Melatonin is able to delay endoplasmic reticulum stress-induced apoptosis in leukocytes from elderly humans

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Received: 5 October 2010 / Accepted: 29 October 2010 / Published online: 18 November 2010
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Abstract The mechanisms regulating neutrophil apoptosis are basically unaffected by the aging process. However, a significant impairment of cell survival occurs in elderly individuals following neutrophil challenge with pro-inflammatory stimuli, such as granulocyte-macrophage colony-stimulating factor (GM-CSF). The goal of the present study was to prove the effects of melatonin supplementation on apoptosis induced by calcium signaling in human leukocytes from elderly volunteers. Treatments with the specific inhibitor of cytosolic calcium re-uptake, thapsigargin, and/or the calcium mobilizing agonist, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), induced mitochondrial membrane depolarization, caspase activation, phosphatidylserine (PS) externalization, and DNA fragmentation in leukocytes from both young and elderly volunteers, although such effects were much more evident in aged leukocytes. Importantly, melatonin treatment substantially preserved mitochondrial membrane potential, reversed caspase activation, reduced

PS exposure and forestalled DNA fragmentation in leukocytes from both age groups. In conclusion, melatonin is able to delay endoplasmic reticulum stress-induced apoptosis in aged leukocytes and may counteract, at the cellular level, age-related degenerative phenomena linked to oxidative stress.

Keywords Melatonin · Aging · Leukocytes · Apoptosis

Introduction

Aging is characterized by a general decline in physiological functions that leads to increased morbidity and mortality (Miyoshi et al. 2006). Although aging is an extremely complex, multifactorial process that has been the subject of considerable speculation (Semsei 2000), accumulated evidence identifies oxidative stress as a source of damage to cellular structure and function. Oxidative stress is a condition in which the redox balance between oxidants and antioxidants is disrupted, thereby tilting the equilibrium towards an oxidized state (Sies 1985). The free radical theory of aging proposes that the organismal deterioration that occurs as a result of increasing longevity is specially a consequence of the persistent accumulation of free radical-mediated damage to essential molecules, which gradually compromises the function of cells, of tissues, and, eventually, of the organism itself (Reiter et al. 2008). Consequently,

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aging may be viewed as a process of irreversible injuries associated with accumulated oxidative debris.

Severe alterations of the immune system function occur during aging, related to a decrease of B cells that may be responsible of a decreased response to exogenous antigens, and to an increase of activated T cells, that may be associated with an increased frequency of autoimmune phenomena, related to cell-mediated immune response (Gavazzi and Krauze 2002; Larbi et al. 2005; Mazzocchi et al. 2010). A growing body of evidence suggests that a key role is played by impaired neutrophil activity, and the neutrophil functions most strongly altered with aging are chemotaxis, phagocytosis, respiratory burst, and killing (Antonaci et al. 1984; Wenisch et al. 2000; Butcher et al. 2001). Despite the strict analogies between these alterations and the impaired functional capacities displayed by apoptotic neutrophils (Whyte et al. 1993), some studies have recently reported that advanced age does not affect either spontaneous or Fas-induced apoptotic events (Larbi et al. 2005; Tortorella et al. 1998). Nevertheless, neutrophils from aged individuals show a diminished rescue capacity when challenged with pro-inflammatory stimuli, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), lipopolysaccharides (LPS), or interleukin-2 (IL-2) (Tortorella et al. 1998, 2006).

Apoptosis (or programmed cell death) is characterized by morphological changes such as DNA fragmentation and disintegration of the cell into apoptotic bodies that can be removed by phagocytosis (Zhang et al. 2003). Two major pathways have been described regulating apoptosis: the extrinsic pathway, in which cell plasma membrane receptors act as the starting point of the apoptotic process, and the intrinsic pathway, in which mitochondria play a central role. Numerous reports suggest that aging is accompanied by alterations in the apoptotic behavior of a variety of cell types and tissues (Zhang et al. 2003). Nonetheless, it remains to be established whether enhanced levels of apoptosis serve as a self-protective mechanism to remove increased numbers of dysfunctional cells as a result of aging, or whether they play a destructive role, causing excessive cell death and the decline of organ function (Zhang et al. 2002).

The indole melatonin (*N*-acetyl-5-methoxytryptamine) is mainly secreted in the pineal gland, although it has been also detected in many other tissues. It is a

highly lipophilic molecule that crosses cell membranes to easily reach subcellular compartments including mitochondria, where it seems to accumulate in high concentrations (Reiter et al. 2001). Melatonin is able to prevent oxidative stress both through its free radical scavenging effect and by directly increasing antioxidant activity (Reiter and Tan 2003), and different studies have demonstrated its protective role against oxidative damage induced by drugs, toxins, and different diseases (León et al. 2004; Espino et al. 2010a, b). It is known that endogenous melatonin production diminishes in elderly persons (Reiter 1992) and that the total antioxidative capacity of serum correlates well with its melatonin levels in humans (Benot et al. 1999). Moreover, melatonin shows beneficial anti-aging effects in rats, preventing lipid peroxidation and other mechanisms related to oxidative stress (Poeggeler 2005; Paredes et al. 2009). Therefore, the age-related decrease of melatonin secretion may play a role in the elevated oxidative damage observed in the elderly population (Reiter et al. 2002).

Since several pro-inflammatory mediators failed to rescue apoptotic leukocytes from aged individuals and no information exists on whether melatonin may influence the mechanism of programmed cell death in aged leukocytes, the aim of the present study was to prove the effects of melatonin supplementation on apoptosis evoked by endoplasmic reticulum stress in human leukocytes from both young and elderly volunteers.

Materials and methods

Chemicals

Melatonin, RPMI-1640 medium, Ficoll-Histopaque separating medium, bovine serum albumin, Triton-X-100 and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) were obtained from Sigma Chemical (St. Louis, MO, USA). Thapsigargin and JC-1 were obtained from Molecular Probes (Eugene, OR, USA). Annexin V-fluorescein isothiocyanate conjugate was from Immunostep (Salamanca, Spain). In situ cell death detection kit was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Hoechst 33342 was purchased from Calbiochem (San Diego, CA, USA). All others reagents were of analytical grade.

Human leukocytes isolation

After informed consent was obtained venous blood was drawn from healthy young (20–30 years old) and elderly (65–75 years old) volunteers of both genders according to a procedure approved by Local Ethical Committees and in accordance with the Declaration of Helsinki. Human leukocytes were separated from whole blood using Ficoll-Histopaque density centrifugation. After centrifugation at 600 g for 30 min, peripheral blood mononuclear cells were isolated from the Histopaque-1077/1119 upper interphase and maintained in RPMI-1640 medium for 1 h to allow the adherence of monocytes so as to obtain a pure lymphocyte preparation, as previously described (Otton et al. 2007). Similarly, peripheral blood polymorphonuclear cells were harvested from the lower interphase and residual erythrocytes were then lysed by short treatment of neutrophil pellet with an ice-cold isotonic NH_4Cl solution (155 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA, pH 7.4) so as to get a neutrophil-enriched preparation, as described elsewhere (Genestier et al. 2005). Cell purity was routinely above 98% and 97% in lymphocytes and neutrophils, respectively, after Diff-Quick staining. Once purified, both neutrophils and lymphocytes were collected and washed in phosphate-buffered saline (PBS) and centrifuged at 480 g for 15 min. The supernatant was then discarded and the cell pellet was gently resuspended in RPMI-1640 medium.

Melatonin treatment

Melatonin was dissolved in absolute ethanol, further diluted with PBS (final ethanol concentration <0.2%) and added to the medium at the concentration to be tested, as previously described (Luchetti et al. 2009).

Where indicated, human leukocytes were pre-incubated with 1 μM melatonin or the vehicle (control) for 1 h, and then stimulated with 10 nM fMLP and/or 1 μM thapsigargin for 1 h.

Measurement of mitochondrial membrane potential

Human leukocytes (10^5 – 10^6 cells/mL) were loaded with the cationic dye JC-1 (10 $\mu\text{g/mL}$, 15 min, 37°C). After dye loading, cells were centrifuged and

resuspended in fresh RPMI medium. JC-1 accumulates in mitochondria forming red fluorescent aggregates at high membrane potentials. However, at low membrane potential, JC-1 exits mainly in the green fluorescent monomeric form. Fluorescence was recorded using an automatic plate reader (Infinite M200, Tecan Austria GmbH, Groedig, Austria). JC-1-loaded cells were excited at 488 nm and emission was detected at 590 nm (JC-1 aggregates) and 525 nm (JC-1 monomers). Data are presented as JC-1 emission ratios (590/525) and expressed as fold-change over the pre-treatment level (experimental/control). Since no significant differences were found between young and aged cells in control conditions, just one bar was displayed to represent control conditions in both groups.

Assay for caspase activity

The determination of caspase-3 and -9 activities was based on a method previously published (Bejarano et al. 2009) with minor modifications. Stimulated or resting cells were pelleted and washed once with PBS. After centrifugation, cells were resuspended in PBS at a concentration of 1×10^7 cells/mL. 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 is composed by 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 (Infinite M200) with excitation wavelength of 360 nm and emission at 460 nm. Preliminary experiments reported that caspase-3 or -9 substrate cleaving was not detected in the presence of the inhibitors of caspase-3 or -9, DEVD-CMK, or z-LEHD-FMK, respectively. The data were calculated as fluorescence units/milligram protein and presented as fold-increase over the pre-treatment level (experimental/control). Since no significant differences were found between young and aged cells in control conditions, just one bar was displayed to represent control conditions in both groups.

Determination of phosphatidylserine (PS) externalization

The PS exposure of resting and stimulated leukocytes (1×10^6 cells/mL) was determined according to a procedure published elsewhere (Bejarano et al. 2008). Briefly, cells were stimulated in HEPES-buffered saline, and cell suspensions (500 μ L) were transferred to 500 μ L of ice-cold 1% (w/v) glutaraldehyde in PBS for 10 min. Cells were then incubated for 10 min with annexin V-fluorescein isothiocyanate conjugate (0.6 μ g/mL) in PBS supplemented with 0.5% (w/v) bovine serum albumin and 2 mM CaCl_2 . Cell staining was measured by using an automatic plate reader (Infinite M200). Samples were excited at 488 nm and emission was recorded at 516 nm. Data were calculated as fluorescence per milligram of protein and expressed as fold-increase over the pre-treatment level (experimental/control). Since no significant differences were found between young and aged cells in control conditions, just one bar was displayed to represent control conditions in both groups.

In situ detection of DNA fragmentation by TUNEL and Hoechst staining

At the end of the treatments, human leukocytes were harvested and washed once with PBS. The cells were then fixed with 4% paraformaldehyde (in PBS, pH 7.4, at least 6 h, RT) and air-dried on slides for 24 h. Afterwards, 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 and TUNEL reaction mixture (50 μ L) was added and the cells were incubated (1 h, 37°C) in a humidified chamber. The cells were washed again with PBS and counterstained with Hoechst 33342 (1 μ g/mL) in PBS 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 a FITC filter. Cells with TUNEL-positive nuclei were considered apoptotic. Hoechst staining was used to determine the total number of cells in a field. A minimum of five fields per slide was used to calculate percent of apoptotic cells, which was expressed as fold-increase over the pre-treatment level (experimental/control).

Statistical analysis

Data are expressed as means $\pm$ standard error of mean (SEM) of the numbers of determinations. To compare the different treatments, statistical significance was calculated by one-way analysis of variance followed by the Tukey's multiple comparison test. $P < 0.05$ was considered to indicate a statistically significant difference.

Fig. 1 Melatonin counteracts the mitochondrial membrane depolarization induced by calcium mobilizing agents in aged leukocytes. Neutrophils (**a**) and lymphocytes (**b**) from both young (black bars) and elderly (grey bars) individuals were pre-incubated with 1 μ M melatonin (Mel) or the vehicle for 1 h, and then stimulated with 1 μ M thapsigargin (TG) or 10 nM fMLP for 1 h to check the mitochondrial membrane potential. Mitochondrial membrane potential was estimated as described under the “Materials and methods” section. Values are presented as means $\pm$ SEM of six separate experiments and expressed as fold-change over the pre-treatment level (experimental/control). Asterisks $P < 0.05$ compared to control values. Sharp signs $P < 0.05$

Results

Melatonin inhibits the intracellular calcium overload-induced mitochondrial disruption in leukocytes from elderly subjects

Intracellular calcium has been suggested to play an important role in the induction of apoptosis in response to a number of pathological conditions (Orrenius et al. 2003) and our previous data strongly indicate that increases in intracellular calcium are required for thapsigargin and/or fMLP-induced apoptosis in human leukocytes (Espino et al. 2010b). To evaluate the effect

of intracellular calcium overload on mitochondrial membrane potential, human leukocytes were treated with both the specific inhibitor of cytosolic calcium re-uptake, thapsigargin, and the calcium mobilizing agonist, fMLP. The treatment of human neutrophils with 1 μ M thapsigargin or 10 nM fMLP for 1 h induced a significant mitochondrial membrane depolarization in both young and elderly subjects as detected by the decrease in JC-1 fluorescence ratio ($P<0.05$; Fig. 1a). Similar results were found when human lymphocytes were treated with 1 μ M thapsigargin for 1 h ($P<0.05$; Fig. 1b). Interestingly, the mitochondrial membrane depolarization in thapsigargin- and/or fMLP-

Fig. 2 Caspase activation is reversed by melatonin in aged leukocytes. Cells from both young (black bars) and elderly (grey bars) individuals were pre-incubated with 1 μ M melatonin (Mel) or the vehicle for 1 h, and then stimulated with 1 μ M thapsigargin (TG) or 10 nM fMLP for 1 h to check caspase-9 (a and c) and caspase-3 (b and d) activities. Caspase activity was

estimated as described under the “Materials and methods” section. Values are presented as means $\pm$ SEM of six separate experiments and expressed as fold-increase over the pre-treatment level (experimental/control). Asterisks $P<0.05$ compared to control values. Sharp signs $P<0.05$

treated leukocytes from aged individuals was significantly higher compared to those in young subjects ($P<0.05$; Fig. 1). However, when human leukocytes were pre-incubated with 1 μM melatonin for 1 h, both thapsigargin and fMLP exerted a negligible effect on mitochondrial membrane potential (Fig. 1), thereby indicating that melatonin is able to reverse endoplasmic reticulum stress-induced mitochondrial membrane depolarization in both young and elderly leukocytes. Moreover, in control cells, melatonin was also able to ameliorate mitochondrial membrane potential in human leukocytes from young volunteers (Fig. 1).

Caspase activation is delayed by melatonin pre-incubation in aged leukocytes

Since caspase-9 is an initiator caspase that is involved in the initial steps of mitochondrial apoptosis (Li et al. 1997), we checked the caspase-9 activity in the presence of thapsigargin or fMLP so as to prove whether the mitochondrial membrane depolarization induced by thapsigargin or fMLP is related to mitochondrial apoptosis. As shown in Fig. 2a, the treatment of neutrophils with 1 μM thapsigargin or 10 nM fMLP for 1 h produced a substantial increase of caspase-9 activity ($P<0.05$). Likewise, 1 μM thapsigargin was also able to stimulate the activation of caspase-9 in human lymphocytes ($P<0.05$; Fig. 2c). Moreover, the caspase-9 activity in thapsigargin- and/or fMLP-treated leukocytes from elderly subjects was substantially higher compared to those in young individuals ($P<0.05$; Fig. 2a, c). Intriguingly, the pre-incubation of leukocytes with 1 μM melatonin for 1 h was able to completely counteract the stimulatory effect of thapsigargin and fMLP on caspase-9 activity not only in leukocytes from young volunteers, but also in those from aged subjects ($P<0.05$; Fig. 2a, c). However, melatonin alone was unable to modify the spontaneous caspase-9 activation.

To examine the effect of intracellular calcium overload on caspase-3 activation, 1 μM thapsigargin and/or 10 nM fMLP were again administered to human leukocytes for 1 h. Our results showed that both thapsigargin and fMLP were able to increase the caspase-3 activity in human neutrophils ($P<0.05$; Fig. 2b). Similar findings were obtained when human lymphocytes were treated with 1 μM thapsigargin for 1 h ($P<0.05$; Fig. 2d). Curiously, thapsigargin and/or

fMLP caused much higher caspase-3 activation in leukocytes from aged subjects than in those from young individuals ($P<0.05$; Fig. 2b, d). Furthermore, it is worth noting that the pre-incubation of leukocytes with 1 μM melatonin for 1 h almost entirely forestalls the stimulatory effect of thapsigargin and fMLP on caspase-3 activity in both young and elderly subjects ($P<0.05$; Fig. 2b, d). Nevertheless, melatonin alone was not able to alter the spontaneous caspase-3 activation in leukocytes from both young and aged individuals.

Fig. 3 Effects of melatonin on PS exposure in aged leukocytes. Neutrophils (a) and lymphocytes (b) from both young (black bars) and elderly (grey bars) individuals were pre-incubated with 1 μM melatonin (Mel) or the vehicle for 1 h, and then stimulated with 1 μM thapsigargin (TG) or 10 nM fMLP for 1 h to check PS externalization. PS exposure was estimated as described under the “Materials and methods” section. Values are presented as means $\pm$ SEM of six separate experiments and expressed as fold-increase over the pre-treatment level (experimental/control). Asterisks $P<0.05$ compared to control values. Sharp signs $P<0.05$

Fig. 4 Melatonin delays cell death in aged leukocytes. Neutrophils (**a**) and lymphocytes (**b**) from elderly subjects were pre-incubated with 1 μ M melatonin (*Mel*) or the vehicle for 1 h, and then stimulated with 1 μ M thapsigargin (*TG*) or 10 nM fMLP for 1 h to check the proportion of cells depicting DNA fragmentation. DNA fragmentation was estimated as described under the “Materials and methods” section. *Right and central panels*: pictures of Hoechst 33342 nuclear stain (*blue*) and TUNEL-positive cells (*green*), respectively. *Left panel* differential interference contrast (*DIC*) images of cultured cells using a 40 $\times$ objective. *Scale bars* 15 μ m. Values inside the central panel are presented as means $\pm$ SEM of three separate experiments and expressed as fold-increase over the pre-treatment level (experimental/control). *Asterisks* $P<0.05$ compared to control values. *Sharp signs* $P<0.05$ regarding *TG* alone. *Double crosses* $P<0.05$ regarding fMLP alone

Fig. 4 (Continued)

Melatonin reverses the induction of both early and late apoptosis markers in leukocytes from elderly volunteers

To further investigate the effect of intracellular calcium overload on apoptosis, it was also evaluated the plasma membrane translocation of PS residues, which reflects a relatively early apoptotic stage. As shown in Fig. 3a, the treatment of human neutrophils with 1 μ M thapsigargin or 10 nM fMLP for 1 h caused a substantial rise in PS externalization ($P < 0.05$). Additionally, when human lymphocytes were treated with 1 μ M thapsigargin for 1 h, a statistically significant increase was found in the percentage of annexin V-positive cells ($P < 0.05$;

Fig. 3b). Moreover, the proportion of thapsigargin- and/or fMLP-treated leukocytes depicting membrane phospholipids externalization was substantially higher in elderly subjects compared to those in young individuals ($P < 0.05$; Fig. 3). Remarkably, the pre-incubation of leukocytes with 1 μ M melatonin for 1 h was able to prevent the stimulatory effect of thapsigargin and fMLP on PS externalization not only in leukocytes from young volunteers, but also in those from aged individuals ($P < 0.05$; Fig. 3). Again, melatonin alone was unable to vary the spontaneous PS exposure.

Lastly, as TUNEL assay is a well-established method for detection of DNA cleavage, a relatively

late apoptotic marker (Heatwole 1999), we assessed the amount of DNA fragmentation in the presence of thapsigargin or fMLP in order to verify whether the mitochondrial membrane depolarization induced by both calcium mobilizing agents, in turn, leads to cell death. Treatment of human neutrophils with 1 μ M thapsigargin or 10 nM fMLP for 1 h produced a substantial increase in the proportion of cells depicting DNA fragmentation in elderly subjects ($P<0.05$; Fig. 4a). Similar results were found when aged lymphocytes were treated with 1 μ M thapsigargin for 1 h ($P<0.05$; Fig. 4b). Importantly, in all cases, fMLP and/or thapsigargin induced much higher proportion of cells depicting DNA fragmentation in leukocytes from aged subjects than in those from young individuals ($P<0.05$; Table 1), thereby reflecting that aged leukocytes are much more vulnerable to apoptosis induced by endoplasmic reticulum stress. Besides, it is worth noting that the pre-incubation of leukocytes with 1 μ M melatonin for 1 h significantly weakens the stimulatory effect of thapsigargin and fMLP on DNA fragmentation in both young and elderly volunteers ($P<0.05$; Table 1 and Fig. 4, respectively), thus substantiating that melatonin is able to delay endoplasmic reticulum stress-induced cell death in leukocytes from elderly humans.

Discussion

The free radical theory of aging proposes that aging and some related diseases are, at least in part, a consequence of oxidative stress (Harman 1992). Actually, several observations suggest a possible

implication of ROS as signaling molecules in apoptosis and although a direct connection between apoptosis and aging has not been established, some data suggest that oxidative stress may elicit its effects on aging via regulation of apoptosis (Zhang and Herman 2002; Pollack and Leeuwenburgh 2001).

Melatonin and its metabolites are potent scavengers of damaging free radicals (Tan et al. 1993; Terrón et al. 2001; Reiter et al. 2009) able to counteract apoptosis (Juknat et al. 2005; Mayo et al. 1998) and we have previously demonstrated that melatonin anti-apoptotic actions in human leukocytes are likely related to its free-radical scavenging properties (Espino et al. 2010a, b). In the current work, we first showed that an excessive intracellular calcium load results in a less efficient capacity of leukocytes from old subjects to escape from apoptosis, whilst melatonin supplementation provides a cell survival advantage against intracellular calcium overload.

Melatonin exposure in the elderly delays endoplasmic reticulum stress-induced leukocyte apoptosis, thus suggesting that the beneficial consequences resulting from melatonin administration likely depend on its effect on mitochondrial physiology. On the contrary, the age-related failure to rescue neutrophils from apoptotic cell death induced by pro-inflammatory mediators, such as GM-CSF, seems to be strictly related to an impairment of GM-CSF-dependent PI3-K/Akt and ERK1/2 activation (Tortorella et al. 2006). In fact, we showed that melatonin is able to reverse the loss of mitochondrial membrane potential, as well as the subsequent caspase activation, evoked by intracellular calcium overload. In this regard, both *in vitro* and *in*

Table 1 Effect of melatonin on DNA fragmentation

	Neutrophils		Lymphocytes	
	Young	Elderly	Young	Elderly
Mel	1.1 $\pm$ 0.2	1.1 $\pm$ 0.1	0.9 $\pm$ 0.1	1.3 $\pm$ 0.3
TG	7.3 $\pm$ 0.3* **	10.2 $\pm$ 0.9*	3.4 $\pm$ 0.0* **	4.8 $\pm$ 0.5*
TG+Mel	3.2 $\pm$ 0.3* ***	3.3 $\pm$ 0.1* ****	1.2 $\pm$ 0.1***	1.8 $\pm$ 0.3***
fMLP	7.2 $\pm$ 0.5* **	15.4 $\pm$ 1.9*	–	–
fMLP+Mel	3.2 $\pm$ 0.4* ****	2.4 $\pm$ 0.5****	–	–

Proportion of cells depicting DNA fragmentation is presented as mean $\pm$ SEM of three separate experiments and expressed as fold-increase over the pre-treatment level (experimental/control)

* $P<0.05$ regarding its corresponding control value; ** $P<0.05$ regarding its corresponding value in aged cells; *** $P<0.05$ regarding its corresponding TG alone value; **** $P<0.05$ regarding its corresponding fMLP alone value

vivo experiments have shown that melatonin can influence mitochondrial homeostasis. Thus, melatonin stabilizes mitochondrial inner membrane (García et al. 1999) thereby improving electron transport chain activity. As a matter of fact, melatonin increases the activities of the brain and liver mitochondrial respiratory complexes I and IV in a time-dependent manner (Martín et al. 2000). Moreover, melatonin maintains the efficiency of oxidative phosphorylation and stimulates ATP synthesis while protecting the mitochondria from oxidative damage (León et al. 2005; Carretero et al. 2009). Finally, recent finding indicates that melatonin is able to prevent mitochondrial cardiolipin oxidation/depletion, which controls several processes involved in mitochondrial bioenergetics, in mitochondrial steps of cell death, as well as in mitochondrial membrane stability and dynamics (Paradies et al. 2010). Nevertheless, we cannot rule out that the delay of leukocyte apoptosis exerted by melatonin is produced via melatonin receptors, since melatonin treatment in murine senescence models has been reported to favor the pro-survival pathways, such as sirtuins or PI3K/Akt signalling, as well as modulate the intrinsic apoptotic pathway, thus increasing pro-survival factors and reducing pro-death proteins or enzymes (Gutierrez-Cuesta et al. 2008; Tajés Orduña et al. 2009).

In conclusion, melatonin is able to delay calcium overload-induced leukocyte apoptosis in advanced age likely due to its antioxidant properties. Therefore, owing to its well-documented protective effects along with its low toxicity to humans, molecular mechanisms involved in melatonin protection should be further investigated to clarify its potential therapeutic use for ageing and age-related disorders.

Acknowledgements This work was supported by Junta de Extremadura (PRI07-A024) and MEC-Fondo Social Europeo de Desarrollo Regional grants (BFU2007-60091 and BFU2010-15049). I. Bejarano and S.D. Paredes are beneficiaries of grants from Consejería de Economía, Comercio e Innovación – Fondo Social Europeo (Junta de Extremadura, PRE06070 and POS07012, respectively). J. Espino is beneficiary of grant from MEC (AP2009-0753).

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