Melatonin as a potential tool against oxidative damage and apoptosis in ejaculated human spermatozoa

It is assumed somatic cells can die in the apoptotic, the autophagic, or the necrotic way; however, the mechanisms of sperm death are not clear. Here, ejaculated human spermatozoa were evaluated for apoptosis and reactive oxygen species production in the absence or presence of melatonin, and we concluded that melatonin reverses sperm apoptosis due to its free radical scavenging actions. (Fertil Steril® 2010;94:1915–7. ©2010 by American Society for Reproductive Medicine.)

Recent publications have suggested that human spermatozoa have the ability to undergo apoptosis or apoptosis-like conditions (1–3). Although calcium is a key regulator of cell survival, the sustained elevation of intracellular calcium plays a role in cell death (4). In addition, apoptosis also can be stimulated by oxidative stress per se, as it has been demonstrated in several cell types (5).

Melatonin is known to regulate seasonal and circadian rhythms of mammals (6), but emerging evidence suggests that melatonin possesses protective effects against free radicals and apoptosis, which may be also caused by its metabolites (7, 8). Therefore, the aim of this study was to evaluate the effect of melatonin on caspase activation, phosphatidylserine (PS) exposure, and reactive oxygen species (ROS) production induced by both the calcium-mobilizing agonist P (9) and the oxidant agent H_2O_2, as well as to analyze the potential protective actions of the pineal gland hormone on ejaculated human spermatozoa viability.

Javier Espino, M.Sc.[a]
Ignacio Bejarano, M.Sc.[a]
Águeda Ortiz, B.Sc.[b]
Graciela M. Lozano, Ph.D.[b]
Juan F. García, M.D.[b]
José A. Pariente, Ph.D.[a]
Ana B. Rodríguez, Ph.D.[a]
[a] *Department of Physiology, Faculty of Science, University of Extremadura, Badajoz, Spain*
[b] *Extremadura Centre for Human Assisted Reproduction, Badajoz, Spain*

Received November 3, 2009; revised and accepted December 29, 2009; published online February 12, 2010.
J.E. has nothing to disclose. I.B. has nothing to disclose. A.O. has nothing to disclose. G.M.L. has nothing to disclose. J.F.G. has nothing to disclose. J.A.P. has nothing to disclose. A.B.R. has nothing to disclose.
Supported by Merck, S.L. and MEC-DGI (BFU2007-60091). J. Espino and I. Bejarano are supported by Fundación Salud 2000 and Junta de Extremadura (PRE06070) fellowships, respectively.
Reprint requests: Ana B. Rodríguez, Ph.D., Department of Physiology, Faculty of Science, University of Extremadura, 06071 Badajoz, Spain (FAX: 34-924-289388; E-mail: moratino@unex.es).

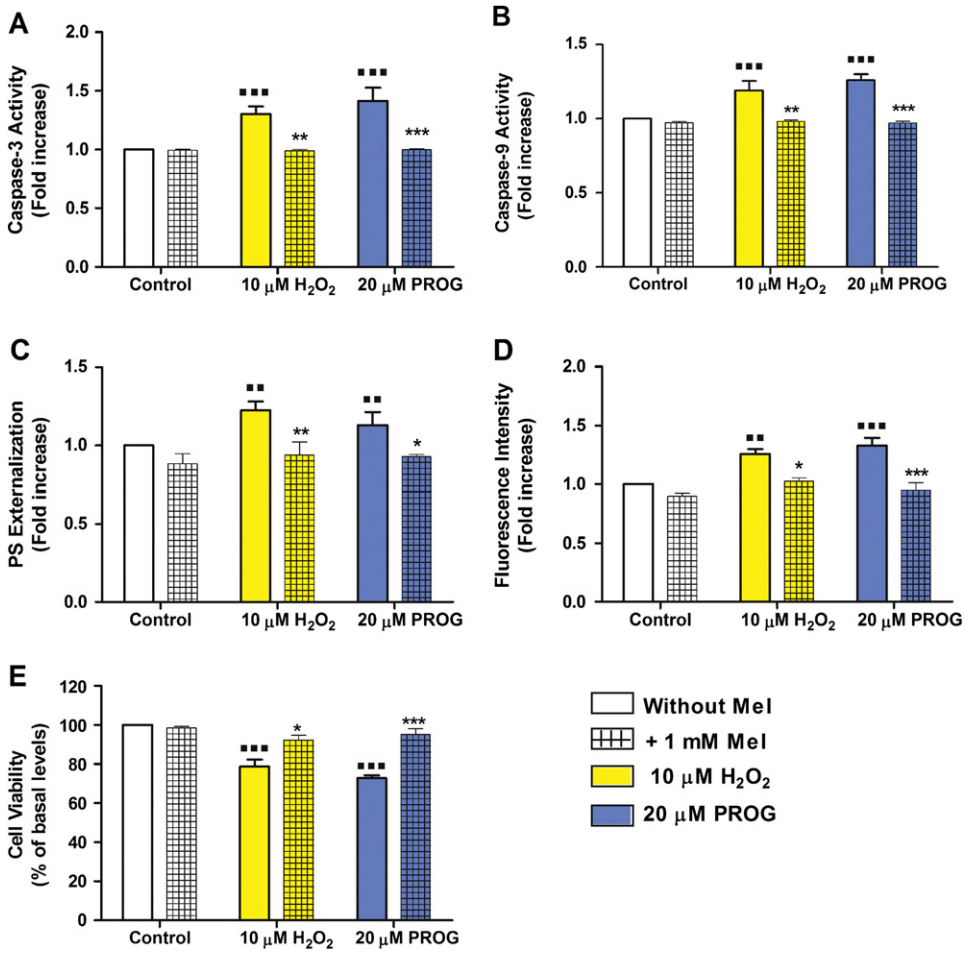
Human semen was obtained from 20 healthy donors according to World Health Organization guidelines at the Extremadura Center of Human Assisted Reproduction (Badajoz, Spain). This study was approved by the Institutional Review Board (IRB) of the University of Extremadura and by the Ethics Committee of the Infantile Hospital (Badajoz, Spain) and was carried out in accordance with the Declaration of Helsinki. Informed consent was obtained from all of the donors. Samples were collected by masturbation after 4 or 5 days of sexual abstinence.

Our findings showed that the treatments of human spermatozoa with both 10 μM of H_2O_2 and 20 μM of P for 60 minutes significantly increased both caspase-3 (Fig. 1A) and caspase-9 activities (Fig. 1B). The activities of caspase-3 and caspase-9 were calculated from the cleavage of their respective specific fluorogenic substrate. To examine the effects of melatonin on caspase activation induced by H_2O_2 and P, samples were preincubated with 1 mM of melatonin for 30 minutes. After apoptosis was induced in both control and melatonin-treated cells with 10 μM of H_2O_2 and 20 μM of P for 60 minutes. Melatonin inhibited the increase of caspase-3 (Fig. 1A) and caspase-9 (Fig. 1B) activities evoked by both H_2O_2 and P toward control values. The incubation with melatonin alone was unable to modify caspase activity with regard to control. To investigate the effect of melatonin on an early apoptotic event, we checked the apoptotic state of spermatozoa by testing for PS exposure. The PS externalization was determined by incubating spermatozoa with annexin V-fluorescein isothiocyanate and cell staining was measured by using a fluorescence spectrophotometer. As shown in Figure 1C, treatment of spermatozoa with 10 μM of H_2O_2 or 20 μM of P for 60 minutes induced a significant increase in PS externalization. However, the pretreatment with 1 mM of melatonin for 30 minutes significantly reduced either H_2O_2- or P-evoked PS externalization, which indicates that melatonin is able to avoid PS exposure, one of the main events in ejaculated human spermatozoa apoptosis. In addition, pretreatment of sperm cells with melatonin alone did not modify PS externalization.

The effect of mitochondrial calcium overload induced by P on oxidative metabolism was also evaluated by using the fluorescent dye dihydrorhodamine 123 (10). To that end, ejaculated human spermatozoa were treated with both P and H_2O_2, a commonly

FIGURE 1

Effects of melatonin on caspase activity, phosphatidylserine (PS) exposure, mitochondrial reactive oxygen species levels, and sperm viability. Cells were preincubated with 1 mM of melatonin (Mel) for 30 minutes, and then apoptosis was induced in both control and melatonin-treated cells with 10 μM of H_2O_2 and 20 μM of P (PROG) for 60 minutes. Caspase-3 (**A**) and caspase-9 (**B**) activity, PS exposure (**C**), mitochondrial reactive oxygen species levels (**D**), and sperm viability (**E**) were estimated as described in the text. Values are presented as means ± SD of four to six separate experiments and expressed as fold increase above pretreatment levels (untreated samples) (experimental/control) or percentage of basal levels (time = 0) One-way analysis of variance followed by Tukey's test was used for multiple comparisons. ■■$P<.01$ and ■■■$P<.001$ compared with control values. *$P<.05$, **$P<.01$, and ***$P<.001$ compared with H_2O_2 or P alone.



Espino. Correspondence. Fertil Steril 2010.

used tool to induce oxidative stress in experimental models, which was used as a positive control. Figure 1D shows that the treatment with 20 μM of P for 60 minutes caused a significant increase in mitochondrial ROS levels in ejaculated human spermatozoa, which were even higher than those evoked by the treatment with H_2O_2. Interestingly, when ejaculated human spermatozoa were previously incubated with 1 mM of melatonin for 30 minutes, mitochondrial ROS production was significantly restored, due to the free radical scavenging properties exhibited by the pineal gland hormone melatonin. Again, pretreatment of cells with melatonin alone evoked a slight, nonsignificant decrease in ROS production. To test whether oxidative stress induced by the treatments contributes to the fate of the cell, we examined the effect of P and H_2O_2 on sperm viability, which was assessed by 93-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT; Sigma, Scharlau, Spain) assay. In both P- and H_2O_2-treated spermatozoa, there was a significant diminution in cell survival with respect to the values obtained under control (untreated cells) conditions (Fig. 1E). Once more, pretreatment with melatonin (1 mM, 30 minutes) forestalled cell death provoked by both treatments, emphasizing the protective role of melatonin against cell death.

The discovery of melatonin as antioxidant (11) has stimulated many studies that have uniformly documented the ability of this molecule to detoxify harmful reactants and reduce molecular damage (12, 13). However, to our knowledge, we are the first to describe the protective role of melatonin against oxidative damage and apoptosis in ejaculated human spermatozoa, which is the result of its direct or indirect free radical scavenging action. Thus, we have found that ejaculated human spermatozoa exposed for a short time to melatonin reversed caspase-3 and caspase-9 activation as well as PS externalization evoked by H_2O_2 and P, restored

the increased mitochondrial ROS production induced by both treatments, and forestalled the diminution in sperm viability.

Studies have indicated that human spermatozoa significantly increased levels of ROS production in response to repeated cycles of centrifugation involved in conventional preparation techniques used for assisted reproductive techniques (ART) (14). Therefore, there is a great interest in protecting spermatozoa from ROS and apoptosis during manipulation and cryopreservation. We propose the use of melatonin, which has an uncommonly low toxicity profile, as a powerful antioxidant and antiapoptotic agent in ejaculated human spermatozoa, as demonstrated in the current study, to supplement sperm preparation media to improve ART outcome. In this regard, previous studies provided evidence that melatonin may also have inhibitory effects on sperm motility in vitro (15) or occasionally decrease sperm concentration and motility below the normal range in healthy men (16). However, effects of melatonin on sperm motility are still controversial, as Fujinoki (17) has recently proven a melatonin-enhanced hyperactivation of hamster sperm. Thus, characterization of the role of melatonin on apoptosis and oxidative damage in ejaculated human spermatozoa may be helpful in preserving high rates of functional spermatozoa after capacitation, or even cryopreservation, techniques for their use in any assisted reproduction program. Further studies are required to clarify molecular mechanisms responsible for the protective actions shown by melatonin against apoptosis and oxidative damage in ejaculated human spermatozoa and to test whether melatonin supplementation is feasible to obtain a successful ART outcome.

REFERENCES

1. Grunewald S, Paasch U, Said TM, Sharma RK, Glander HJ, Agarwal A. Caspase activation in human spermatozoa in response to physiological and pathological stimuli. Feril Steril 2005;83:1106–12.
2. Bejarano I, Lozano GM, Ortiz A, García JF, Paredes SD, Rodríguez AB, et al. Caspase 3 activation in human spermatozoa in response to hydrogen peroxide and progesterone. Fertil Steril 2008;90:1340–7.
3. Lozano GM, Bejarano I, Espino J, González D, Ortiz A, García JF, et al. Relationship between caspase activity and apoptotic markers in human sperm in response to hydrogen peroxide and progesterone. J Reprod Dev 2009;55:615–21.
4. Demaurex N, Distelhorst C. Cell biology. Apoptosis—the calcium connection. Science 2003;300:65–7.
5. Brookes PS, Yoon Y, Robotham JL, Andrews MW, Sheu SS. Calcium, ATP, and ROS: a mitochondrial love–hate triangle. Am J Physiol Cell Physiol 2004;287:C817–33.
6. Reiter RJ, Tan DX, Manchester LC, Paredes SD, Mayo JC, Sainz RM. Melatonin and reproduction revisited. Biol Reprod 2009;81:445–56.
7. Peyrot F, Ducrocq C. Potential role of tryptophan derivatives in stress responses characterized by the generation of reactive oxygen and nitrogen species. J Pineal Res 2008;45:235–46.
8. Hardeland R, Tan DX, Reiter RJ. Kynuramines, metabolites of melatonin and other indoles: the resurrection of an almost forgotten class of biogenic amines. J Pineal Res 2009;47:109–26.
9. Espino J, Mediero M, Bejarano I, Lozano GM, Ortiz A, García JF, et al. Reduced levels of intracellular calcium releasing in spermatozoa from asthenozoospermic patients. Reprod Biol Endocrinol. doi:10.1186/1477-7827-7-11.
10. Jou MJ, Peng TI, Yu PZ, Jou SB, Reiter RJ, Chen JY, et al. Melatonin protects against common deletion of mitochondrial DNA-augmented mitochondrial oxidative stress and apoptosis. J Pineal Res 2007;43:389–403.
11. Tan DX, Chen LD, Poeggeler B, Manchester LC, Reiter RJ. Melatonin: a potent, endogenous hydroxyl radical scavenger. Endocrine J 1993;1:57–60.
12. Reiter RJ, Paredes SD, Manchester LC, Tan DX. Reducing oxidative/nitrosative stress: a newly-discovered genre for melatonin. Crit Rev Biochem Mol Biol 2009;44:175–200.
13. Paredes SD, Bejarano I, Terrón MP, Barriga C, Reiter RJ, Rodríguez AB. Melatonin and tryptophan counteract lipid peroxidation and modulate superoxide dismutase activity in ringdove heterophils in vivo. Effect of antigen-induced activation and age. Age (Dordr.) 2009;31:179–88.
14. Agarwal A, Ikemoto I, Loughlin KR. Relationship of sperm parameters with levels of reactive oxygen species in semen specimens. J Urol 1994;152:107–10.
15. Irez TO, Senol H, Alagöz M, Basmaciogullari C, Turan F, Kuru D, et al. Effects of indoleamines on sperm motility in vitro. Hum Reprod 1992;7:987–90.
16. Luboshitzky R, Shen-Orr Z, Nave R, Lavi S, Lavie P. Melatonin administration alters semen quality in healthy men. J Androl 2002;23:572–8.
17. Fujinoki M. Melatonin-enhanced hyperactivation of hamster sperm. Reproduction 2008;136:533–41.