Melatonin protects human spermatozoa from apoptosis via melatonin receptor– and extracellular signal–regulated kinase-mediated pathways

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Objective: To evaluate whether the protective effect of melatonin on H_2O_2-induced caspase activation and DNA fragmentation depends on the interaction between melatonin and its surface receptors.
Design: Laboratory study.
Setting: Center for assisted human reproduction at a Spanish hospital.
Patient(s): Twenty-one healthy donors.
Intervention(s): Human spermatozoa were treated with increasing concentrations of hydrogen peroxide (H_2O_2; 1μM, 10 μM, 100 μM, 1mM) and preincubated with 1 mM melatonin.
Main Outcomes Measure(s): Activation of caspase-3 and -9 as well as DNA fragmentation were examined by fluorescence methods.
Result(s): Our findings showed that H_2O_2 induced a significant increase in caspase-9 and caspase-3, which was dose independent. Conversely, pretreatment with melatonin reduced H_2O_2-mediated caspase activation in a dose-dependent way. Moreover, the antiapoptotic effects of melatonin in ejaculated human spermatozoa may involve membrane melatonin receptor MT1. In addition, we found that the survival-promoting pathway extracellular signal–regulated kinase (ERK) is likely to have a role in the protective actions of melatonin in ejaculated human spermatozoa. Finally, we confirmed these results further by demonstrating that melatonin prevention of H_2O_2-induced DNA fragmentation is dependent on both MT1 receptor and ERK signaling.
Conclusion(s): These results indicate that the stimulation with melatonin triggers a set of events culminating in cell death prevention in ejaculated human spermatozoa. (Fertil Steril® 2011;95:2290–6. ©2011 by American Society for Reproductive Medicine.)

Key Words: Melatonin, apoptosis, spermatozoa, MT receptor, MAPK signaling

Considering the morphologic and biochemical criteria, it is assumed that somatic cells can die in an apoptotic, autophagic, or necrotic manner (1, 2); however the mechanisms of sperm death are not clear. Although the mature spermatozoa are transcriptionally inactive and the typical apoptosis features seen in somatic cells are absent, recent publications have suggested that human spermatozoa have the ability to undergo apoptosis or apoptosis-like conditions (3–6). We have demonstrated previously that hydrogen peroxide (H_2O_2) and P are able to induce a mitochondria-dependent apoptosis in ejaculated human spermatozoa, which requires rises in cytosolic calcium concentration ($[Ca^{2+}]_c$) and calcium entry into mitochondria (4). Nevertheless, apoptosis also can be stimulated by oxidative stress, as it has been demonstrated in several cell types (7). Oxidative stress-induced sperm damage may occur when the intracellular reactive oxygen species (ROS) levels are in excess of the cells' scavenging capacity (8). Although physiologic levels of ROS are required for normal sperm function (9), spermatozoa have a limited amount of cellular cytoplasm in which scavenging enzymes are found, thus making them highly susceptible to ROS damage (10).

The pineal gland hormone melatonin may be involved in the protection of different cell types against damage-induced apoptosis (11, 12). Melatonin acts on practically all cell types by binding specific receptors and interactors, such as the plasma membrane receptors MT1/MT2 (13), thereby eliciting specific signal transduction pathways (14). Furthermore, melatonin and its metabolites potently scavenge ROS (15), thus altering redox-sensitive events and preventing oxidative damage. The effects of melatonin and its metabolites on ROS fluxes and mitochondrial protection (16) are expected to influence the activation of fundamental signaling pathways such as stress-activated/mitogen-activated protein kinases (SAP/

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MAPKs). These protein kinases are mediators of the signal transduction from the cell surface to the nucleus, to control virtually all the physiologic processes in eukaryotic cells (17, 18). In this regard, it has been proposed recently that extracellular signal–regulated kinase (ERK) plays a central role in the decision-making process that determines whether cells will live or die in response to apoptotic stimuli (19).

Because the sperm processing procedures used for assisted reproductive techniques (ART) aimed to improve sperm quality by increasing the number of progressive motile and morphologically normal sperm cells, protection of functional spermatozoa is a vital component of ART. Although a melatonin-enhanced motility has been recently reported in human spermatozoa (20), little is known about the protective effects of melatonin against apoptosis and oxidative damage in ejaculated human spermatozoa (21). For this reason, the goal of this study was to evaluate whether the protective effect of melatonin on sperm apoptosis induced by the oxidizing agent H_2O_2 depends on the interaction between melatonin and its surface receptors. In addition, we also analyzed whether melatonin sperm protection is linked to ERK, phosphoinositide 3-kinase (PI3K) activation, or both.

MATERIALS AND METHODS

Reagents

Melatonin, H_2O_2, 2-benzyl-N-acetyltryptamine (luzindole), PD98059, LY294002, nonidet-P-40 substitute (NP40), MES hydrate, HEPES, CHAPS, dithiothreitol (DTT), RPMI 1640 medium, and N-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (AC-DEVD-AMC) were purchased from Sigma (Madrid, Spain). The MT2-specific antagonist 4-phenyl-2-propionamidotetralin (4P-PDOT) was acquired from Tocris (Bristol, UK). N-acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (AC-LEHD-AMC) was obtained from Bachem (Bubendorf, Switzerland). The in situ cell death detection kit was acquired from Roche Diagnostics (Mannheim, Germany). Hoechst 33342 was purchased from Calbiochem (San Diego, CA). All others reagents were of analytical grade.

Sample Collection and Preparation

Human semen was obtained from 21 healthy donors according to World Health Organization guidelines (22) at the Extremadura Center of Human Assisted Reproduction (Badajoz, Spain). This study was approved by the Institutional Review Board of the University of Extremadura and by the ethics committee of the Infantile Hospital (Badajoz, Spain). Samples were collected by masturbation after 4 or 5 days of sexual abstinence. Semen was washed twice in RPMI 1640 medium (250 × *g*, 10 min), the supernatant was discarded, and the pellet was resuspended in RPMI 1640 medium.

Melatonin action on MT1/MT2 receptors was antagonized by luzindole and 4P-PDOT. Luzindole and 4P-PDOT were added at concentrations of 50 μM and 50 nM, respectively (23). Experiments of SAP/MAPK inhibition were performed by using a selective pharmacologic inhibitor of ERK, namely PD98059. Similarly, a selective pharmacological inhibitor of PI3K, LY294002, was used to inhibit the PI3K/Akt pathway. PD98059 and LY294002 were added at final concentrations of 1 and 10 μM, respectively (24). Each of these inhibitors was added 30 min before the preincubation with melatonin.

Assay for Caspase Activity

Stimulated or resting cells were pelleted and washed once with phosphate-buffered saline (PBS). After centrifugation, cells (4×10^7 cells/ml) were again resuspended in PBS. Fifteen microliters of the cell suspension was added to a microplate and mixed with the appropriate peptide substrate dissolved in a standard reaction buffer, which was composed of 100 mM HEPES, pH 7.25, 10% sucrose, 0.1% CHAPS, 5 mM DTT, 0.001% NP40, and 40 μM of caspase-3 substrate (AC-DEVD-AMC) or 0.1 M MES hydrate, pH 6.5, 10% PEG , 0.1% CHAPS, 5 mM DTT, 0.001% NP40, and 100 μM of caspase-9 substrate (AC-LEHD-AMC). Substrate cleavage was measured with a microplate reader (Tecan Infinite M200) with an excitation wavelength of 360 nm and emission at 460 nm (25). Data were calculated as fluorescence units per milligram of protein and were presented as fold-increase over the pretreatment level (experimental/control).

In Situ Detection of DNA Fragmentation by TUNEL and Hoechst Staining

At the end of a treatment protocol, human spermatozoa were harvested and washed once with PBS. The cells were then fixed with 4% paraformaldehyde (in PBS, pH 7.4, at least 6 hours, room temperature [RT]) and air-dried on slides for 24 hours. Afterward, the air-dried cells were washed twice with PBS and incubated in permeabilization solution (0.1% Triton-X-100 in 0.1% sodium citrate, 15 min, RT). The permeabilization solution was then removed, deoxyuride-5′-triphosphate biotin nick end labeling (TUNEL) reaction mixture (50 μL) was added, and the cells were incubated (1 hour, 37°C) in a humidified chamber. The cells were washed again with PBS and counterstained with Hoechst 33342 (1 μg/mL) for 5 min to identify cellular nuclei. The incidence of apoptosis was assessed under an epifluorescence microscope (BX51, Olympus Spain S.A.U., Barcelona, Spain) using an FITC filter. Cells with TUNEL-positive nuclei were considered apoptotic (26). Hoechst staining was used to determine the total number of cells in each field. A minimum of five fields per slide were used to calculate the percentage of apoptotic cells.

Statistical Analysis

Data are expressed as means ± SEM of the numbers of determinations. Statistical significance was calculated by one-way analysis of variance followed by the Tukey's multiple comparison test. $P<0.05$ was considered statistically significant.

RESULTS

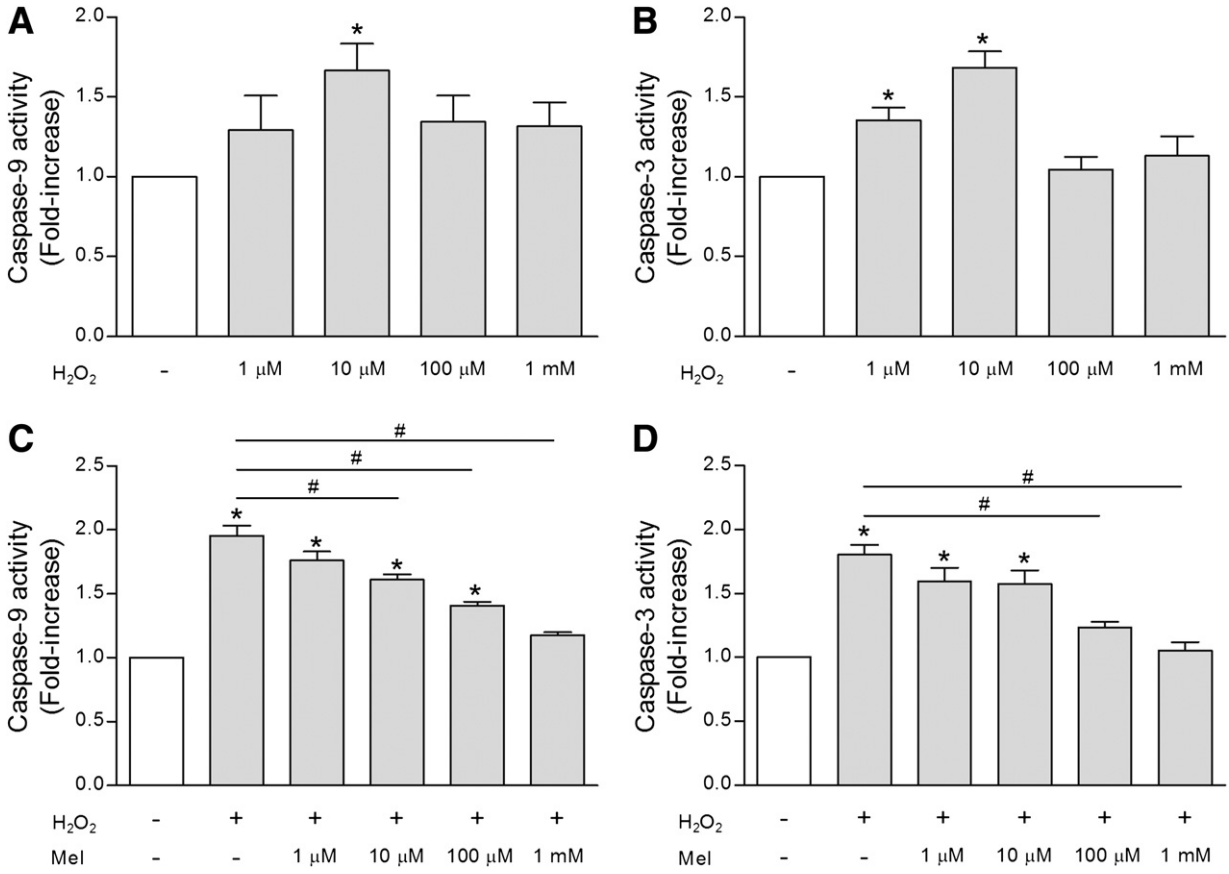
To examine the effect of oxidizing agents on caspase activation, human spermatozoa were treated with increasing concentrations of H_2O_2 (1 μM–1 mM) for 1 hour. Treatment of spermatozoa with H_2O_2 for 1 hour induced caspase-9 activation, an initiator caspase that is involved in the initial steps of mitochondrial apoptosis (27), in a dose-independent trend. Cell stimulation with H_2O_2 caused maximum stimulation at 10 μM (1.66 ± 0.19 fold-increase; $P<0.05$; Fig. 1A). Similar findings were obtained for caspase-3 activation, the maximum effect being produced at 10 μM (1.68 ± 0.11 fold-increase; $P<0.05$; Fig. 1B). Because the dose of 10 μM H_2O_2 displayed the most detrimental effects, in terms of caspase activation, such a dose was used to induce apoptosis in subsequent experiments.

Because we previously proved that melatonin protects against apoptosis and oxidative damage in ejaculated human spermatozoa (21), we also analyzed whether melatonin protection is dose dependent. To this end, increasing doses of melatonin (1 μM–1 mM) were added 1 hour before apoptosis induction by 10 μM H_2O_2. Although the dose of 1 μM melatonin proved to be ineffective, the inhibiting actions of melatonin on caspase-9 activation seem to be dose dependent, the highest dose of melatonin (1 mM) being the most protective (1.17 ± 0.02 vs. 1.95 ± 0.09 fold-increase in H_2O_2-treated spermatozoa in the presence or absence of melatonin, respectively; $P<0.05$; Fig. 1C). Similar results were found regarding H_2O_2-induced caspase-3 activation. Thus, melatonin administration diminished caspase-3 activity evoked by 10 μM H_2O_2, the highest dose of melatonin (1 mM) being the most efficient (1.05 ± 0.07 vs. 1.80 ± 0.08 fold-increase in H_2O_2-treated spermatozoa in the presence or absence of melatonin, respectively; $P<0.05$; Fig. 1D). For this reason, the dose of 1 mM melatonin was used to prevent apoptosis in subsequent experiments.

To investigate whether the protective effect of melatonin on sperm apoptosis actually depends on the interaction between

FIGURE 1

Melatonin reverses hydrogen peroxide (H_2O_2)-induced caspase activation in a dose-dependent manner. (**A** and **B**) Human spermatozoa were stimulated for 1 hour with increasing concentrations of H_2O_2 (1 μM, 10 μM, 100 μM, and 1mM). (**C** and **D**) Spermatozoa were preincubated for 1 hour with increasing concentrations of melatonin (Mel; 1 μM, 10 μM, 100 μM, and 1mM) and then stimulated with 10 μM H_2O_2. Caspase-9 and -3 activity was estimated as described in the Materials and Methods section. Values are presented as means ± SEM of six independent samples and expressed as fold-increase over the pretreatment level (experimental/control). *P<0.05 compared with control values; #P<0.05.



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melatonin and its cell surface receptors, we also analyzed whether luzindole, which specifically antagonizes melatonin binding and activation of MT1/MT2 receptors, is able to counteract the inhibiting actions of 1 mM melatonin on H_2O_2-induced caspase activation. As shown in Figure 2A, in the presence of luzindole (50 μM), melatonin is no longer able to forestall H_2O_2-induced caspase-9 activation (1.89 ± 0.09 vs. 1.17 ± 0.02 fold-increase in the presence or absence of luzindole, respectively; P<0.05). Similar findings were obtained regarding caspase-3 activity (Fig. 2B). These results show that the signal transduction elicited by MT1/MT2 receptor stimulation affects the protective effect of melatonin on sperm apoptosis.

As an alternative, we also studied the involvement of melatonin receptors on melatonin anti-apoptotic actions. 4P-PDOT, which selectively antagonizes melatonin binding and activation of the MT2 receptor, was added at the concentration of 50 nM. In the presence of 4P-PDOT, melatonin is able to counteract H_2O_2-induced caspase-9 activation (1.30 ± 0.06 vs. 1.17 ± 0.02 fold-increase in the presence or absence of 4P-PDOT, respectively; Fig. 2A). Similar results were obtained regarding caspase-3 activity (Fig. 2B); therefore, MT2 receptor does not appear to be involved in the protective effect of melatonin on sperm apoptosis, thereby suggesting that the antiapoptotic effects displayed by melatonin are likely mediated through the MT1 receptor.

To confirm the involvement of the signal transduction, we analyzed a different downstream step of a more generalized nature—that is, activation of ERK, PI3K, or both, which may be contrasted by the inhibitors PD98059 and LY294002, respectively. Therefore, in the presence of PD98059 (1 μM), the inhibitory effects of melatonin on H_2O_2-induced caspase-9 activation were abolished (1.82 ± 0.09 vs. 1.17 ± 0.02 fold-increase in the presence or absence of PD98059, respectively; P<0.05; Fig. 3A). However, LY294002 (10 μM) was unable to modify the inhibitory effects of melatonin on H_2O_2-evoked caspase-9 activation (1.24 ± 0.03 vs. 1.17 ± 0.02 fold-increase in the presence or absence of LY294002, respectively; Fig. 3B). These results highlight the possible implication of the survival-promoting pathway ERK on the protective actions of melatonin in human spermatozoa, whereas PI3K seems to lack a role in this system.

Because TUNEL assay is a well-established method for detecting DNA cleavage, a relatively late apoptotic marker (26), we assessed the proportion of spermatozoa depicting DNA fragmentation in the presence of H_2O_2 to verify whether caspase activation induced by the oxidant agent, in turn, leads to cell death. Treatment of

FIGURE 2

Melatonin receptors mediated inhibiting effects of melatonin on hydrogen peroxide (H_2O_2)-induced caspase activation. Human spermatozoa were preincubated with 1 mM melatonin (Mel) for 1 hour in the absence or the presence of its MT1/MT2 receptor antagonists luzindole (Luz) and 4P-PDOT, and then stimulated with 10 μM H_2O_2 to check caspase-9 (**A**) and caspase-3 (**B**) activity. Luzindole and 4P-PDOT were added at the concentration of 50 μM and 50 nM, respectively, 30 min before other treatments. Caspase activity was estimated as described in the Materials and Methods section. Values are presented as means ± SEM of six independent samples and expressed as fold-increase over the pretreatment level (experimental/control). *P<0.05 compared with control values; #P<0.05.

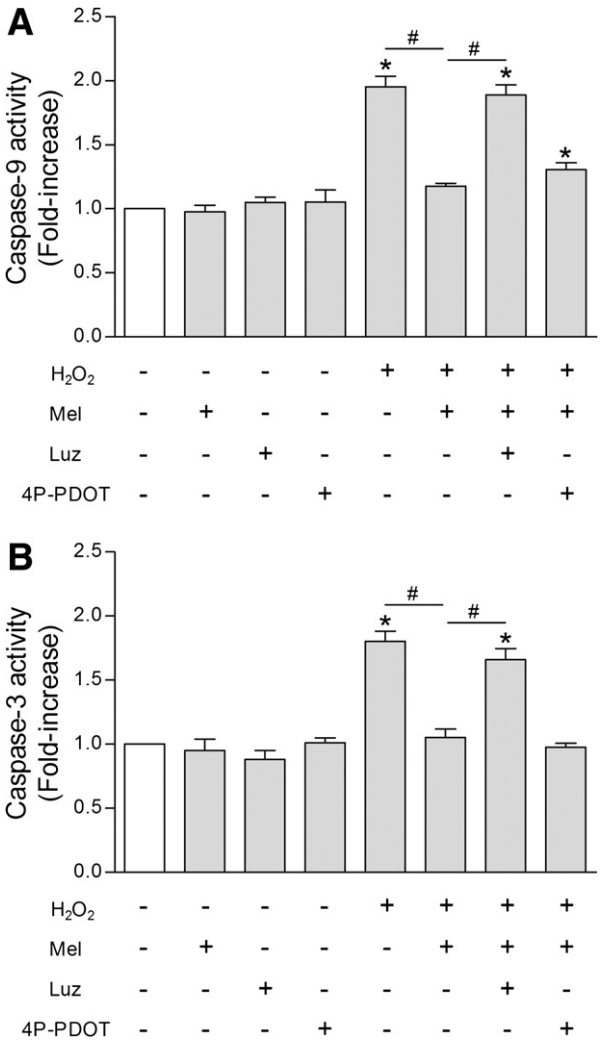

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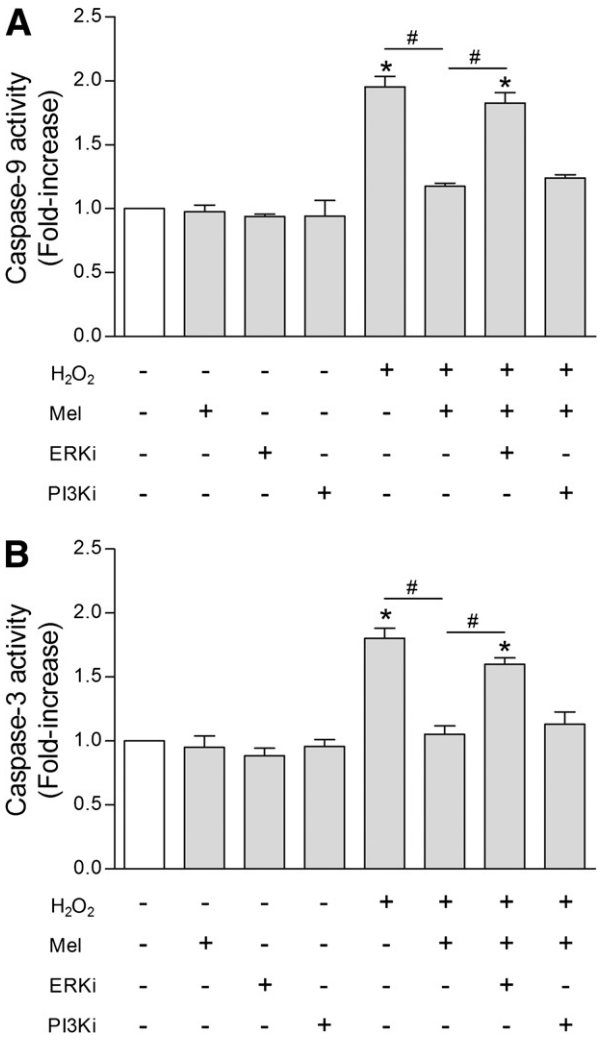
FIGURE 3

MAPK signaling mediated the inhibiting effects of melatonin on hydrogen peroxide (H_2O_2)-induced caspase activation. Human spermatozoa were preincubated with 1 mM melatonin (Mel) for 1 hour in the absence or presence of the kinase inhibitors PD98059 (ERKi) and LY294002 (PI3Ki), and then stimulated with 10 μM H_2O_2 to check caspase-9 (**A**) and caspase-3 (**B**) activity. PD98059 and LY294002 were added at concentrations of 1 μM and 10 μM, respectively, 30 min before other treatments. Caspase activity was estimated as described in the Materials and Methods section. Values are presented as means ± SEM of six independent samples and expressed as fold-increase over the pretreatment level (experimental/control). *P<0.05 compared with control values; #P<0.05.



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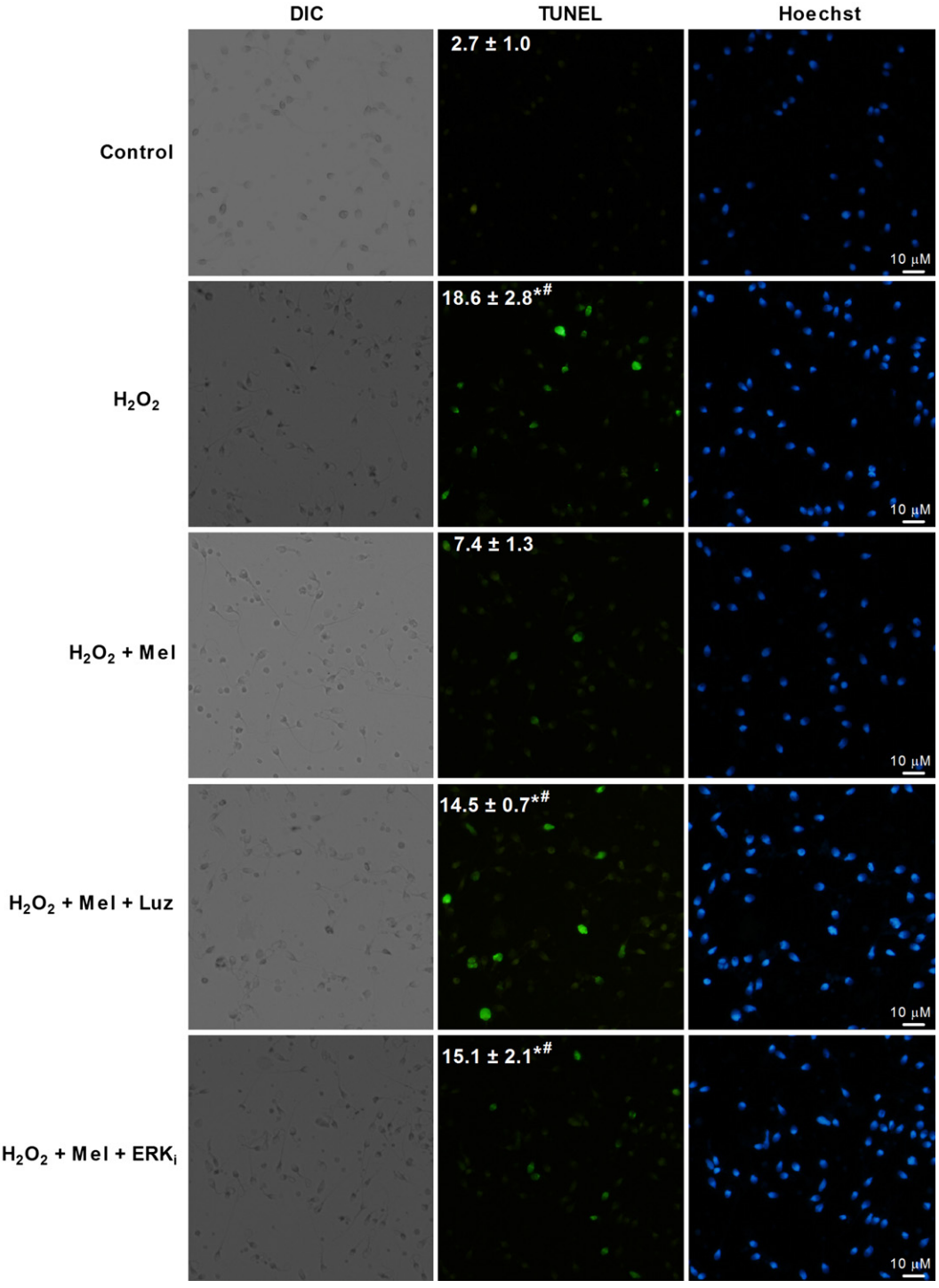
spermatozoa with H_2O_2 (10 μM for 1 hour) substantially enhanced the proportion of spermatozoa depicting DNA fragmentation (18.6 ± 2.8% vs. 2.7 ± 1.0% in the presence or absence of H_2O_2, respectively; P<0.05; Fig. 4), thus showing that the oxidant agent H_2O_2 succeed in triggering an apoptotic program. Interestingly, the preincubation of human spermatozoa with melatonin (1 mM for 1 hour) was able to completely counteract the stimulatory effect of H_2O_2 on DNA fragmentation (7.4 ± 1.3%; Fig. 4). In the presence of either the MT1/MT2 receptor antagonist luzindole or the ERK inhibitor PD98059, melatonin is no longer able to reverse H_2O_2-induced DNA fragmentation (14.5 ± 0.7 and 15.1 ± 2.1% in the presence of luzindole and PD98059, respectively; P<0.05; Fig. 4). These findings indicate that the signal transduction elicited by MT1 receptor stimulation by melatonin triggers a set of events culminating in cell death prevention.

FIGURE 4

Melatonin counteracts H_2O_2-mediated DNA fragmentation through melatonin receptor- and ERK-dependent pathways. Human spermatozoa were preincubated with melatonin (Mel; 1 mM) for 1 hour in the absence or presence of and luzindole (Luz) and PD98059 (ERKi), and then stimulated with H_2O_2 (10 μM) to check the proportion of cells depicting DNA fragmentation. Luzindole and PD98059 were added at concentrations of 50 μM and 1 μM, respectively, 30 min before other treatments. DNA fragmentation was estimated as described in the Materials and Methods section. *Left panel*: differential interference contrast (DIC) images of cultured cells. *Center* and *right* panels: pictures of TUNEL-positive cells (*green*) and Hoechst 33342 nuclear stain (*blue*), respectively. Scale bars, 10 μm. Values inside the central panel are presented as means ± SEM of three separate experiments and expressed as a percentage of cells depicting DNA fragmentation. *$P<0.05$, regarding control group; #$P<0.05$, regarding H_2O_2 + Mel group.



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DISCUSSION

In the last decade, several studies have investigated apoptosis in ejaculated sperm. The existence of a caspase-dependent apoptotic pathway in ejaculated human spermatozoa has been extensively demonstrated (4, 28), and the evaluation of caspase activation has been suggested as a sensitive assay for detection of sperm apoptosis (29). Other somatic apoptotic markers, such as DNA fragmentation and phosphatidylserine (PS) exposure, have been used to detect sperm apoptosis (5, 6). In the present study, we have shown that increasing concentrations of the oxidant agent H_2O_2 induce activation of caspase-3 and caspase-9 in a dose-independent fashion. Moreover, we also demonstrated that H_2O_2-mediated caspase activation, in turn, leads to cell death, because H_2O_2-treated spermatozoa depicted increased DNA fragmentation, a late detectable event on nucleus during apoptosis. These results are consistent with previous findings reporting an H_2O_2-induced apoptosis that is dependent on the mitochondrial pathway and mediated by the mitochondrial calcium overload (4, 5). Likewise, Mahfouz et al. (30) have recently described that exposure to H_2O_2 significantly reduced sperm viability and increased the percentage of dead sperm in neat sperm, as well as in immature and mature sperm fractions.

Emerging evidence suggests that the pineal gland hormone may be involved in some important processes, such as the protection of different cell types against damage-induced apoptosis (11, 12). In this sense, we have recently described the protective effects of melatonin against apoptosis and oxidative damage in ejaculated human spermatozoa (21). In addition, we have report herein that the antiapoptotic actions displayed by melatonin in human spermatozoa are clearly dose dependent, the highest dose of melatonin tested (1 mM) being the most efficient to prevent apoptotic events.

Regardless of whether we initially indicated that melatonin protection in human spermatozoa was likely due to its free-radical scavenger properties, we have proved that antiapoptotic actions of melatonin in ejaculated human spermatozoa may also involve MT1 membrane melatonin receptor. In fact, the inhibiting actions of melatonin on H_2O_2-induced apoptosis was abolished by the addition of the MT1/MT2 melatonin receptor antagonist luzindole to the incubation medium, whereas the addition of the specific MT2 antagonist 4P-PDOT was incapable of altering the protective effects of melatonin. In this regard, previous reports have described that melatonin antagonizes apoptosis via membrane melatonin receptors in several cell types (23, 31). We have also found that the ERK defense pathway is likely to have a role in the protective actions of melatonin on sperm apoptosis. Again, our findings are consistent with previous reports postulating a connection between melatonin's antiapoptotic actions and MAPK signaling in several cell types (32, 33).

There is a great interest in protecting spermatozoa from ROS and apoptosis during their manipulation and cryopreservation for ART purposes. In this sense, it has been reported that ART may show significant improvement in in vitro supplementation of antioxidants or metal chelators to achieve a better success (34). For this reason, melatonin may be used as a powerful free-radical scavenger and antiapoptotic agent to supplement sperm preparation media and to obtain successful ART outcome. Nonetheless, our findings cannot be generalized back to the male population, because this study was performed only with normozoospermic men. Additional studies using subfertile men should be conducted.

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