

# Melatonin enhances hydrogen peroxide-induced apoptosis in human promyelocytic leukaemia HL-60 cells

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**Abstract** Melatonin is an indoleamine secreted by the pineal gland that shows multiple tasks. This ubiquitously acting free radical scavenger has recently been shown to stimulate the production of reactive oxygen species (ROS) in tumour cells, making them undergo apoptosis, whilst it prevents apoptosis in healthy cells. The mechanisms by which melatonin exerts these dual actions are, however, not yet clearly understood. Thus, the aim of this study was to further investigate how melatonin can enhance oxidative stress-induced apoptosis in a leukaemia cell line. The results show that melatonin increased the apoptotic effects of H<sub>2</sub>O<sub>2</sub> in human myeloid HL-60 cells as assessed by cellular viability, mitochondrial permeability transition induction, mitochondrial membrane depolarization, ROS generation, caspases 3, 8 and 9 activity, phosphatidylserine externalization, and DNA fragmentation techniques. When healthy leucocytes were exposed to H<sub>2</sub>O<sub>2</sub>, melatonin increased the viability of the cells. Taken together, the findings indicate that melatonin is a potential physiological tool capable of protecting healthy cells from chemotherapy-induced ROS production as well as inducing tumour cell death. Because cancer cells manifest increased oxidative stress as a result of their elevated metabolism, the use of melatonin may be useful in impairing their ROS buffering capacity.

**Keywords** Melatonin · Hydrogen peroxide · Apoptosis · Leukaemia cells

## Introduction

Melatonin is an indolamine mainly synthesized by the pineal gland. It possesses a wide spectrum of biological effects and is ubiquitously distributed from unicells to human [1, 2]. An increasing number of studies have reported the important role of melatonin in preventing apoptosis in healthy cells [3]. Evidence shows that melatonin decreases apoptosis in healthy cells because of its antioxidant and free radical scavenging abilities [4, 5]. However, the mechanisms by which melatonin exerts its oncostatic actions are not fully understood yet. Surprisingly, melatonin is able to stimulate reactive oxygen species (ROS) production in tumour cells [6, 7], making them undergo apoptosis [8]. However, this remains controversial since some reports claim that melatonin is able to prevent apoptosis of tumour cells both in vitro [9, 10] and in vivo [11, 12]. Melatonin has been reportedly shown to promote apoptotic cell death through the generation of ROS in several cancer cells including human myeloid HL-60, CMK, Jurkat, Molt-4 tumour cells [7, 13] lymphoma cells [14] and HT-29 human colorectal cancer cells [15]. Moreover, the treatment with melatonin reduced the antioxidant defenses in tumour cells depleting glutathione and increasing ROS production [16].

It is well known that some of the most common chemotherapeutic agents induce ROS generation, increasing their toxicity to the cells. This is the case of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) [17]. Since some tumours develop resistance to ROS induced by chemotherapeutics [18] and taking into account the dual antioxidant and pro-oxidant actions of melatonin in normal and tumour cells, respectively, this molecule could be used in chemotherapy treatments to induce ROS and increase their effects within the tumour cell, protecting normal cells from ROS-induced

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cell death. The rationale behind is that anticancer agents simultaneously applied with melatonin seem to cause less damage in normal cells [19] and to enhance the efficacy of the treatment [20], although little is known about this issue [21].

Thus, the aim of this study was to further investigate how melatonin can enhance oxidative stress-induced apoptosis in a leukaemia cell line. For that purpose, we have investigated the effects of melatonin on H<sub>2</sub>O<sub>2</sub>-induced apoptosis in human myeloid HL-60 cells in culture, analysing cellular viability, mitochondrial permeability transition (MPT) induction, mitochondrial membrane depolarization, ROS generation, caspases 3, 8 and 9 activity, phosphatidylserine (PS) externalization and DNA fragmentation.

## Materials and methods

### Materials

HL-60 15-12 cell lines (ECACC No 88120805) were purchased from The European Collection of Cell Cultures (ECACC) (Dorset, U.K.). Foetal bovine serum (FBS) and penicillin/streptomycin were obtained from HyClone (Aalst, Belgium). L-Glutamine and RPMI 1640 medium were obtained from Cambrex (Verviers, Belgium). Melatonin, *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (AC-DEVD-AMC) and Ficoll-Histopaque separating medium were obtained from Sigma (Madrid, Spain). *N*-acetyl-Leu-Glu-His-Asp-7-amido-4-trifluoromethylcoumarin (AC-LEHD-AMC), *Z*-Asp-Glu-Val-Asp-chloromethylketone (*Z*-DEVD-CMK), *N*-acetyl-Leu-Glu-His-Asp-chloromethylketone (Ac-LEHD-CMK), *N*-acetyl-Leu-Glu-Thr-Asp-AFC (Ac-LETD-AFC) and *Z*-Ile-Glu-Thr-DL-Asp-fluoromethylketone (*Z*-IETD-FMK) were obtained from Bachem (Bubendorf, Switzerland). Dihydrorhodamine-123 (DHR-123) and tetramethylrhodamine methyl ester (TMRM) were obtained from Molecular Probes (Oregon, USA). In situ cell death detection kit was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Annexin binding buffer, propidium iodide (PI) and annexin V-FITC were obtained from Immunostep (Salamanca, Spain). Hoechst 33342 was purchased from Calbiochem (San Diego, CA, USA). All other reagents were of analytical grade.

### Cellular culture

HL-60 cells are a human promyelocytic leukaemia cell line. Cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1.25% DMSO at 37°C under a humidified condition of

95% air and 5% CO<sub>2</sub>. Cells were routinely plated at a density of 3 × 10<sup>5</sup> cells/ml into fresh flasks, and the viability was <95% in all the experiments as assayed by the trypan-blue exclusion method. Cells were pretreated with 1 mM melatonin for 16 h and then treated with 500 µM H<sub>2</sub>O<sub>2</sub> for 2 h at 37°C, under 5% CO<sub>2</sub>. Melatonin was dissolved in absolute ethanol to give a 200 mM stock solution which was further diluted in RPMI 1640 medium to yield a 1-mM melatonin concentration resulting in a 0.5% ethanol concentration after being added to the cells. This concentration of ethanol did not modify the cell viability, and it was used as control value.

### Human leucocytes isolation

Venous blood was drawn from healthy volunteers (20–55 year-old) of both genders under informed consent according to a procedure approved by Local Ethical Committees and in accordance with the Declaration of Helsinki. Human leucocytes 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 [22]. 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<sub>4</sub>Cl solution (155 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, 0.1 mM EDTA, pH 7.4) so as to get a neutrophil-enriched preparation, as previously described [22]. 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.

### Cell viability assay

The metabolic activity of the cells was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described [7]. In brief, cells were seeded in a 96-well plate at a density of 5 × 10<sup>5</sup> cells per well. Then, cells were treated with melatonin at the indicated doses and times. After the treatments, the medium was removed and MTT was added into each well, and then incubated for 60 min at 37°C. Treatments were carried out in triplicate. Optical density was measured in an automatic microplate reader (Tecan Infinite M200) at a test wavelength of 490 nm and reference wavelength of

650 nm to negate the effect of cell debris. The data are presented as percentage above control (untreated samples).

#### MPT pore opening assay

To test the effects of melatonin on H<sub>2</sub>O<sub>2</sub>-induced MPT pore, cells were loaded with calcein-AM in the presence of cobalt chloride to quench fluorescence from all cellular domains except from within mitochondria. Although this protocol does not distinguish between calcein efflux and cobalt influx, it is consistent with induction of the permeability transition pore [23]. Opening was induced by 500  $\mu$ M H<sub>2</sub>O<sub>2</sub> in cells pretreated with 1 mM melatonin for 16 h or vector. MPT pore opening was indicated by a reduction in mitochondrial calcein signal and was measured with an automatic microplate reader (Tecan Infinite M200) with excitation wavelength of 488 nm and emission at 515 nm. Treatments were carried out in triplicate. Data are expressed as fractional changes of emitted fluorescence above the baseline values.

#### Determination of mitochondrial membrane potential

The quantification of mitochondrial membrane depolarization was carried out by measuring the fluorescence intensity of the membrane-permeant cationic probe TMRM. The resulting signal of TMRM-stained cells thus represents only the probe accumulated in intact mitochondria. A collapse on mitochondrial membrane is indicated by a decrease in output fluorescence. Briefly, cells ( $1 \times 10^6$  cells/ml) were washed once with PBS and loaded with 1  $\mu$ M TMRM by incubation at 37°C for 30 min. The fluorescence intensity of TMRM was measured in an automatic microplate reader (Tecan Infinite M200). Excitation was set at 443 nm and emission at 575 nm. Treatments were carried out in triplicate. The data are presented as fold increase over the pretreatment level (experimental/control).

#### Measurement of intracellular ROS levels

Intracellular ROS production was quantified by measuring the fluorescence intensity of rhodamine-123 (Rh-123). Briefly, cells ( $1 \times 10^6$  cells/ml) were washed once with PBS and loaded with 2  $\mu$ M DHR-123 by incubation at 37°C for 30 min. This probe is a non-fluorescent, cell-permeable compound; once inside the cell, it turns fluorescent upon oxidation to yield Rh-123, fluorescence being proportional to ROS generation. The fluorescence intensity of Rh-123 was measured in an automatic microplate reader (Tecan Infinite M200). Excitation was set at 488 nm and emission at 543 nm. Treatments were carried out in

triplicate. The data are presented as fold increase over the pre-treatment level (experimental/control).

#### Assay for caspase activity

To determine caspases 3, 8 and 9 activities, stimulated or resting cells were pelleted and washed once with PBS. After centrifugation, cells were resuspended in PBS at a concentration of  $1 \times 10^7$  cells/ml; 15  $\mu$ l of the suspension were 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  $\mu$ M caspase-3 substrate AC-DEVD-AMC or 100  $\mu$ M caspase-8 substrate AC-LETD-AFC. For caspase-9, the reaction buffer was composed of 0.1 M mes hydrate, pH 6.5, 10% PEG, 0.1% CHAPS, 5 mM DTT, 0.001% NP40 and 100  $\mu$ M of caspase-9 substrate AC-LEHD-AMC. Substrate cleavage was measured with a microplate reader (Tecan Infinite M200) with excitation wavelength of 360 nm and emission at 460 nm. Preliminary experiments reported that caspases 3, 8 or 9 substrate cleaving was not detected in the presence of the inhibitors of caspases 3, 8 or 9, Z-DEVD-CMK, Z-IETD-FMK or AC-LEHD-CMK, respectively. Treatments were carried out in triplicate. The data were calculated as fluorescence units/mg protein and presented as fold increase over the pre-treatment level (experimental/control).

#### In situ detection of DNA fragmentation by TUNEL and Hoechst staining

At the end of the treatments, HL-60 cells 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  $\mu$ 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  $\mu$ 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 were used to calculate the percentage of apoptotic cells, which was expressed as fold-increase over the pre-treatment level (experimental/control).

## Annexin V staining

Cells were harvested and washed twice with PBS and centrifuged at  $500\times g$  for 5 min, then the supernatant was discarded, and the pellet was resuspended in 95  $\mu\text{l}$  annexin V-binding buffer at a density of  $10^5$ – $10^6$  cells/ml containing annexin V-FITC. Cells were analysed by cytometry (Cytomyx FC-500 Beckman-coulter) after addition of PI. Each sample was tested 3–5 times in independent experiments. Annexin V binds to those cells that express PS on the outer layer of the cell membrane, and PI stains the cellular DNA of those cells with a compromised cell membrane. This allows for live cells (unstained with either fluorochrome) to be discriminated from early apoptotic cells (stained only with annexin V) late apoptotic or necrotic cells (stained with both annexin and PI) [23].

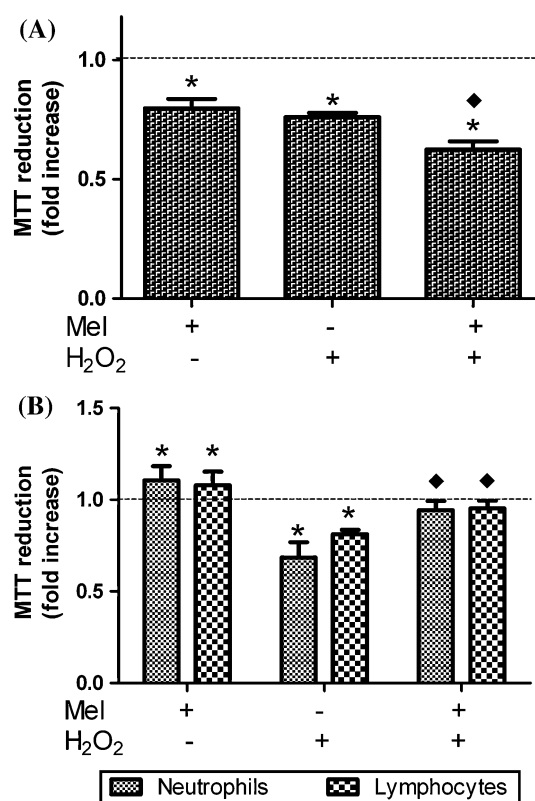
## Statistical analysis

Data are presented as mean  $\pm$  standard error of mean (SEM) and analysis of statistical significance was performed using Student's *t*-test.  $2\alpha < 0.05$  was considered to indicate a statistically significant difference ( $P < 0.05$ ).

## Results

### Melatonin improves the viability of healthy cells and augments cell death in tumour cells

In order to compare the effects of melatonin on the mitochondrial activity (i.e., metabolic activity) on  $\text{H}_2\text{O}_2$ -exposed cells, both tumour cells (HL-60 cells) and healthy cells (human neutrophils and lymphocytes) were used to perform the MTT reduction assay. As shown in Fig. 1a, a decrease in the MTT reduction was observed when the cultured cells were incubated with 1 mM melatonin for 16 h and/or 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 2 h with respect to untreated cells. In addition, melatonin decreased the HL-60 cells viability induced by  $\text{H}_2\text{O}_2$  (Fig. 1a;  $P < 0.05$ ). On the other hand, the effects of  $\text{H}_2\text{O}_2$  on healthy cells after the pre-treatment with melatonin were opposite. As shown in Fig. 1b 1 mM melatonin for 16 h increased the viability of leucocytes with respect to the untreated cells ( $P < 0.05$ ). Although the metabolic activity dropped because of the treatment with  $\text{H}_2\text{O}_2$  (Fig. 1b;  $P < 0.05$ ), melatonin was able to prevent this drop and restored the metabolic activity of neutrophils and lymphocytes to values similar to those obtained at the controls (Fig. 1b;  $P < 0.05$ ). These results emphasize the dual behaviour of the indolamine when in the presence of healthy leucocytes or leukaemia cells, supporting the idea that melatonin may reduce the side

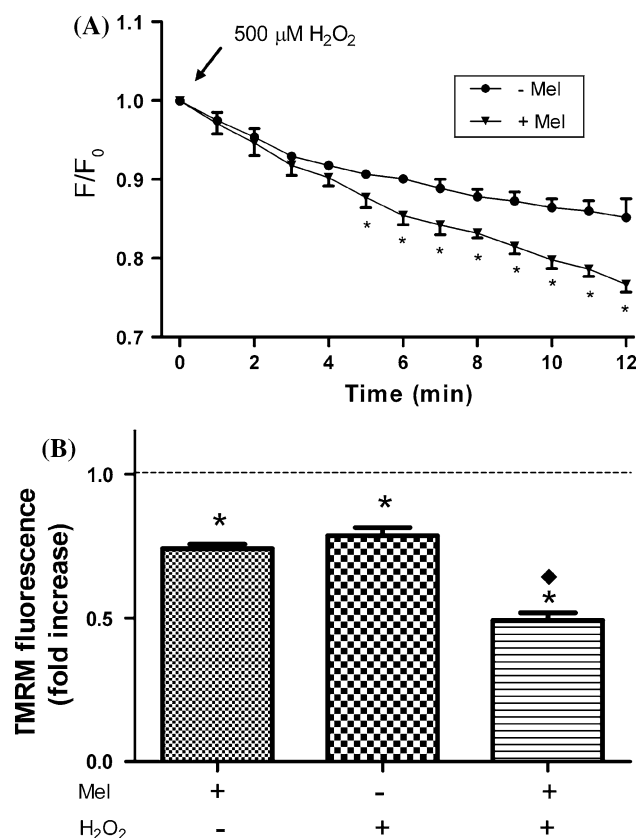


**Fig. 1** Melatonin improves the viability of healthy cells and augments cell death in tumour cells. HL-60 cells (a) or neutrophils and lymphocytes (b) were treated with 1 mM melatonin (Mel) or the vehicle (0.5% ethanol) for 16 h and then were stimulated with 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 2 h. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was estimated as described under Materials and Methods section. Values are presented as means  $\pm$  SEM of 5–8 separate experiments, which were carried out in triplicate, and expressed as fold-increase over the pre-treatment level (experimental/control). \* $P < 0.05$  compared to control values. <sup>♦</sup> $P < 0.05$  compared to melatonin or  $\text{H}_2\text{O}_2$  alone values

effects of drugs in healthy cells in terms of production of oxidative stressors and act at the same time as a cytotoxic agent in tumour cells.

### Melatonin enhances the $\text{H}_2\text{O}_2$ -induced mitochondrial disruption in tumour cells

Opening of MPT pore is considered a key event in cell death. We previously reported that both melatonin and  $\text{H}_2\text{O}_2$  compromise mitochondrial stability in leukaemic cells [24, 25]. To check the effects of melatonin pre-treatment on mitochondria integrity compromised by  $\text{H}_2\text{O}_2$ , we performed MPT pore opening assay and TMRM loading. MPT causes mitochondria to release apoptogenic molecules from intermembrane space into cytoplasm [26]. Figure 2a displays a higher slope in calcein fluorescence decrease induced by 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  when cells were previously treated with 1 mM melatonin for 16 h. Therefore,



**Fig. 2** Melatonin enhances the  $\text{H}_2\text{O}_2$ -induced mitochondrial disruption in HL-60 cells. **a** Cells were pre-incubated with 1 mM melatonin (Mel) or the vehicle (0.5% ethanol) for 16 h and then were stimulated with 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  (as the arrow indicated). Mitochondrial permeability transition (MPT) induction assay was performed as described under “Materials and methods” section. Changes in fluorescence were expressed as means  $\pm$  SEM of 4–6 separate experiments of fractional changes of emitted fluorescence ( $F/F_0$ ). \* $P < 0.05$  compared to  $\text{H}_2\text{O}_2$  alone. **b** Cells were pre-incubated with 1 mM melatonin (Mel) or vehicle (0.5% ethanol) for 16 h and then were stimulated with 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 2 h. Mitochondrial membrane potential and cell treatment were carried out as described under “Materials and methods” section. Values are presented as means  $\pm$  SEM of 5–8 separate experiments, which were carried out in triplicate, and expressed as fold-change over the pre-treatment level (experimental/control). \* $P < 0.05$  compared to control values.  $\blacklozenge P < 0.05$  compared to melatonin or  $\text{H}_2\text{O}_2$  alone

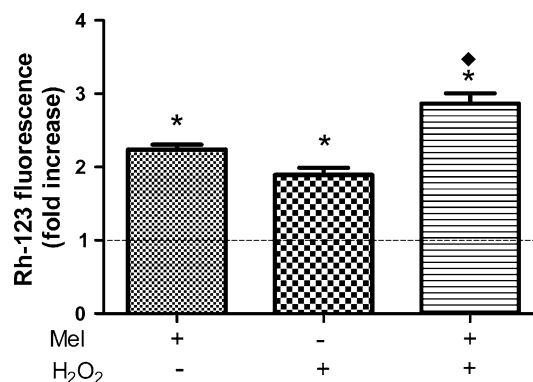
MPT pore in tumour cells becomes more sensitive to  $\text{H}_2\text{O}_2$  induction when cells are previously treated with melatonin. It is also well known that MPT leads to the loss of mitochondrial membrane potential ( $\Delta\psi$ ) [26]. Figure 2b shows a significant loss of  $\Delta\psi$ , indicated by a decrease in TMRM fluorescence when cells were exposed to 1 mM melatonin for 16 h, and then 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  was added for 2 h, with respect to the values obtained when cell were incubated with either melatonin or  $\text{H}_2\text{O}_2$  values ( $P < 0.05$ ). These findings support the hypothesis that melatonin enhances the  $\text{H}_2\text{O}_2$ -evoked mitochondrial disruption in tumour cells.

### Melatonin increases $\text{H}_2\text{O}_2$ -induced ROS generation in tumour cells

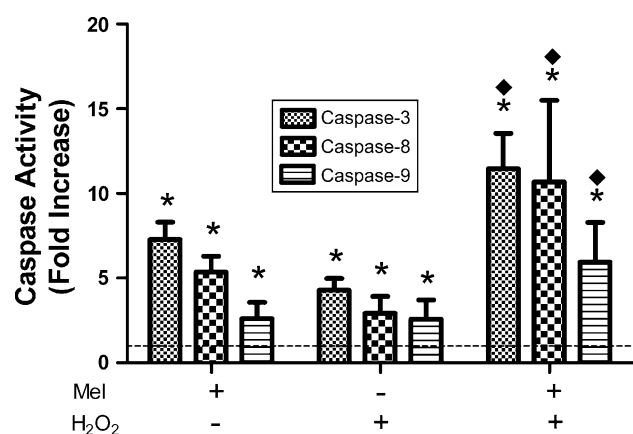
Since ROS are generated in mitochondria because of the uncoupling of electron transport chain as a step of apoptosis intrinsic pathway, and consequently ROS can induce oxidative damage of cell macromolecules which leads to apoptosis, we also evaluated the effect of melatonin on the production of intracellular ROS induced by  $\text{H}_2\text{O}_2$ . As shown in Fig. 3, treatment of cells for 16 h with 1 mM melatonin or for 2 h with 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  raised the production of intracellular ROS, as revealed by the increase in Rh-123 fluorescence. Cell pre-treatment with 1 mM melatonin caused a significant elevation of  $\text{H}_2\text{O}_2$ -induced ROS generation with respect to the control, and to when cells were treated with melatonin or  $\text{H}_2\text{O}_2$  alone ( $P < 0.05$ ).

### Caspase activation by $\text{H}_2\text{O}_2$ is enhanced by melatonin in tumour cells

Caspase-3 plays an essential executioner role in apoptosis in which caspases 8 and 9 are involved as critical upstream activators. To study the effects of cell pre-treatment with melatonin on  $\text{H}_2\text{O}_2$ -induced caspase activation, we treated HL-60 cells with 1 mM melatonin for 16 h and then 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  were added for 2 h. Our results showed that melatonin augmented substantially the  $\text{H}_2\text{O}_2$ -induced caspase-3 activity ( $P < 0.05$ ; Fig. 4). We observed similar results when the activities of activators caspases 8 and 9 were evaluated ( $P < 0.05$ ; Fig. 4). In addition, when



**Fig. 3** Melatonin increases  $\text{H}_2\text{O}_2$ -induced ROS generation in HL-60 cells. Dihydrorhodamine-123-loaded HL-60 cells were pre-incubated with 1 mM melatonin (Mel) or vehicle (0.5% ethanol) for 16 h and then were stimulated with 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 2 h. Intracellular ROS production was determined by changes of rhodamine-123 (Rh-123) fluorescence as described in “Materials and methods” section. Values are presented as means  $\pm$  SEM of 5–8 separate experiments, which were carried out in triplicate, and expressed as fold-increase over the pretreatment level (experimental/control). \* $P < 0.05$  compared to control values.  $\blacklozenge P < 0.05$  compared to melatonin or  $\text{H}_2\text{O}_2$  alone



**Fig. 4** Caspase activation by H<sub>2</sub>O<sub>2</sub> is enhanced by melatonin. HL-60 cells were treated with 1 mM melatonin (Mel) or vehicle (0.5% ethanol) for 16 h and then were stimulated with 500 μM H<sub>2</sub>O<sub>2</sub> for 2 h, caspases 3, 8 and 9 activities were estimated as described under “Materials and methods” section. Values are presented as means ± SEM of 5–8 separate experiments and expressed as fold-increase over pretreatment levels (experimental/control). \**P* < 0.05 compared to control values. ♦*P* < 0.05 compared to melatonin or H<sub>2</sub>O<sub>2</sub> alone

leukaemia cells were treated with melatonin or H<sub>2</sub>O<sub>2</sub> separately, caspases 3, 8 and 9 activities increased significantly compared to the controls (*P* < 0.05; Fig. 4).

Melatonin augments the H<sub>2</sub>O<sub>2</sub>-induced DNA fragmentation in tumour cells

Chromatin cleavage is an idiosyncrasy of apoptosis, given that, during apoptosis, some endogenous nucleases are activated cleaving DNA into internucleosomal fragments of ~200 pb. TUNEL assay is a well-established method for detection of DNA cleavage, a relatively late apoptotic marker [25]. We assessed the amount of DNA fragmentation in the presence of H<sub>2</sub>O<sub>2</sub> to verify whether melatonin enhanced the programmed cell death. Treatment of HL-60 cells with both 1 mM melatonin for 16 h and 500 μM H<sub>2</sub>O<sub>2</sub> for 2 h produced a substantial increase in the proportion of cells depicting DNA fragmentation (*P* < 0.05; Fig. 5a). Importantly, a higher proportion of cells depicting DNA fragmentation was found when cells were pre-incubated with 1 mM melatonin before H<sub>2</sub>O<sub>2</sub> treatment as compared to H<sub>2</sub>O<sub>2</sub> treatment alone (*P* < 0.05; Fig. 5a), thereby reflecting that melatonin pre-treated leukaemia cells are much more vulnerable to apoptosis induced by H<sub>2</sub>O<sub>2</sub>.

Melatonin moves HL-60 cells apoptosis forward later apoptotic stages caused by H<sub>2</sub>O<sub>2</sub>

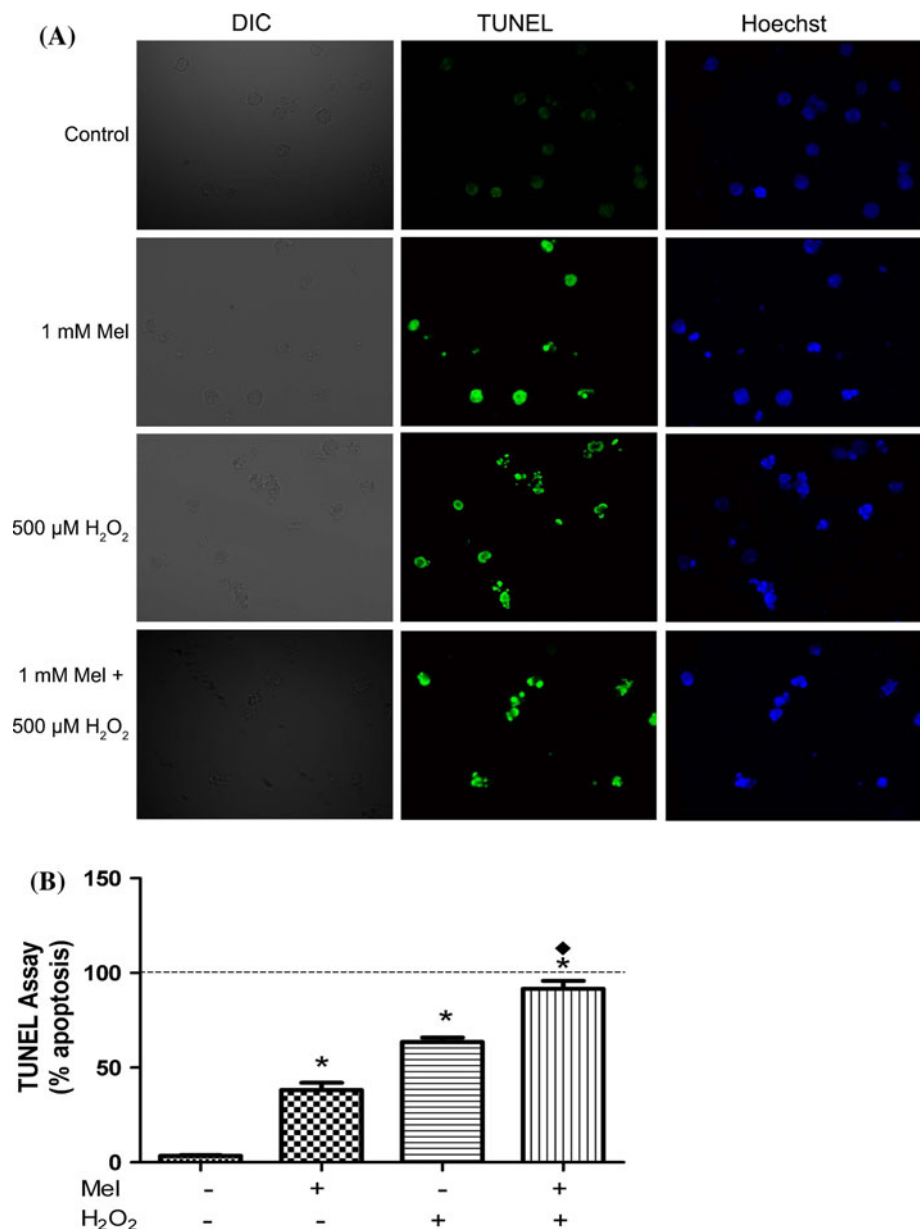
Lastly, to check the different cell apoptotic stages under melatonin and H<sub>2</sub>O<sub>2</sub> treatments, HL-60 cells annexin

V-FITC/PI staining was performed. Figure 6 show different regions where cells are located depending on PS externalization (annexin V positivity) and porosity of membrane (PI positivity). Despite apoptotic and necrotic cells show annexin V/PI positivity, a higher permeability for PI is easily differentiated in necrosis cases, showing a population of cells with upper location than late apoptosis (Fig. 6). Control samples (Fig. 6; upper-left section) showed most cells in the living cell region. The low percentage of cells showing positivity was due to spontaneous cell death. Cells treated with 1 mM melatonin displayed an increase in annexin V/PI positivity in both the late apoptosis and necrosis regions (Fig. 6; upper-right section). Although an increase in the percentage of cells at the necrosis region was observed, similar results were obtained when cells were treated with 500 μM H<sub>2</sub>O<sub>2</sub> for 2 h (Fig. 6; lower-left section). When cells were treated with 1 mM melatonin for 16 h and 500 μM H<sub>2</sub>O<sub>2</sub>, double-stained cells displayed a location mainly at the necrosis area (Fig. 6; lower-right section), being a low percentage of cells located at the living cell or early apoptosis regions. These results strongly suggest that the toxicity of H<sub>2</sub>O<sub>2</sub> may be raised by melatonin.

## Discussion

The presence of melatonin regulates the expression of clock genes which are responsible of haemorrhhythmicity. Lack of regulation of internal clock gene could make cancer appear [27]. In addition, an increasing number of studies report that melatonin induces a significant, strong inhibition in tumour cells. This may be also the case of its precursor [28]. As previously described, melatonin disrupts mitochondrial function and generates intracellular ROS [7, 24]. A recent report supports the fact that melatonin-induced ROS was one of the main effects to evoke apoptosis in HL-60 cells, given that other antioxidant agents were able to reduce apoptotic events [11]. Experimental evidence shows that tumour cells are under an elevated oxidative stress, compared to healthy cells. Such a huge stress is due to metabolic alterations and subsequent increased ROS production [29–31]. A moderate increase in ROS promotes cellular proliferation and mutations, and moreover contributes to develop anticancer drug resistance. The activity of antioxidant intracellular enzymatic defenses, including superoxide dismutase (SOD), glutathione-*S*-transferase (GST) or glutathione peroxidase is elevated in cancer tissues [32]. In fact, the expression of antioxidant enzymes, like SOD, GST or catalase, shows an adaptational increase under the elevated oxidative stress in cancer cells [33]. It is thought that the increase in the amount of antioxidant agents may contribute to anticancer drug resistance during the disease progression. Since ROS are

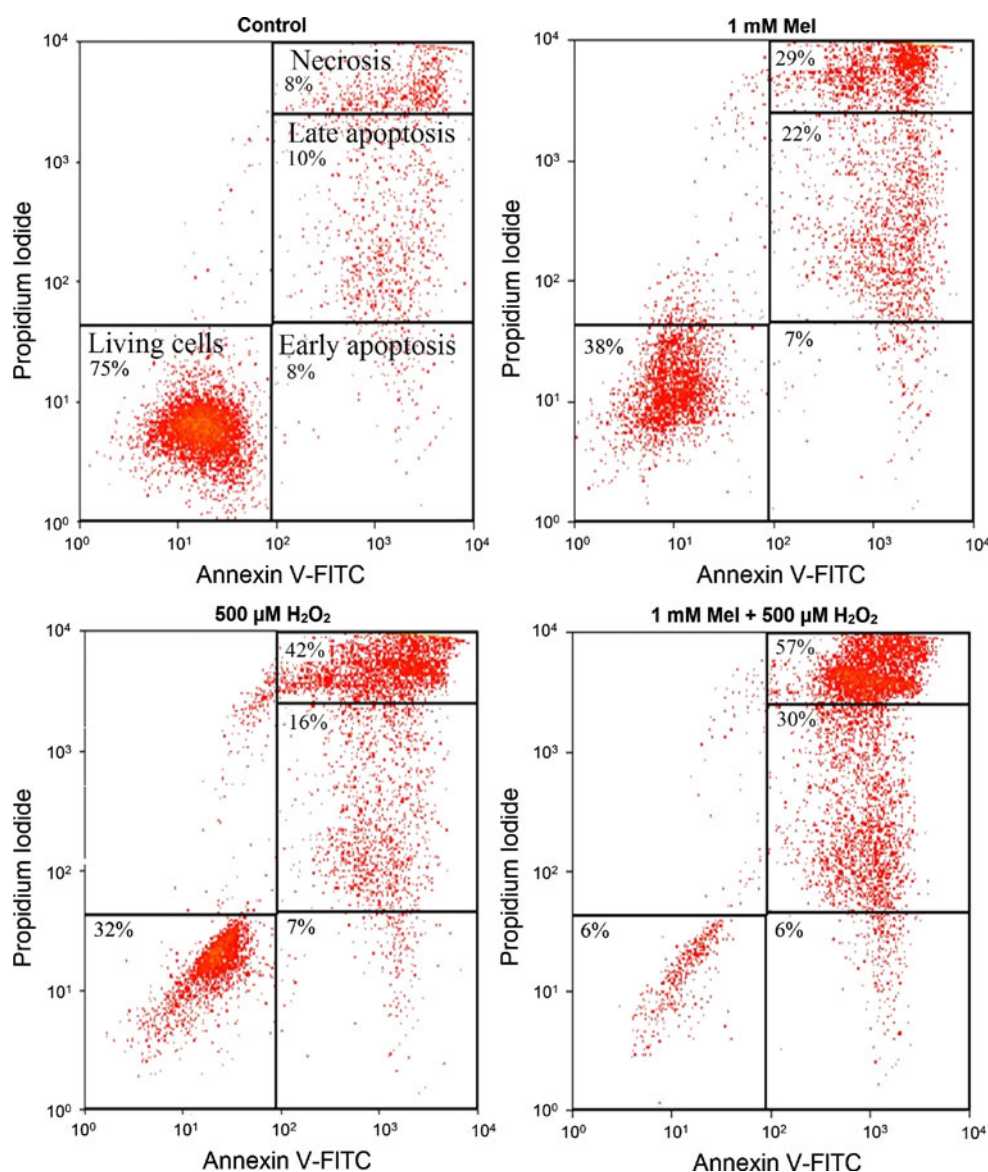
**Fig. 5** Melatonin augments the  $H_2O_2$ -induced DNA fragmentation in HL-60 cells. Cells were pre-treated with 1 mM melatonin (Mel) or vehicle (0.5% ethanol) for 16 h and then were stimulated with 500  $\mu M$   $H_2O_2$  for 2 h. To evaluate the proportion of cells depicting DNA fragmentation, TUNEL assay was performed and DNA fragmentation was estimated as described under “Materials and methods” section. **a** 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  $\mu m$ . **b** 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). \* $P < 0.05$  compared to control values.  $\blacklozenge P < 0.05$  compared to melatonin or  $H_2O_2$  alone



able to produce strong cellular injuries, a further ROS insult could provide a chance to evoke cell death much more efficiently, when the antioxidant intracellular machinery has reached its maximum, and even counteract drug resistance, which has been reported for some cancers [17]. Melatonin has been shown not only to generate intracellular ROS in tumour cells [7, 16], but also to increase the apoptotic effects of anticancer agents that generate intracellular ROS. In order to discard actions of anticancer drugs different from those mediated by ROS generation, 500  $\mu M$   $H_2O_2$  was used in this study to emulate chemotherapeutically caused oxidative stress in HL-60 cells pre-treated with 1 mM melatonin. Our findings revealed a reinforcing apoptotic action of the indoleamine, when

leukaemia HL-60 cells were treated with the oxidative agent. Such intensification is due, at least in part, to the supplementary effects of melatonin on oxidative stress. It is well known that mitochondria produce ROS when  $\Delta\psi$  decreases by MPT induction. Other processes, including deficiency of mitochondrial antioxidant occur simultaneously with MPT. Such damage in mitochondria causes the release of apoptotic factors that trigger caspase-9 activation. In this regard, treated HL-60 cells with melatonin may represent a further oxidative insult, which is increased when an oxidative agent, like  $H_2O_2$ , is added with the indoleamine. Melatonin and  $H_2O_2$  have been previously shown to induce mitochondrial damage [25, 34], but interestingly, in our study, when cells were treated

**Fig. 6** Melatonin moves cell leukaemia apoptosis forward the later apoptotic stages caused by  $H_2O_2$ . Cells were pre-treated with 1 mM melatonin (Mel) or vehicle (0.5% ethanol) for 16 h and then were stimulated with 500  $\mu M$   $H_2O_2$  for 2 h. To evaluate the stages of apoptosis, cells were stained with annexin V-FITC and propidium iodide (PI) and analysed by flow cytometry as described under “Materials and methods” section. Data are representative of three independent experiments



simultaneously with both substances, a significant elevation in mitochondrial malfunction parameters (MPT induction, loss of  $\Delta\psi$ , intracellular ROS production, diminished metabolic capacity and higher activation of caspase-9) could be detected in comparison to the mitochondrial injury provoked by these substances alone. Taken together, these results strongly indicate that mitochondria were seriously affected when combining both treatments, which activated the intrinsic apoptotic pathway. In addition, it was also observed that the extrinsic pathway was also activated through caspase-8 activity when leukaemia cells were treated. These results are in agreement with those of Martín-Renedo and colleagues [35] who reported a melatonin-induced increase in caspase-8 activity in hepatocarcinoma cells. Also, a recent study has shown that increases in intracellular ROS regulate the activation

of caspase-8 during apoptosis [36]. In our study, increases in caspase-8 activity were obtained in cells treated with either  $H_2O_2$  or melatonin or in combination of both agents. Melatonin likely increases the  $H_2O_2$ -induced oxidative stress causing an increased activation of caspase-8. This caspase-8 activation might be due to the strong pro-oxidant activity in tumour cells, as shown by Wolfler et al. [11] in Jurkat cells. In addition, melatonin has been shown to lead to the promotion of Fas-induced cell death. Pre-incubation with the indolamine may augment the susceptibility of cells to additional oxidant agents, including  $H_2O_2$ . In fact, melatonin has been reported to increase Fas-receptor expression in cultured cancer endothelial cells [37].

It is known that the initiator caspases 8 and 9 are able to activate the executioner caspase-3, evoking an irreversible apoptotic stage that leads to cell death. Our results showed



the activation of initiator caspases and the resulting increase in caspase-3 activity in the presence of H<sub>2</sub>O<sub>2</sub> in melatonin-pretreated cells. The toxic effect of melatonin in tumour cell lines has been widely reported [15, 34, 35]. In this study, a higher number of cells with fragmented DNA, i.e., the number of apoptotic cells, were observed when cells were treated with both H<sub>2</sub>O<sub>2</sub> and melatonin, as shown by TUNEL assay techniques. As expected, most HL-60 cells were found at the final apoptotic stages (late apoptosis or necrosis). This indicates that the treatment had a relevant toxicity in leukaemia cells. The findings of this study in leukaemia cells are contrary to those described by Chetsawang et al. [38] in neuroblastoma, where it was suggested that melatonin displays a protector role in tumour cells similar to that exerted in normal cells. An MTT assay was carried out to evaluate the metabolic capacity of healthy leucocytes, and therefore their viability, with melatonin and H<sub>2</sub>O<sub>2</sub> treatments. In agreement with Juknat et al. [39], we observed that the pre-treatment with melatonin was able to reverse H<sub>2</sub>O<sub>2</sub> toxic effects both in lymphocytes and neutrophils. However, when HL-60 cells were pre-treated with melatonin before H<sub>2</sub>O<sub>2</sub>, the viability diminished considerably.

In summary, melatonin augmented oxidative stress in tumour cells and reduced oxidative stress in healthy cells. Many chemotherapeutic agents generate ROS as one of their mechanisms to kill cancer cells. Cancer cells possess increased oxidative stress levels as a result of their elevated metabolism. Consequently, the expression of antioxidant enzymes and their activities are severely increased. Our findings indicate that melatonin may be a potential tool able to protect healthy cells from chemotherapy-induced ROS and saturate, at the same time, the antioxidant buffering capacity of cancer cells due to the generation of ROS induced by the indoleamine in this type of cells.

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