m}}$), manifesting a significant difference just only after swim-up was performed in the presence of melatonin ($\text{Pr}^{\text{m}}\text{Sw}^{\text{m}}$) (Figure 6). The heterogeneity of oligozoospermic samples did not allow us to obtain a clear tendency of the fragmentation pattern in our study (data not shown).

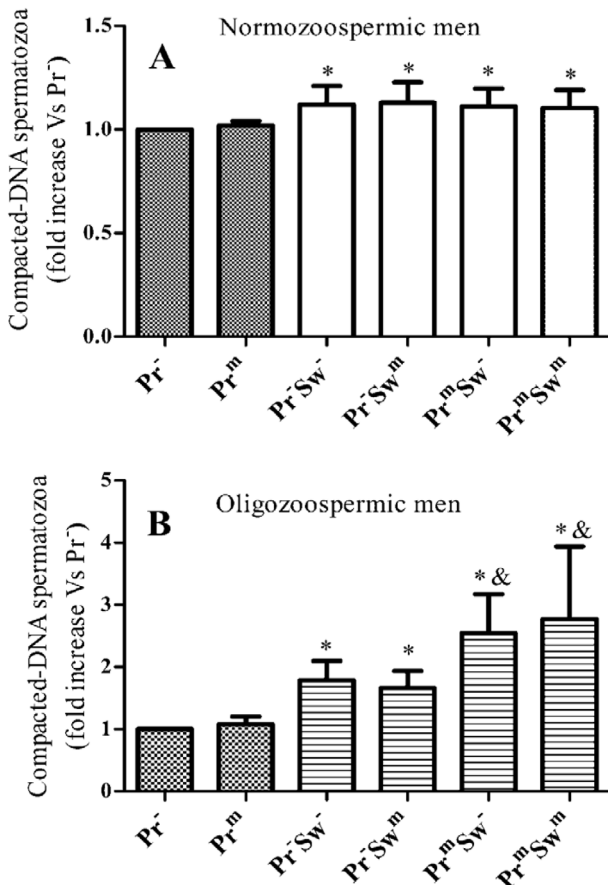


Figure 5. Quantification of sperm cells containing compacted DNA. DNA compaction was determined by PI content as described in Section 2. Values are presented as mean \pm SEM of 20 separate experiments and expressed as fold increase over the non-pretreatment level (Pr^-). *: $P < 0.05$ compared to Pr^- values. &: $P < 0.05$ compared to $\text{Pr}^{\text{m}}\text{Sw}^-$ and $\text{Pr}^{\text{m}}\text{Sw}^{\text{m}}$.

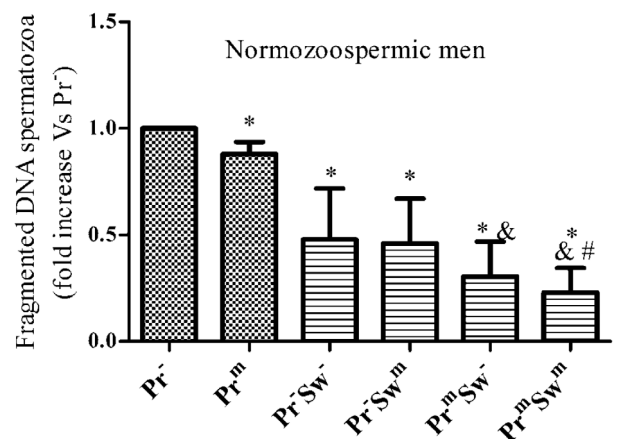


Figure 6. Quantification of sperm cells with fragmented DNA. DNA fragmentation was determined by PI content as described in Section 2. Values are presented as mean \pm SEM of 20 separate experiments and expressed as fold increase over the non-pretreatment level (Pr^-). *: $P < 0.05$ compared to Pr^- values. &: $P < 0.05$ compared to Pr^{m} . #: $P < 0.05$ compared to $\text{Pr}^{\text{m}}\text{Sw}^-$ and $\text{Pr}^{\text{m}}\text{Sw}^{\text{m}}$.

4. Discussion

The precise transmission of paternal genetic information largely depends on the integrity of DNA in the sperm, which is widely recognized as a marker of male infertility. High levels of DNA fragmentation in sperm cells are closely related to low levels of natural fertility (Agarwal and Said, 2003; Giwercman et al, 2010). Despite the fact that some studies did not find any relationship between DNA fragmentation and clinical outcomes of IVF-intracytoplasmic sperm injection (IVF-ICSI) (Gandini et al., 2004; Esbert et al., 2011), seemingly, excessive levels of ROS induce oxidative stress in the intracellular environment with dramatic effects on the outcome of ART, thereby lowering fertilization, implantation, and pregnancy rates (Lozano et al., 2009). The complete avoidance of ROS generation during ART is still unknown; however, new procedures to attenuate the ROS generated by critical steps during sperm preparation are worth exploring. A metaanalysis carried out by Collins et al. (2008) revealed a significant association between DNA fragmentation and poor success of IVF-ICSI. Moreover, a positive correlation has been found between levels of ROS and DNA fragmentation in sperm of infertile men (Moustafa et al., 2004; Fraczek and Kurpysz, 2005). In this sense, given the high antioxidant capacity of melatonin, it could guarantee a protective environment not only in vitro, as we are showing here (Figures 5 and 6), when the semen fluid is removed to collect spermatozoa, but also in vivo as it generates a milieu of lower levels of ROS (Bejarano et al., 2014). Certain levels of melatonin in the spermatozoa environment from spermatogenesis to capacitation could be an effective strategy to avoid ROS-induced infertility. It is worth taking into account that low levels of ROS act as second messengers of capacitation. Although, unlike other antioxidant compounds, melatonin reduces ROS levels, it does not completely remove them (DeLamirande and Gagnon, 1984). Nonetheless, the relation between DNA fragmentation and infertility is still unclear.

Regarding the processing of semen samples, a significant increase in sperm DNA fragmentation has been shown after incubations at 37 °C for 1 h (Balasuriya et al., 2014). This fact is important to consider during the liquefaction of semen and the short-term storage of semen in an IVF-ICSI cycle involving incubation at the aforesaid temperature (Balasuriya et al., 2014). Therefore, our present findings support supplementation with protective agents against unwanted damage, melatonin being an innocuous and effective candidate to combat DNA damage induced by oxidative stress, both in vivo and in vitro (Espino et al., 2010; Bejarano et al., 2014).

Andrology laboratory techniques, such as semen cryopreservation or repeated centrifugation cycles, have unwanted effects on germ cells (e.g., excessive levels of

ROS), thus causing oxidative stress in these cells. These effects produce irreversible damage to DNA (Kumar et al, 2011). Furthermore, during the performance of semen preparation techniques, the natural protection against oxidative threats is removed. Experimental evidence indicated that in vitro storage of sperm cells prior to insemination increases the resistance to nuclear decondensation in the oocyte. This phenomenon is due to an increase in cross-linking within the sperm cells' histones by the formation of disulfide bonds, thereby increasing embryonic mortality (Luchetti et al., 2009). These events make sense in a context of seminal plasma removal as well as an increased oxidative environment that favors formation of disulfide bonds. Taken together, these facts highlight the importance of minimizing the production of exogenous stress during sperm preparation in ART.

The spermatozoon is a peculiar cell with a large number of mitochondria and high metabolism, especially during its hyperactivation and capacitation, which generates abundant ROS (Lopez et al., 2009). The protective role of melatonin on mitochondria regarding activation of the intrinsic apoptosis pathway is well known (Acuña-Castroviejo et al., 2001; Espino et al., 2010; Radogna et al., 2015). Melatonin could lead to both direct ROS scavenging at high concentrations (millimolar) or increased activity and expression of antioxidant enzymes observed at low concentrations (nanomolar) (Martin et al., 2006; Reiter et al., 2008; Reiter et al., 2009), thus preventing oxidative damage both in vivo and in vitro (Espino et al., 2011; Bejarano et al., 2014). Moreover, it has been found that urinary 6-sulfatoxymelatonin and total antioxidant capacity levels positively correlate with a relevant improvement in seminogram values (Ortiz et al., 2011). Considering these aspects, infertile males were selected for the present study to evaluate the effect of incubation of semen samples with melatonin on sperm quality during storage and preparation for their use in ART.

Caspase-3 is a cysteine-protease that plays a crucial role in the execution phase of apoptosis. This protease is activated by both intrinsic and extrinsic pathways, leading to cell death. Therefore, active caspase-3 is an excellent indicator of cell death. Interestingly enough, the active caspase-3 is located exclusively in the midpiece of the sperm (Oehninger et al., 2003), where mitochondria are mainly located. We previously described that oxidative stress causes apoptosis in sperm cells via the mitochondrial pathway (Bejarano et al., 2008). Herein, it has been shown that the use of melatonin during incubation and sperm preparation for ART prevents both the rise in caspase-3 activity and the subsequent DNA fragmentation in the sperm samples. In both groups, normal and oligozoospermic, melatonin preincubation prior to sample processing (storage at 37 °C,

30 min) to use in ART was enough to decrease caspase-3 activity (Figure 3). Particularly, oligozoospermic samples displayed a reduction in caspase-3 activation in sperm cells after swim-up when cells were pretreated and/or migrated with melatonin (Figure 3B). Normozoospermic samples did not undergo any significant change in the caspase-3 activity when swim-up was performed in the presence of 1 mM melatonin (Figure 3A). Although it seems controversial, one should keep in mind that the good quality of normozoospermic samples could not help acknowledge improvements regarding the nontreated sample Pr (Figure 3). This fact indicates that melatonin could either prevent activation of caspase-3 or promote the migration of those sperm cells that have not suffered any activation of caspase-3. Despite the fact that the actual mechanism needs to be studied, melatonin undoubtedly prevents iatrogenic activation of caspase-3 and the consequent DNA fragmentation, in agreement with Espino et al. (2010), who reported that melatonin could revert the apoptotic events triggered by oxidative stress or increased levels of intracellular Ca^{2+} .

In human sperm cells, CD46 expression is strictly located in the inner acrosomal membrane (Anderson et al., 1989) and it is exposed only when the acrosome reaction occurs (D'Cruz and Haas, 1992). CD46 plays therefore an essential role in the process of fusion between the sperm and the oocyte membrane (Taylor et al., 1994). This molecule can be identified as a marker of the fertilizing capacity of sperm. Based on this, the expression of CD46 was analyzed in the samples in order to observe the effect of melatonin on the sperm preparation procedures. In both groups, normal and oligozoospermic, melatonin increased the ratio of adequate sperm (Figure 4). It is known that the acrosome reaction requires the presence of physiological levels of ROS. ROS at physiological levels are required to assure fertilization, given that mature sperm cells are exceptionally sensitive to oxidative stress (Agarwal et al., 2014b). This is consistent with previous results that indicated that oxidative stress impairs sperm motility and increases the tyrosine nitrosylation and S-glutathionylation of key sperm proteins, which might be involved in the pathological mechanism leading to the deterioration of sperm functions (Morielli and O'Flaherty, 2015). After subjecting the melatonin-preincubated samples (Pr^{m}) to swim-up, both migrated ($\text{Pr}^{\text{m}}\text{Sw}^{\text{m/-}}$) normozoospermic and oligozoospermic samples showed an increase in the ratio of CD46 with respect to those samples without melatonin treatment (PrSw) (Figure 4). This prompted us to hypothesize an association between oxidative stress and loss of capacitation during swim-up, which could become an obstacle for the acrosome reaction, the consequent CD46 exposure, and therefore sperm cells' functionality. In this scenario, melatonin could shield oxidative side effects during the ART procedures in vitro.

Chromatin compaction confers DNA with protection from oxidative damage. The mammalian sperm nucleus acquires high condensation during the late stages of spermatogenesis. When sperm DNA contains reduced compaction, chromatin is much more sensitive to oxidative stress and deletions, frame-shift mutations, DNA cross-links, and chromosomal rearrangements (Schulte et al., 2010). During the passage of sperm through the male genital tract, it undergoes the structural and biochemical changes needed to achieve optimal fertilizing capacity (Rodriguez et al., 1985). Decreased sperm chromatin compaction has been associated with subfertility in several species (Hekmatdoost et al., 2009). In normozoospermic samples, preincubation with melatonin (Pr^{m}) had no effect on DNA compaction (Figure 5A). By the same token, preincubation with melatonin in oligozoospermic samples had no influence on the ratio of sperm DNA compaction before swim-up (Figure 5B). On the other hand, in normozoospermic samples, the collection of motile sperm by swim-up showed an increase in the ratio of sperm cells with compacted DNA, independently of the preincubation and/or migration with melatonin (Figure 5A). Unlike in normozoospermic cases, the effect of melatonin on compacted-DNA spermatozoa became more visible in the oligozoospermic group (Figure 5). This may be due to the high compaction ratio among normozoospermic patients, which does not allow additional increases in the ratio of sperm cells with compacted DNA. An elevated heterogeneity of oligozoospermic patients in sperm DNA compaction was found (data not shown). As expected, the addition of melatonin to the fresh semen had no effect in the ratio of sperm cells with compacted DNA in both normal and oligozoospermic patients (Figure 5B). However, a significant increase was displayed when samples were swim-up, and the ratio of compaction was even higher when samples were preincubated with melatonin (Figure 5B), which, according to previous studies, is a potential improvement in fertility (Hekmatdoost et al., 2009). Obviously, melatonin cannot improve DNA compaction during the short time of sperm preparation for ART; however, these results could indicate that melatonin enhances the migration of sperm cells with compacted DNA (Figure 5B) and even prevents DNA fragmentation (Figure 6), as previously shown by Espino et al. (2011) when describing the molecular mechanisms involved in melatonin protection from oxidative stress. In any case, melatonin improves the quality of sperm samples at the DNA level for ART.

ART in vitro procedures facilitate ROS and oxidative stress development, which would harmfully affect sperm quality and accordingly diminish ART success. In order to minimize the risk of iatrogenic induction of DNA damage,

antioxidant protection of the sperm must be considered. For example, it could be useful to supplement either the seminal plasma for short-term in vitro storage or culture media for swim-up or centrifugation. Based on the findings shown, melatonin is promising as an adjunctive therapy in the treatment of infertility. Its antioxidant properties and its low toxicity make it an ideal tool to protect sperm from the oxidative damage caused during the preparation of sperm for use in ART. In addition to its antioxidant capability, different studies asserted that melatonin promotes in vitro sperm motility (du Plessis et al., 2010; Ortiz et al., 2011). Nonetheless, further research based on conducting studies with larger populations is required to confirm the potential and applicability of melatonin in ART.

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