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ORIGINAL ARTICLE

Oral melatonin administration and programmed cell death of neutrophils, lymphocytes, and other cell types from rats injected with HL-60 cells

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Summary
Recent years have seen mounting evidence for the role of melatonin in mediating programmed cell death, with a protective, anti-apoptotic effect in healthy cells, but an anti-tumoural, pro-apoptotic action in many tumour cells. In this study, we evaluated the effect of melatonin on the programmed cell death induced by thapsigargin (TG), on lymphocytes and neutrophils, and on various tissues obtained from rats injected with human promyelocytic leukæmia cells (HL-60), treated with melatonin in their drinking water (20 μm), and fed *ad libitum*. Melatonin treatment significantly reduced caspase-3 and -9 activity, and caused the proportions of lymphocytes, neutrophils, and eosinophils to revert to their basal values. No histological differences were observed. In conclusion, melatonin has anti-apoptotic effects on lymphocytes and neutrophils obtained from rats injected with HL-60 leukæmia cells.

Key words: melatonin; neutrophils; lymphocytes; apoptosis; HL-60 cells

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INTRODUCTION

Melatonin is a physiological mediator that is present at all evolutionary levels, from bacteria to humans (Reiter 1991, Hardeland and Fuhrberg 1996). In mammals, melatonin is produced by the pineal gland and by a variety of extrapineal tissues (Panke et al. 1979, Carrillo-Vico et al. 2005, Kobayashi et al. 2005). Among its actions in controlling seasonal reproduction in photoperiodic mammals (Reiter and Fraschini 1969, Reiter 1973), melatonin has a major role in controlling tumour development and growth, and its anti-proliferative activity has been demonstrated both *in vivo* and *in vitro* in different cell systems (Cos et al. 2001, 2006, Berger 2008, Bejarano et al. 2009, Dauchy et al. 2009, Park et al. 2010).

Programmed cell death is a genetically predetermined mechanism that can operate via two molecular pathways – the extrinsic pathway and the

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intrinsic pathway. In the extrinsic pathway (also known as the "death receptor pathway"), programmed cell death is caused by ligand-induced activation of death receptors on the cell surface. Such death receptors include receptor-1 of the tumour necrosis factor (TNF), CD95/Fas (the CD95L/FasL receptor), and receptors-1 and -2 of the TNF-related programmed cell death-inducing ligand (TRAIL). In the intrinsic pathway (also known as the mitochondrial pathway), programmed cell death is the result of a cascade of intracellular events in which mitochondrial permeabilization plays a crucial role (Scaffidi et al. 1998). The proteins of the Bcl-2 family regulate programmed cell death by acting on the mitochondria. Once activated, the pro-apoptotic proteins of this family increase the permeability of the inner mitochondrial membrane (mitochondrial permeability transition pore, mPTP) and open a pore in the outer mitochondrial membrane that allows the release of numerous pro-apoptotic proteins from the intermembrane space (Hajnóczyk et al. 2003), including SMAC/DIABLO (which blocks caspase inhibitors) and cytochrome c. Once in the cytosol, cytochrome *c* activates a protein complex known as the apoptosome, which triggers the activation of caspase-9 (Kroemer et al. 2007, Rasola and Bernardi 2007).

Melatonin influences programmed cell death: in normal cells it exerts an anti-apoptotic effect, in various cancer cell lines it is pro-apoptotic (Jou et al. 2010). For example, melatonin promotes cell death in HL-60 cells (Rubio et al. 2007), lymphoma (Trubiani et al. 2005), HT-29 cell line (García-Navarro et al. 2007) and MCF-7 breast cancer cells (Cucina et al. 2009). The mechanisms by which melatonin acts have not been completely elucidated, although different modes of action have been proposed. Melatonin is a highly lipophilic molecule that readily crosses cell membranes to reach intracellular organelles, including mitochondria (Paradies et al. 2010). Indeed, the evidence points to a melatonin-mitochondria relationship, and the hormone's anti-apoptotic properties in healthy cells has been attributed to its interaction with the mitochondrial transition pore (Petrosillo et al. 2009, Hibaoui et al. 2009).

Caspase-3 and -9 are the principal mediators of programmed cell death, and their activation is widely regarded as an apoptotic marker. Bejarano et al. (2009) have recently shown that stimulation of HL-60 leukæmia cells with millimolar concentrations of melatonin increases the activity of both these caspases, whereas at micromolar concentrations it is ineffective. These results are coherent with previous studies that have observed an activation of caspase-3 by melatonin in that same cell line (Rubio et al. 2007) with the activation occurring via both the extrinsic and the intrinsic pathways.

Given these antecedents, the aim of the present work was to study the effect of oral administration of melatonin on programmed cell death in lymphocytes and neutrophils, and on various tissues obtained from rats injected with HL-60 cells.

MATERIAL AND METHODS

Experimental animals

The experimental animals were male Wistar rats (*Rattus norvegicus*), 4 to 6 weeks of age, supplied by the Animalarium Service, University of Extremadura, housed at a constant temperature of 20±5 °C, and maintained on "Panlab" feed with water *ad libitum*. Four groups were formed: (i) control rats, (ii) control rats treated with melatonin, (iii) leukæmia cell injected rats, and (iiii) leukæmia cell injected rats treated with melatonin. The animals were housed individually in 25×15×15 inch cages (Panlab), in a room of 2.86×3.80×2.85 metres, indirectly ventilated, with 50% relative humidity and artificial lighting, and were exposed to a 12-hour light/12-hour dark photoperiod (dark period from 20:00 to 08:00).

HL-60 cells

Rats were injected with a line of human promyelocytic leukæmia cells (HL-60), cultured in 75 cm^3 flasks with RPMI-1640 medium supplemented with 10% (v/v) foetal bovine serum (heat inactivated), 1.25% DMSO, 1% L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin, at 37 °C and 100% humidity, in an atmosphere containing 5% CO_2. The cultures presented doubling times of about 48 hours. Once about 75–80% of confluence had been reached, the medium was changed under sterile conditions in a laminar flow hood. Cell counts were performed in Neubauer chambers, and viability was measured using trypan blue stain. This stain allows easy identification of dead cells which take up the dye and appear blue with uneven cell membranes, while live cells repel the dye and appear translucent and colourless.

The treatment solution

A stock solution of melatonin was prepared freshly every 3 or 4 days containing 348 mg of melatonin dissolved in 10 ml of 96% ethanol, and stored at –20 °C. The working solution was prepared by adding 288 µl of this stock solution to 500 ml of water, giving a final concentration of the hormone of 20 µg/ml. Melatonin (Sigma, St Louis, USA) was

administered in tap water for a period of 16 weeks. Water bottles were covered with aluminium foil to protect from light.

Inoculation with HL-60 cells

The rats were inoculated with HL-60 cells by intraperitoneal injection of 10×10^7 cells suspended in 2 ml of PBS. A 2-ml syringe was used, with a 0.8×40 mm needle. To obtain the cells, the culture medium was withdrawn from the flasks, and the cell suspension was centrifuged in 50 ml Falcon tubes for 5 minutes at 150 g. The supernatant containing the culture medium was discarded, and the pellet was resuspended in PBS, adjusting the cell concentration to that of the study.

Determination of the leukocyte formula

The leukocyte count is based on counting the number of white blood cells per unit volume present in a blood sample. The leukocyte formula gives an idea of the relative proportion of the different types of leukocytes in a blood sample: neutrophils, eosinophils, basophils, lymphocytes, and monocytes.

To count the leukocytes, a drop obtained from the tail was deposited on one end of a slide, and the end of another slide was placed on this drop until, by capillary action, it had spread along the edge. A smear was formed by dragging the top slide over the other, and the smear was then air-dried.

These samples were fixed in methanol for 5 minutes, and then stained with hæmatoxylin-eosin using 5 passes in each of the stains.

Cell counts were made from the stained samples under 100× oil immersion optical microscopy.

Collection of lymphocytes and polymorphonuclear cells

The animals of the three groups were anaesthetized with diethyl ether and killed by decapitation, collecting the blood immediately from the neck blood vessels into Falcon tubes containing 0.5 ml of PBS and 0.5 ml of lithium heparin. Aliquots of 2 millilitres of this heparinized blood were put into tubes prepared with two density gradients (separating media) – Histopaque 1.077 and 1.119. A volume of 2 ml of 1.119 was put into the bottom of each tube, and then 2 ml of 1.077 on top of this, adding it very gently to avoid mixing. Then the blood was added, again very gently down the walls of the tube to avoid it mixing with the density gradients. The preparation was centrifuged at 600 g for 30 minutes. The lymphocyte and polymorphonuclear cell rings were collected, and rinsed twice with PBS (480 g for 10 minutes). The supernatant was discarded, and the tube with the precipitate was tapped to separate the cells from the walls and the bottom of the tube. Finally, the precipitate was resuspended in 1 ml of Hank's medium.

Determination of caspase-3 and -9

Once isolated, the lymphocytes and polymorphonuclear cells were subjected to a programmed cell death treatment with thapsigargin (TG) for 1 hour.

Caspase activity was measured using a peptide with an aspartic acid residue associated with a fluorescent compound of the type AMC (7-amino-4-methylcoumarin). These compounds are normally of the coumarin type.

After the programmed cell death treatment, the cell suspension was centrifuged at 500 g for 10 minutes, the supernatant was discarded, and the precipitate resuspended in 500 µl of lysis buffer. This suspension was subjected to sonication, with two 4-second pulses at 40%. The application of ultrasound to the cells ruptures them, releasing the cytosolic content into the medium. The cells were then incubated on ice at 4 °C for 15 to 20 minutes, the use of low temperatures being to halt enzyme activity. This was followed by centrifugation at 14 000 g for 15 minutes at 4 °C.

To 2 ml of reaction buffer (which contained the caspase-3 substrate AC-DEVD-AMC or the caspase-9 substrate AC-LEHD-AMC), 50 µl of the supernatant were added for the determination of caspase-3, or 150 µl of supernatant in the case of caspase-9. The tubes were incubated for 25 to 30 minutes at 37 °C in darkness. During this time, caspase activity present in the supernatant releases the AMC fluorophore from the caspase substrate, which can easily be determined by spectrofluorimetry. The spectrofluorimeter used was a Shimadzu RF-5301 PC (Shimadzu Scientific Instruments, Japan), which accepts samples of 2 ml. In both cases (caspase-3 and caspase-9), excitation was at 360 nm, and measurement at 460 nm. Additionally, a blank tube was measured, containing 5 µl of trial buffer added to 2 ml of reaction buffer. All treatments were performed in duplicate.

Histological determinations

The dissected organs were fixed in 10% formalin for one week at 4 °C. They were then rinsed in phosphate buffered saline (PBS1×) at 4 °C for 2 hours with mechanical shaking, and dehydrated in increasing concentrations of ethanol. After treatment in xylene to make them transparent, they were embedded in paraffin wax for transversal sectioning. Sections were cut on a microtome at 7–10 µm, mounted serially on slides, and kept at 37 °C for one week. Four series of sections were prepared and distributed on four slides to ensure the representativeness of the sample.

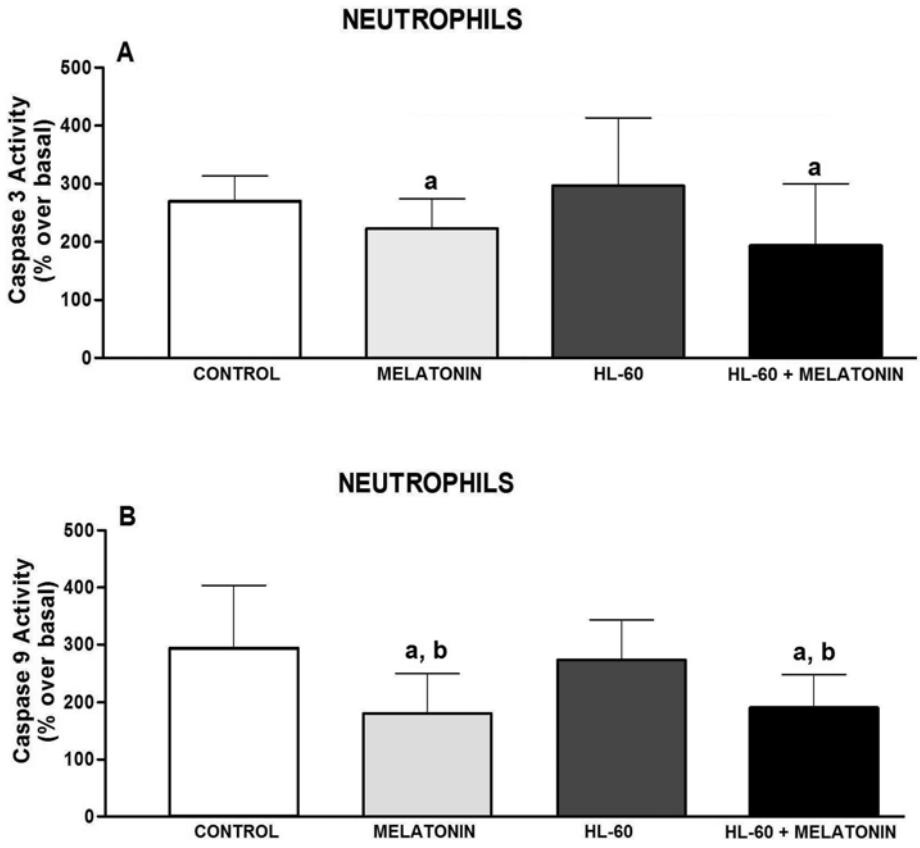

Fig. 1A, B. **Thapsigargin induced caspase activity in neutrophils** from control rats, control rats treated with melatonin, leukæmia cell (HL-60) injected rats, and from leukæmia cell (HL-60) injected rats treated with melatonin. The cells were incubated with 1 μm thapsigargin (TG) for 60 minutes. The activities of caspase-3 (*A*) and caspase-9 (*B*) were estimated as described in Materials and methods. The data represent the mean ± standard error of 10 separate experiments; (*a*) statistically significant as compared with HL-60 injected rats; (*b*) statistically significant as compared with control animals.

The sections were subjected to a series of changes in different solutions and for different times depending on the sample type; in particular, two changes in xylene, and successive passes in alcohols of increasing concentration, and distilled water (dH_2O). Two series of sections (two slides) from each sample were stained with hæmatoxylin-eosin and two with Masson trichromate, except the brain samples for which Nissl staining was used.

Statistical analysis

Data are expressed as mean ± S.E.M. of the number of determinations carried out in duplicate. The results were analysed using a non-parametric one-way ANOVA, followed by a *post hoc* Tukey test to compare all pairs of columns, at the significance level $2\alpha = 0.05$.

RESULTS

Figs 1 and 2 show the activity of caspase-3 and caspase-9 induced by thapsigargin in neutrophils (Fig. 1) and lymphocytes (Fig. 2).

In neutrophils, the incubation of 1 μm thapsigargin modified neither caspase-3 (Fig. 1A) nor caspase-9 (Fig. 1B) activity significantly in the cells from HL-60 injected rats compared with the control group. However, these two activities were significantly reduced in the neutrophils obtained from the HL-60 injected rats treated with melatonin compared with those not treated with the hormone.

Similarly, the incubation of 1 μm thapsigargin modified neither caspase-3 (Fig. 2A) nor caspase-9 (Fig. 2B) activity significantly in the lymphocytes from HL-60 injected rats compared with the control group. Again however, treatment with melatonin

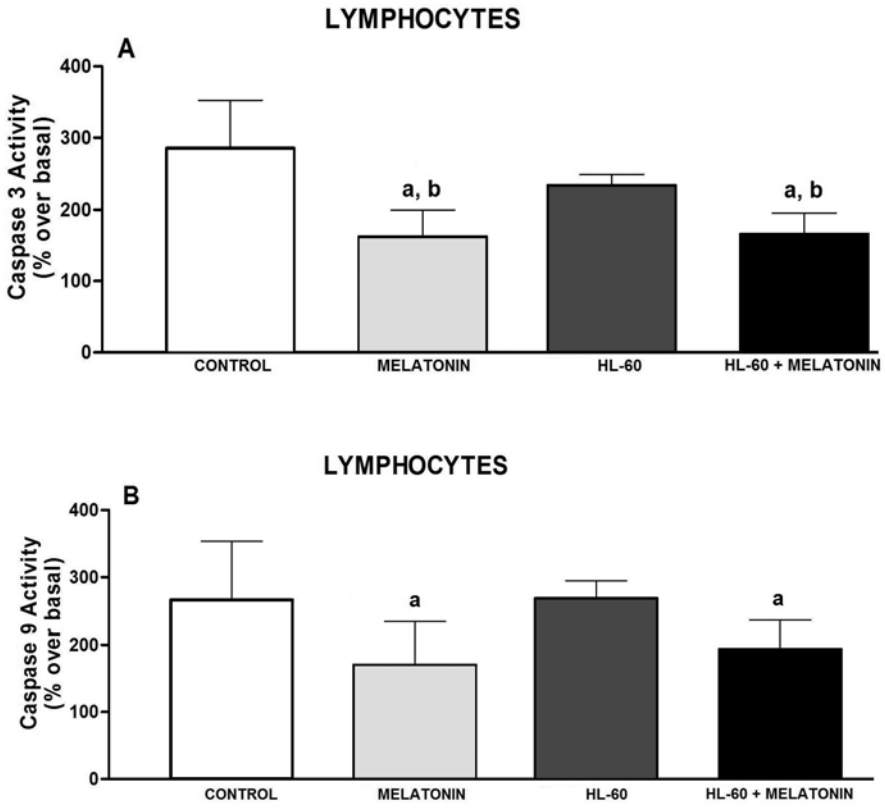

Fig. 2A, B. **Thapsigargin induced caspase activity in lymphocytes** from control rats, control rats treated with melatonin, leukæmia cell (HL-60) injected rats, and from leukæmia cell (HL-60) injected rats treated with melatonin. The cells were incubated with 1 µm thapsigargin (TG) for 60 minutes. The activities of caspase-3 (*A*) and caspase-9 (*B*) were estimated as described in Materials and methods. The data represent the mean ± standard error of 10 separate experiments; (*a*) statistically significant as compared with HL-60 injected rats; (*b*) statistically significant as compared with controls.

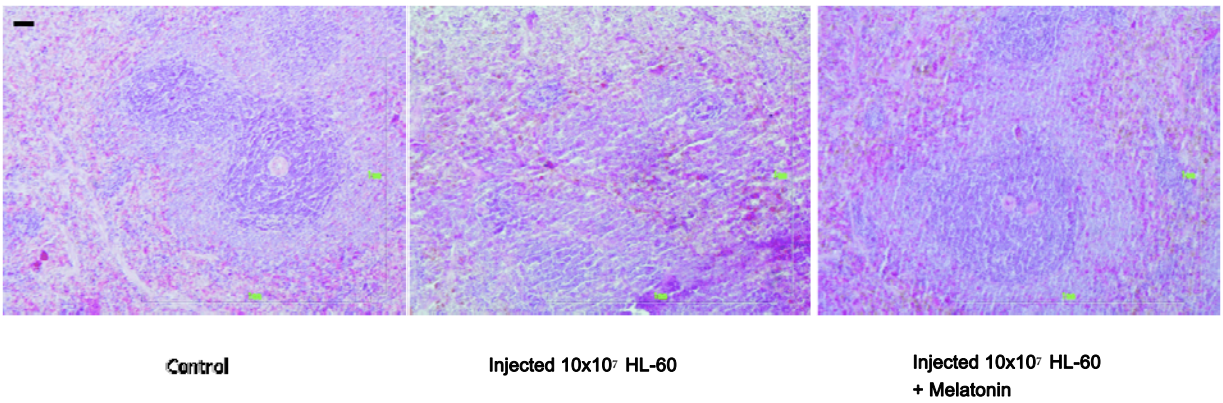

Fig. 3. **Detail of a follicle of the spleen** of a control rat, a leukæmia cell (HL-60) injected rat, and a leukæmia cell injected rat treated with melatonin. The stain used is hæmatoxylin-eosin. Scale Bar = 120 µm.

Table 1. **Percentage values of the leukocyte count obtained after staining a drop of blood** from control rats, control rats treated with melatonin, leukæmia cell (HL-60) injected rats, and leukæmia cell injected rats treated with melatonin.

	CONTROL	MEL	HL-60	HL-60 + MEL
LYMPHOCYTES	0.64±0.02	0.61±0.02	0.79±0.01[a, b]	0.60±0.01
NEUTROPHILS	0.34±0.02	0.36±0.02	0.15±0.01[a, b]	0.39±0.02
EOSINOPHILS	0.02±0.01	0.01±0.01	0.06±0.02[a, b]	0.01±0.01

Each value represents the mean ± standard error of 10 determinations performed in the last week of the study prior to killing the animals; (*a*) statistically significant versus the control group; (*b*) statistically significant versus animals treated with both HL-60 and melatonin.

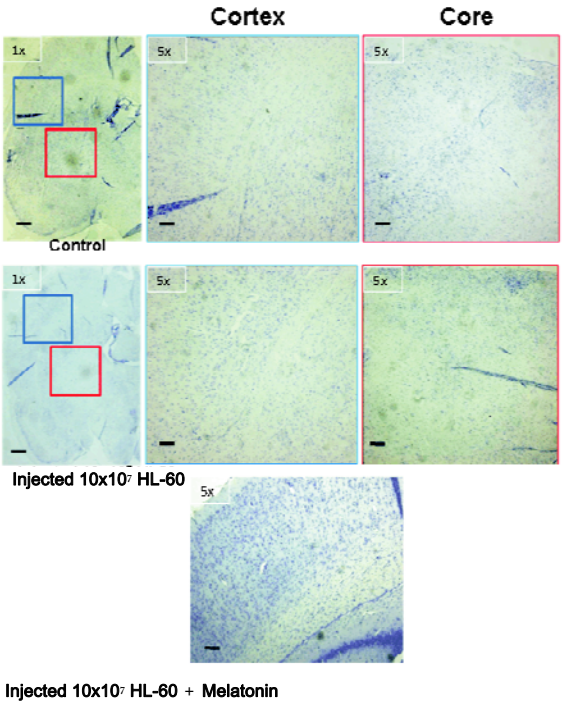

Fig. 4. **Cortex and core of brain samples** from a control rat, a leukæmia cell (HL-60) injected rat, and a leukæmia cell injected rat treated with melatonin. The stain used is cresyl violet (Nissl stain). Scale Bar = 500 µm (5×), 2 mm (1×).

significantly reduced these two activities in the lymphocytes in response to the thapsigargin, compared with the HL-60 injected rats not treated with the hormone.

Table 1 lists the percentages of the leukocyte formulas of blood obtained from control rats, control rats treated with melatonin, leukæmia cell (HL-60) injected rats, and leukæmia cell (HL-60) injected rats treated with melatonin. The lymphocyte and eosinophil percentages in the group of leukæmia cell (HL-60) injected rats were both significantly greater than in the control group and in the leukæmia cell (HL-60) injected rats treated with melatonin. In contrast, there was a significant decrease (statistically significant) in the neutrophil percentage in the blood obtained from the leukæmia cell (HL-60) injected rats with respect to the control group and the group of leukæmia cell (HL-60) injected rats treated with the hormone.

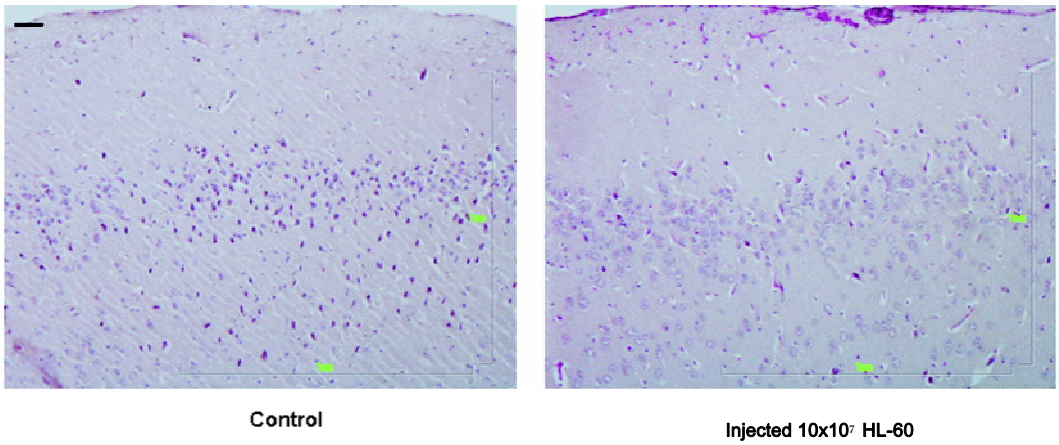

Fig. 5. **Brain samples** from a control rat, a leukæmia cell (HL-60) injected rat, and a leukæmia cell injected rat treated with melatonin. The stain used is hæmatoxylin-eosin. Scale Bar = 120 µm.

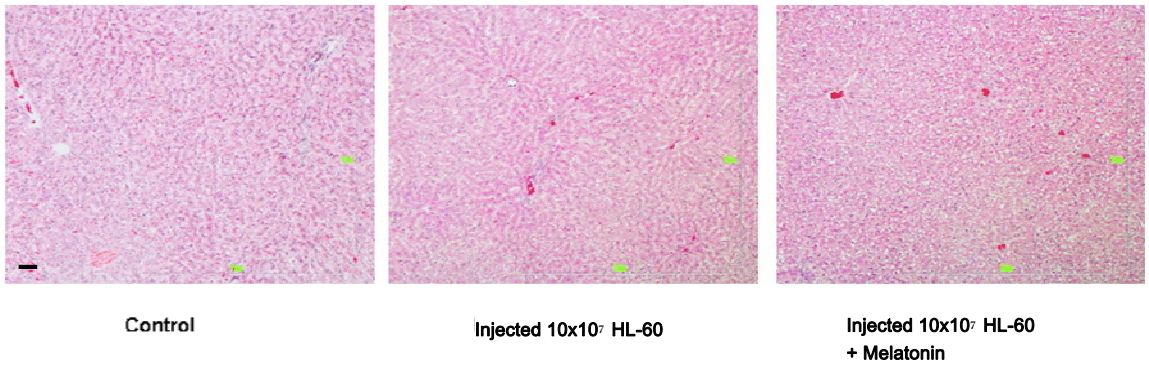

Fig. 6. **Liver samples** from a control rat, a leukæmia cell (HL-60) injected rat, and a leukæmia cell injected rat treated with melatonin. The stain used is hæmatoxylin-eosin. Scale Bar = 120 µm.

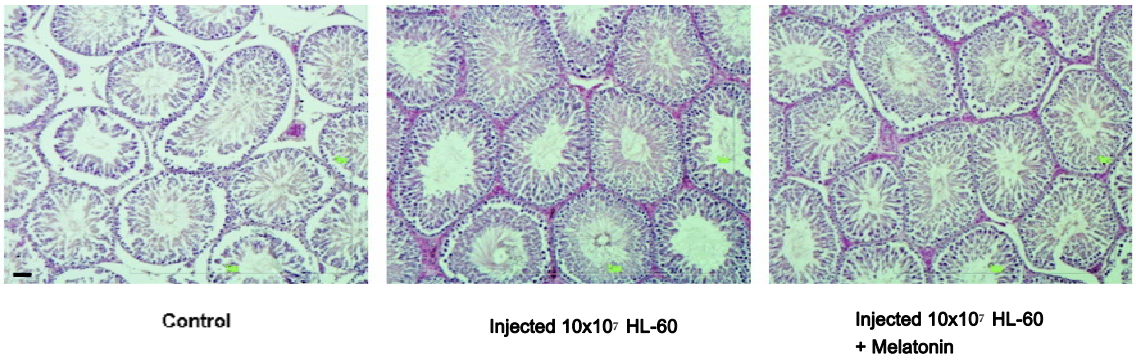

Fig. 7. **Histological sections of testes** of a control rat, a leukæmia cell (HL-60) injected rat, and a leukæmia cell injected rat treated with melatonin. The stain used is hæmatoxylin-eosin. Scale Bar = 120 µm.

Fig. 3 corresponds to the spleen. The staining reveals the white pulp (lymphoid tissue with a bluish colour because the nucleus occupies 90% of the cytoplasm) and the red pulp (mainly erythrocytes, enucleated cells of a more reddish colour). There are no striking changes in this distribution between the three groups, but one observes in the group of animals injected with HL-60 a decrease in size (atrophy) of the lymphoid follicles relative to the control, but not in the case of the animals treated with melatonin. This could correspond to a side effect of the treatment, such as weight loss.

The focus of the study of the brain specimens was on the regions of the subarachnoid space and the spaces of Virchow as being where the appearance of infiltration starts and is most often observed (Figs 4, 5). No involvement was observed of tissue-level structures such as basal ganglia or cortex.

Nor in the liver samples (Fig. 6) were any striking histological differences observed between the three groups. There was no appreciable increase in collagen (green) which would have been indicative of tissue damage, and the typical histological structures were conserved.

The histological analysis of the testes (Fig. 7) showed no significant differences between the three groups, the disposition of the seminiferous tubules being normal, and the cellularity conserved.

DISCUSSION

Melatonin is a molecule that has been well conserved evolutionarily (Tan et al. 2010), is ubiquitous and plays important roles in various physiological processes. Among the physiological effects of this indolamine are the control of circadian and activity-rest and sleep-wake rhythms, and of seasonal reproductive cycles. It also has antioxidant and immunomodulatory activity (Rodríguez et al. 2005, Chahbouni et al. 2010). In cancer treatment, this indolamine is involved in controlling tumour development and growth, presenting an anti-proliferative activity in numerous cancer cell lines (García-Navarro et al. 2007, Wenzel et al. 2005). There have been only a few studies with leukæmia cells, however. In particular, since the anti-proliferative and pre-apoptotic effects of melatonin have been described for leukæmic cells only using *in vitro* models (Büyükavci et al. 2006, Rubio et al. 2007, Bejarano et al. 2009), the present investigation was designed to determine the effect of melatonin on the caspase activity of lymphocytes and neutrophils obtained from Wistar rats injected with the cell line HL-60.

HL-60 cells constitute a leukæmia cell line that has been used for research on how certain types of blood cells are formed. HL-60 cells are also widely investigated to determine the effect of DNA topoisomerase (topo) IIα and IIβ on cell differentiation and programmed cell death (Sugimoto et al. 1998) and is especially useful in studies of dielectrophoresis (Ratanachoo et al. 2002) which require an aqueous environment with round cells in suspension. These cells have also made contributions in studies of programmed cell death and intracellular Ca^{2+} homeostasis (Fang et al. 1998).

Recent reports have documented that treatment with melatonin inhibits programmed cell death in non-tumour cells (Jou et al. 2010), while others have suggested that this indolamine promotes programmed cell death in cancer cells (Sainz et al. 2003). In this sense, our results have shown that, in neutrophils and lymphocytes obtained from leukæmia cell injected rats treated with melatonin, the TG-induced caspase-3 and caspase-9 activities are lower than those in neutrophils and lymphocytes obtained from injected rats untreated with the indolamine. We also observed a reduction of caspase-9 activity in neutrophils and of caspase-3 activity in lymphocytes obtained from leukæmia cell injected rats treated with melatonin relative to the controls. This reduction in caspase activity could be due to the known anti-apoptotic processes promoted by melatonin in immune cells (Sainz et al. 2003).

In this regard, there has recently been reported a decrease in TG- and fMLP- (N-formyl-methionyl-leucyl-phenyalanine) induced programmed cell death in human neutrophils and lymphocytes treated with melatonin (Espino et al. 2010) as has also been described by other workers (Luchetti et al. 2006, Radogna et al. 2008). It appears that melatonin exerts this protective action by blocking the opening of the mPTP (Espino et al. 2010). This same anti-apoptotic process has been described for other cell types: kidney cells (Kunduzova et al. 2003), hippocampal neurons (Shen et al. 2002), cultured mouse striatal neurons (Andrabi et al. 2004), pineal cells (Yoo et al. 2002) and cardiomyocytes (Petrosillo et al. 2009).

The inductor of programmed cell death, TG, is a specific inhibitor of the Ca^{2+}-ATPase of the endoplasmic reticulum, SERCA, with which it blocks the refilling of intracellular Ca^{2+} stores, inducing an increase in the cytosolic Ca^{2+} concentration, and thus causing an overload of Ca^{2+} in the mitochondria. When the mitochondria are overloaded with Ca^{2+}, mitochondrial uncoupling occurs, accompanied by a depolarization of the inner mitochondrial membrane and the production of ROSs (Reactive Oxygen Species) of mitochondrial origin. Also, the

mitochondrial permeability transition pore opens, constituting a megachannel that allows large molecular weight molecules to pass (Korsmeyer et al. 2000). The opening of this pore facilitates the release of pro-apoptotic agents of mitochondrial origin, such as cytochrome c and the Apaf-1 protein, among others, which together with procaspase-9 form a multimolecule complex known as the "apoptosome". This activates caspase-9, an initiator caspase, which in turn activates other executor caspases including caspase-3, triggering the process of programmed cell death (Korsmeyer et al. 2000) and resulting in cell death. In the current study, in the cells obtained from rats treated with melatonin, this process was inhibited, since melatonin blocks the opening of the mPTP, which would explain the decrease of caspase-3 and -9 activity we observed in lymphocytes and neutrophils (Kroemer et al. 2007, Rasola and Bernardi 2007).

We also recorded changes in leukocyte counts (the leukocyte formula), with the percentage of lymphocytes in the group of leukæmia cell (HL-60) injected rats treated with melatonin being lower than both the control group and the untreated group of injected rats, while the percentage of neutrophils was higher than in the other two groups. There was also an increase of eosinophils in the blood of the leukæmia cell (HL-60) injected rats with respect to the other two groups. These facts may reflect the existence of a population of HL-60 cells in the injected animals that, while insufficient to trigger a typical pathology of leukæmia, is capable of causing the atrophy of some tissues, as was observed in the histological study of the spleen.

In HL-60 injected rats, melatonin functions as an inducer of programmed cell death and an inhibitor of tumour development. This was observed in Ehrlich ascites carcinoma (EAC) cells which were injected intraperitoneally into female mice (El-Missiry and Abd El-Aziz 2000), in which oral administration of melatonin reduced the viability and volume of the tumour, and delayed the progression of the cell cycle. Also in rats with colon cancer, melatonin reduced both the multiplicity of tumours and the mitotic index (Anisimov et al. 2000).

Previous *in vitro* studies have shown that melatonin has a time-dependent effect in promoting the programmed cell death of HL-60 cells (Bejarano et al. 2009), confirming earlier results of Rubio et al (2007). Similar findings have been reported for other cell types: B-lymphoma cells (Trubiani et al. 2005, García-Navarro et al. 2007), human HT-29 colorectal cancer cells (Wenzel et al. 2005), HepG2 hepato-carcinoma cells (Martín-Renedo et al. 2008), the colon 38 cancer cell line (Melen-Mucha et al. 1998) and rat pituitary prolactin-secreting tumour cells (Yang et al. 2007).

Rubio et al (2007) also observed the effect of melatonin on cell viability, and found that high concentrations of melatonin (in the millimolar range) significantly diminished the reduction of MTT (3-[4,5/dimethyl/thiazol/2/yl]-2,5 diphenyl trihydro-chloride) staining and the number of HL-60 cells. Bejarano et al (2009) report a time-dependent activation-deactivation of the caspase-9 initiator induced by melatonin treatment, with the maximum effect being 12 hours after stimulation.

Overall, our results show that melatonin plays a crucial role in the inhibition of lymphocyte and neutrophil caspase-3 and -9 activity in control rats, as well as providing the capacity to revert to the basal lymphocyte, neutrophil, and eosinophil proportions in HL-60 injected rats.

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