J. Pineal Res. 2012; 53:91–98
Doi:10.1111/j.1600-079X.2012.00974.x

Journal of Pineal Research

Journal of Pineal Research
Molecular, Biological, Physiological and Clinical Aspects of Melatonin

Melatonin potentiates chemotherapy-induced cytotoxicity and apoptosis in rat pancreatic tumor cells

Abstract: Melatonin has antitumor activity via several mechanisms including its antiproliferative and proapoptotic effects in addition to its potent antioxidant action. Thus, melatonin has proven useful in the treatment of tumors in association with chemotherapeutic drugs. This study was performed to evaluate the effect of melatonin on the cytotoxicity and apoptosis induced by three different chemotherapeutic agents, namely 5-fluorouracil (5-FU), cisplatin, and doxorubicin in the rat pancreatic tumor cell line AR42J. We found that both melatonin and the three chemotherapeutic drugs induce a time-dependent decrease in AR42J cell viability, reaching the highest cytotoxic effect after 48 hr of incubation. Furthermore, melatonin significantly augmented the cytotoxicity of the chemotherapeutic agents. Consistently, cotreatment of AR42J cells with each of the chemotherapeutic agents in the presence of melatonin increased the population of apoptotic cells, elevated mitochondrial membrane depolarization, and augmented intracellular reactive oxygen species (ROS) production compared to treatment with each chemotherapeutic agent alone. These results provide evidence that in vitro melatonin enhances chemotherapy-induced cytotoxicity and apoptosis in rat pancreatic tumor AR42J cells and, therefore, melatonin may be potentially applied to pancreatic tumor treatment as a powerful synergistic agent in combination with chemotherapeutic drugs.

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Key words: melatonin, chemotherapy, cytotoxicity, apoptosis, AR42J cells

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Received November 22, 2011;
Accepted January 2, 2012.

Introduction

Melatonin has diverse physiological functions [1]. It plays a crucial role in regulating circadian rhythms and is involved in immunomodulation and hematopoiesis [2–4]. Melatonin also exerts anticancer activity through either cytostatic mechanisms or cytotoxic action by triggering the apoptotic machinery [5]. A large number of studies have demonstrated that melatonin has important oncostatic properties, because it inhibits cell proliferation in several cancer cell lines, including human B-lymphoma cells [6], human myeloid leukemia cell HL-60 [7], and human neuroblastoma cancer cells [8]. Moreover, evidence shows that melatonin is able to inhibit linoleic acid (LA) uptake by tumor cells and its conversion into the mitogenic factor 13-hydroxyoctadecadienoic acid (13-HODE) via melatonin receptor MT1-dependent suppression of cAMP in human cancer xenografts [9]. In this regard, nude rats under constant light conditions exhibited higher tumor LA uptake and its metabolism to 13-HODE and, consequently, higher growth of breast cancer (MCF-7) xenografts, which involved melatonin suppression [10]. In addition, it has been reported that melatonin and MT1 levels are significantly diminished with the onset of old age and that this reduction in melatonin and its receptor leads to an enhanced mammary tumor growth and a decreased sensitivity to melatonin [11]. As hormone-dependent mammary tumors showed a strong dependence on local expression of sulfatase activity, the indole melatonin, specifically in mammary tumors, could also exert its antitumoral actions by down-regulating the expression of sulfatase activity in malignant tissues [12].

Additionally, melatonin is one of the most potent antioxidant agents [13, 14], with a protective action against chemotherapy-induced cytotoxicity [15, 16], which may be due, at least in part, to an enhanced reactive oxygen species (ROS) generation. The majority of chemotherapeutic drugs are typical activators of the mitochondria-mediated intrinsic pathway of apoptosis [17, 18]. The intrinsic apoptosis pathway is characterized by loss of mitochondrial membrane potential and release into the cytosol of mitochondrial apoptogenic factors, including cytochrome c. The release of proapoptotic factors from mitochondria to the cytosol results in activation of the caspase protease cascade.

Initially, caspase-9 becomes activated, followed by activation of the executioner caspase-3, 6, and 7. Activated executioner caspases cleave specific cellular substrate proteins, promoting the destruction of the cell (for review see [19]).

Melatonin has diverse actions on apoptosis; it protects normal cells from apoptosis [20–22], while it induces apoptosis in many cancer cells [23], including human myeloid HL-60 cells [24], B-lymphoma cells [6], HT-29 human colorectal cancer cells [25, 26], and human pancreatic tumor cells [27]. Various studies have reported that melatonin may be an excellent candidate as an anticancer agent or for combined therapy owing to its antioxidant, oncostatic, and proapoptotic activities in tumor cells [16]. In a recent study in leukemia cells taking from patients with acute myeloid leukemia, it has been reported that treatment with the chemotherapeutic drug etoposide in combination with melatonin resulted in increased elimination of the leukemia cells in culture [28]. Additionally, melatonin potentiates chemotherapy-induced apoptosis in human leukemia HL-60 cells [29], human hepatoma cell lines [30], human Ewing sarcoma cancer cells [31], and ovarian cancer cells [32]. Furthermore, it has also been reported that melatonin protects both normal human peripheral blood lymphocytes and cancer cells, such as HeLa and human leukemia K562 cells, against genotoxic treatment and apoptosis induced by idarubicin (an anthracycline antibiotic used in the treatment of acute leukemia and other malignancies) [33]. Therefore, melatonin could enhance the efficacy of chemotherapy in terms of both tumor regression rate and survival time. Preliminary clinical studies in patients with cancer demonstrate the benefits of melatonin in association with cancer chemotherapeutic agents [34, 35]. However, limited data are available on the effect of melatonin on the cytotoxicity and/or apoptosis evoked by chemotherapeutic drugs in pancreatic tumor cells.

In this study, we evaluated the effect of melatonin on the cytotoxicity and apoptosis induced by different chemotherapeutic agents, namely 5-fluorouracil (5-FU), cisplatin, and doxorubicin, in the rat pancreatic tumor cell line AR42J.

Materials and methods

Chemicals and reagents

AR42J cell line (ECACC No 9300618) derived from exocrine rat pancreatic tumor was purchased from The European Collection of Cell Cultures (ECACC) (Dorset, UK). Fetal bovine serum and penicillin/streptomycin were obtained from HyClone (Aalst, Belgium). L-Glutamine and RPMI 1640 medium were obtained from Cambrex (Verviers, Belgium). Doxorubicin hydrochloride, 5-fluorouracil (5-FU), cis-diammineplatinum (II) dichloride (cisplatin), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma (Madrid, Spain). 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was obtained from Bachem (Barcelona, Spain) and dihydrorhodamine-123 (DHR-123) was from Molecular Probes (Eugene, OR, USA). All other reagents were of analytical grade.

Cell culture

AR42J cells are derived initially from a transplantable tumor of a rat exocrine pancreas. This line is tumorigenic in nude mice and shows a significant secretion of amylase and other exocrine enzymes. AR42J cells (passages 6–12) were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C under a humidified condition of 95% air and 5% CO_2. Cells were routinely plated at a density of 3×10^5 cells/mL into 24-well plate, and the viability was >95% in all experiments as assayed by the trypan blue exclusion method.

Cell viability assay

Cell viability was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, which is based on the ability of viable cells to convert a water-soluble, yellow tetrazolium salt into a water-insoluble, purple formazan product. The enzymatic reduction of the tetrazolium salt occurs only in living, metabolically active cells, but not in dead cells. Cells were seeded in 24-well plates at a density of 2×10^5 cells per well and subsequently exposed to the appropriate treatment at 37°C. After the treatments, the medium was removed, and MTT was added into each well and then incubated for 60 min at 37°C, as previously described [36]. The supernatant was discarded, and DMSO was added to dissolve the formazan crystals. Treatments were carried out in triplicate. Optical density was measured in an automatic microplate reader (Tecan Infinite M200; Tecan Austria GmbH, Groedig, Austria) at a test wavelength of 490 nm and a reference wavelength of 650 nm to nullify the effect of cell debris. Data are presented as percentage above control (untreated samples).

Determination of apoptosis

After treatment, cells were detached with 0.2% trypsin-EDTA, harvested and washed twice with phosphate-buffered saline (PBS), and centrifuged at 500g for 5 min; then the supernatant was discarded, and the pellet was resuspended in 95 μL annexin V-binding buffer containing annexin V-FITC at a density of 10^5–10^6 cells/mL. Cells were analyzed by flow cytometry (Cytomycs FC-500; Beckman-Coulter, Hialeah, FL, USA) after addition of propidium iodide (PI). Each sample was tested 3–5 times in independent experiments. Annexin V binds to those cells that express phosphatidylserine (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) [36].

Measurement of mitochondrial membrane potential

Cells were incubated with 1 μM JC-1 for 15 min at 37°C as previously described [24]. The cationic dye, JC-1, exhibits

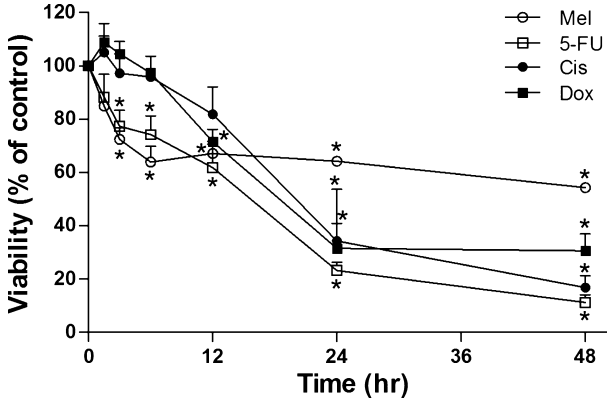

Fig. 1. Tumoricidal activity of melatonin and different chemotherapy drugs in pancreatic AR42J cells. Cells were treated with 1 mM melatonin (Mel), 1 mM 5-fluorouracil (5-FU), 20 $\mu\mu$ cisplatin (Cis), or 1 μM doxorubicin (Dox) for 1.5, 3, 6, 12, 24, and 48 hr, and then cell viability was assessed by the MTT reduction assay as described in the Material and Methods section. Values are presented as means ± S.E.M. of four separate experiments carried out in triplicate and expressed as percentage (%) above control. $^{*}P < 0.05$ compared to control values (time = 0 hr).

potential-dependent accumulation in mitochondria. It indicates mitochondria depolarization by a decrease in the red-to-green fluorescence intensity ratio. After incubation with JC-1, the dye was removed, and the cells were washed in PBS. The green JC-1 signal was measured at the excitation wavelength of 485 nm and the emission wavelength of 535 nm, and the red signal, at the excitation wavelength of 540 nm and the emission wavelength of 590 nm. Fluorescence changes were analyzed using a fluorescence spectrophotometer (RF-5301-PC; Shimadzu, Kyoto, Japan). Treatments were carried out in triplicate. Data are presented as emission ratios (590/535). Changes in mitochondrial membrane potential were quantitated as the integral of the decrease in JC-1 fluorescence ratio.

Measurement of intracellular reactive oxygen species (ROS) production

Cells were loaded with 2 μM dihydrorhodamine-123 (DHR-123) by incubation at 37°C for 30 minutes as previously described [37]. This probe is a nonfluorescent cell-permeable compound. Once inside the cell, it turns fluorescent upon oxidation to yield rhodamine-123 (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. Data are presented as fold increase over the pretreatment level (experimental/control).

Statistical analysis

Data are presented as mean ± standard error of mean (S.E.M) for each group. Significance of differences between

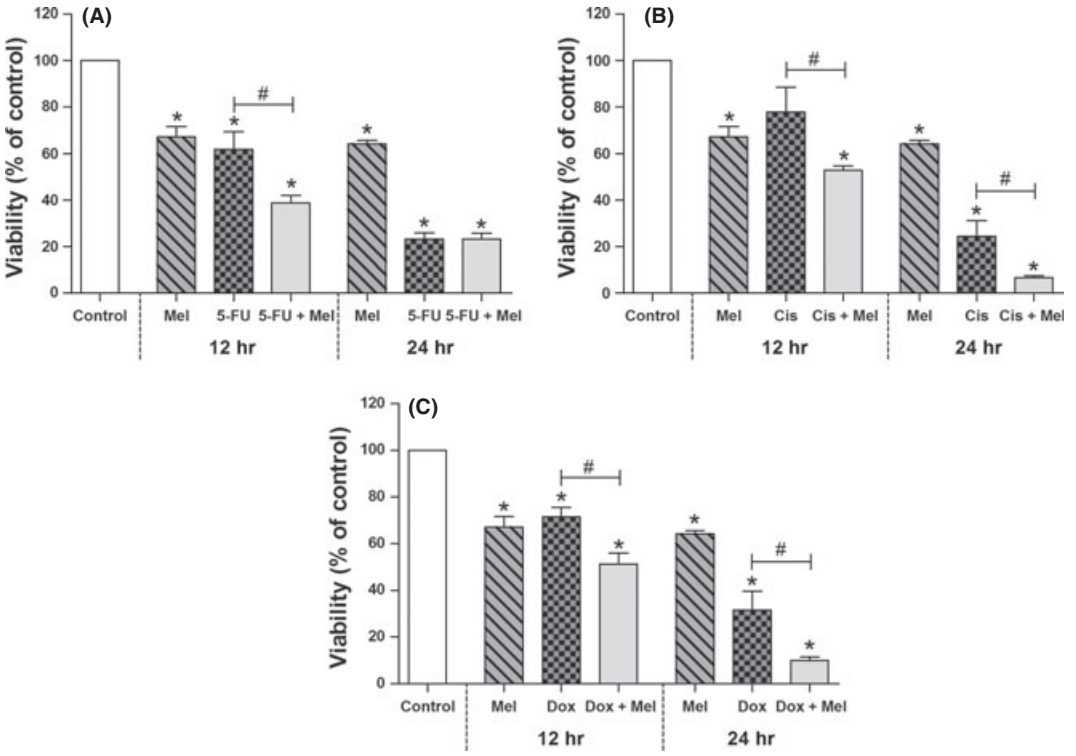

Fig. 2. Effect of melatonin on chemotherapy-induced cytotoxicity induced in pancreatic AR42J cells. Cells were treated with 1 mM melatonin (Mel) (A, B, C), 1 mM 5-fluorouracil (5-FU) (A), 20 μM cisplatin (Cis) (B), or 1 μM doxorubicin (Dox) (C) separately or combined, or the vehicle (control) for 12 and 24 hr, and then cell viability was assessed by the MTT reduction assay as described in the Material and Methods section. Values are presented as means ± S.E.M. of six separate experiments carried out in triplicate and expressed as percentage (%) above control. $^{*}P < 0.05$ compared to control values. $^{\#}P < 0.05$ compared to melatonin group.

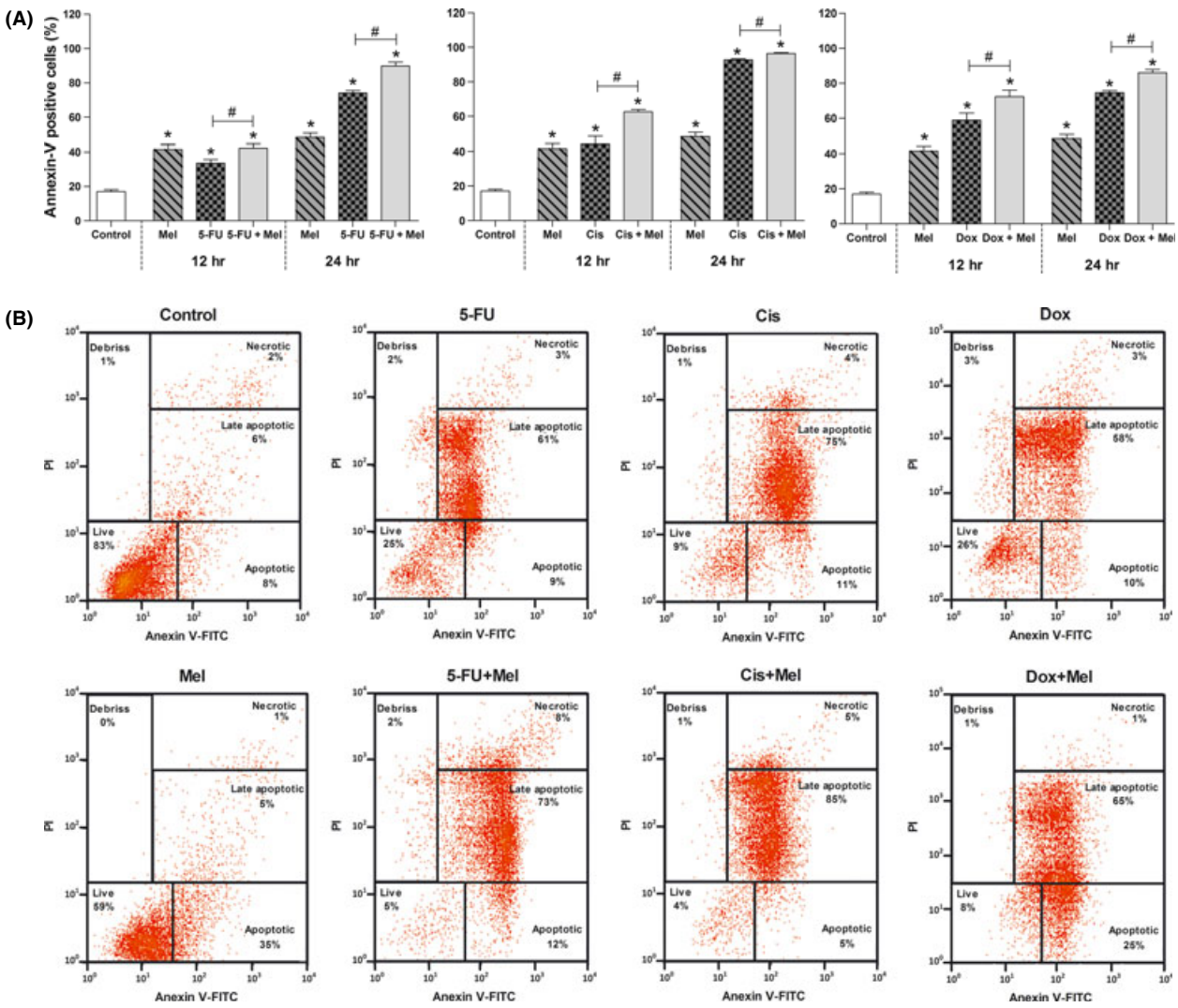

Fig. 3. Apoptotic cell death triggered by chemotherapy agents is strengthened by their combination with melatonin. Pancreatic AR42J cells were treated with 1 mM melatonin (Mel), 1 mM 5-fluorouracil (5-FU), 20 μM cisplatin (Cis), or 1 μM doxorubicin (Dox) separately or combined, or the vehicle (control) for 12 and 24 hr, and then apoptosis was analyzed as described in the Material and Methods section. (A) Histograms showing the percentage of annexin V-positive cells. Values are presented as means ± S.E.M. of 3–5 separate experiments and expressed as percentage (%). (B) Representative plots displaying the redistribution of phosphatidylserine (annexin V-positive cells) in the presence of propidium iodide (PI) after 24 hr of treatment with the indicated combinations of drugs. $^{*}P < 0.05$ compared to control values. $^{\#}P < 0.05$ compared to melatonin group.

two groups was evaluated using Student's *t*-test. For multiple comparisons, one-way analysis of variance (ANOVA) was used followed by post hoc Tukey's test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Figure 1 show the viability of AR42J cells incubated with 1 mM melatonin, 1 mM 5-fluorouracil, 20 μM cisplatin, or 1 μM doxorubicin for different periods of time (from 1,5 to 48 hr). In previous studies, we have shown that melatonin is able to decrease the cell viability in tumor leukocytes [37]. As shown in Fig. 1, both melatonin and the three chemotherapeutic agents induced a significant and time-dependent decrease in cell viability of the pancreatic tumor cell line AR42J. In all cases, the highest cytotoxic effect was reached after 48 hr of incubation.

Parallel assays were performed to examine the possible potentiating effect of melatonin on chemotherapy-mediated cytotoxicity in AR42J cells, which is treated with each chemotherapeutic agent in the absence or presence of melatonin for 12 and 24 hr. Fig. 2 showed a further decrease in cell viability when melatonin (1 mM) was combined with 1 mM 5-fluorouracil (Fig. 2A), 20 μM cisplatin (Fig. 2B), or 1 μM doxorubicin (Fig. 2C) compared to each chemotherapy treatment alone, indicating that melatonin can enhance cytotoxicity of the chemotherapeutic drugs in AR42J cells.

Externalization of PS is one of the most important markers of apoptosis [24]. To investigate the potentiating effect of melatonin and the three chemotherapeutic drugs

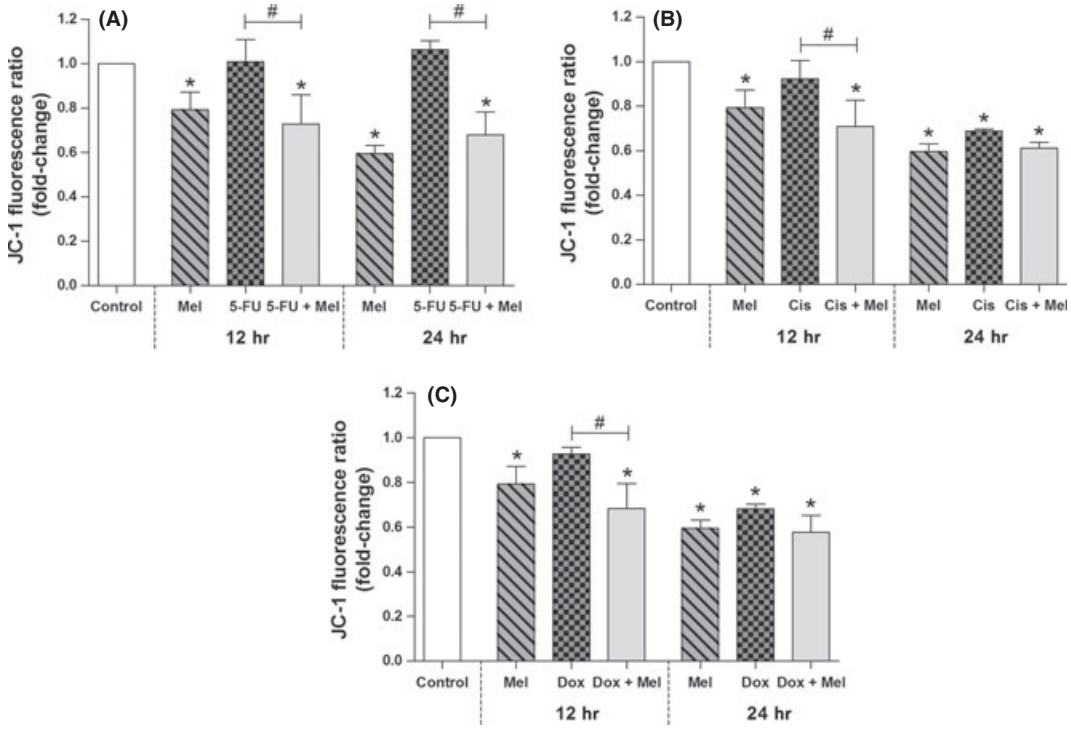

Fig. 4. Mitochondrial membrane depolarization induced by chemotherapy agents is potentiated by their combination with melatonin. JC-1-loaded pancreatic AR42J cells were treated with 1 mM melatonin (Mel) (A, B, C), 1 mM 5-fluorouracil (5-FU) (A), 20 μM cisplatin (Cis) (B), or 1 μM doxorubicin (Dox) (C) separately or combined, or the vehicle (control) for 12 and 24 hr, and then mitochondrial membrane potential was estimated as described in the Material and Methods section. Values are presented as means ± S.E.M. of six separate experiments carried out in triplicate and expressed as fold change over the pretreatment level (experimental/control). $^{*}P < 0.05$ compared to control values. $^{\#}P < 0.05$ compared to melatonin group.

on apoptosis in AR42J cells, translocation of PS was evaluated using annexin V and propidium iodide (PI) double staining. When double-stained pancreatic AR42J cells were analyzed by flow cytometry, the results revealed that single-agent exposure to 1 mM melatonin, 1 mM 5-fluorouracil, 20 μM cisplatin, or 1 μM doxorubicin for 12 and 24 hr induced a significant increase in the percentage of apoptotic cells (Fig. 3A and 3B) compared to control cells. In addition, when AR42J cells were treated with each of the chemotherapeutic drugs in combination with melatonin, there was a further rise in the population of apoptotic cells (Fig. 3A, B), which was statistically significant when compared with the values of each individual treatment, suggesting that melatonin augments chemotherapy-induced apoptosis.

Basically, similar results were obtained when measuring mitochondrial membrane potential. Treatment of cells for 12 and 24 hr with 1 mM melatonin or the chemotherapeutic agents administered separately induced a mitochondrial membrane depolarization as detected by the decrease in the mitochondrial-specific, voltage-sensitive dye JC-1 fluorescence ratio (Fig. 4). Treatment of cells with 1 mM 5-fluorouracil exerted a negligible effect on mitochondrial membrane potential (Fig. 4A). Additionally, combined treatment of each chemotherapeutic drug with 1 mM melatonin increased the mitochondrial depolarization compared to the single treatments (Fig. 4), except when cells were incubated for 24 hr with 20 μM cisplatin or 1 μM doxorubicin in the presence of melatonin (Fig. 4B, C).

Surprising recent findings indicate that melatonin is able to produce intracellular ROS in tumor cells, as detected by increases in fluorescence of oxidation-sensitive intracellular probes [37, 38]. To determine whether the potentiating effect of melatonin on the chemotherapeutic drugs is because of a synergistic effect on the production of intracellular ROS, DHR-123-loaded AR42J cells were treated with melatonin and the three chemotherapeutic agents, separately or combined, for 12 and 24 hr. As shown in Fig. 5, treatment of cells with 1 mM 5-fluorouracil, 20 μM cisplatin, or 1 μM doxorubicin caused a detectable intracellular ROS generation as revealed by the increase in Rh-123 fluorescence. In AR42J cells, we found no significant effect of melatonin by itself on ROS production at the dose of 1 mM for 12 and 24 hr. In contrast, combined treatment of melatonin with each chemotherapeutic agent further increased intracellular ROS generation compared to each chemotherapy treatment alone (Fig. 5). These results imply that melatonin could enhance the intracellular ROS production induced by chemotherapeutic drugs, at least, in pancreatic AR42J cells.

Discussion

The results presented in this paper demonstrate a potentiating effect of melatonin on the anticancer activity against

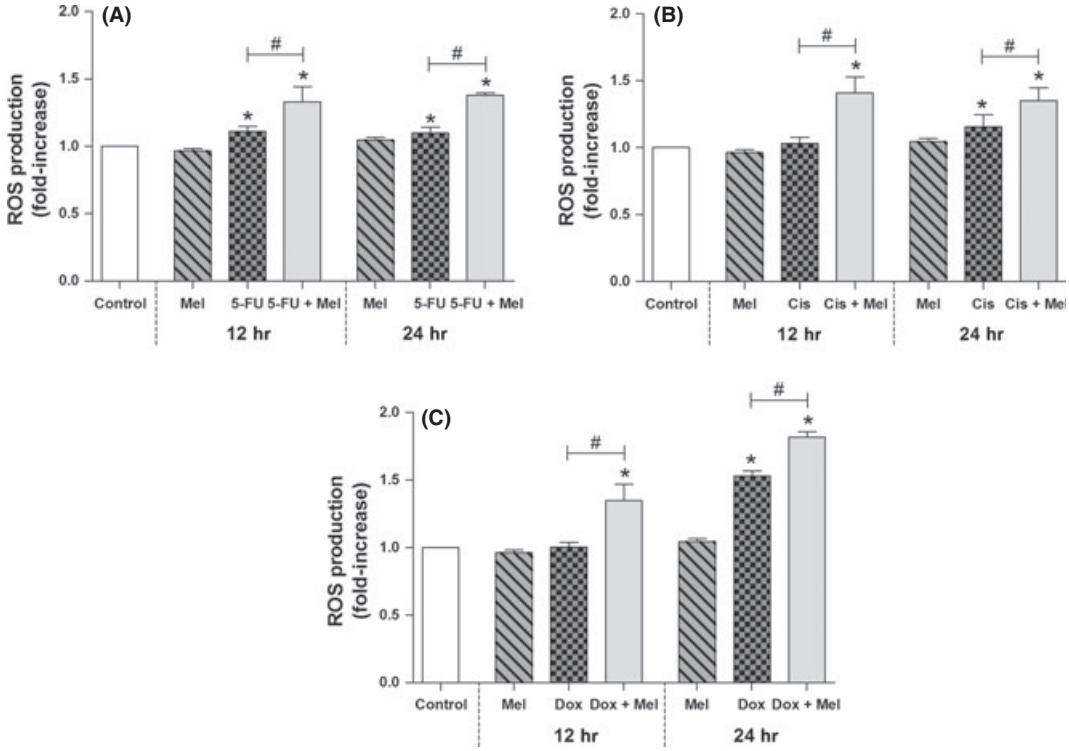

Fig. 5. Combination of melatonin with chemotherapy drugs potentiates intracellular reactive oxygen species (ROS) production in pancreatic AR42J cells. DHR-123-loaded cells were treated with 1 mM melatonin (Mel) (A, B, C), 1 mM 5-fluorouracil (5-FU) (A), 20 μM cisplatin (Cis) (B), or 1 μM doxorubicin (Dox) (C) separately or combined, or the vehicle (control) for 12 and 24 hr, and then intracellular ROS production was assessed as described in the Material and Methods section. Values are presented as means ± S.E.M. of six separate experiments carried out in triplicate and expressed as fold increase over the pretreatment level (experimental/control). $^{*}P < 0.05$ compared to control values. $^{\#}P < 0.05$ compared to melatonin group.

rat pancreatic tumor AR42J cells showed by 5-fluorouracil, cisplatin, and doxorubicin. Such an effect is mainly because of the potentiation of chemotherapeutic-induced apoptosis. These results suggest that chemotherapy combined with melatonin may increase the therapeutic effect of anticancer drugs.

AR42J cells are derived from azaserine-induced malignant nodules from the rat pancreas and represent a cancerous version of the pancreatic epithelium [39]. Melatonin is known to possess antitumor properties in diverse types of cancer, including pancreatic, liver, and prostate cancer cells [27, 30, 31, 40]. Synergistic effect of melatonin on anticancer activity of the three chemotherapeutic agents used in this work is because of an elevated cell death induction, which was time dependent in all cases. Combined treatment of each chemotherapeutic drug with melatonin for 12 and 24 hr markedly increases the cytotoxicity in AR42J cells, compared with the values of treatments alone. Similar results have been previously reported in several cancer cell lines, including human Ewing sarcoma cancer cells [31], ovarian cancer cells [32], human hepatoma cell lines [30], human leukemia HL-60 cells [29], mouse hepatoma cell line HEPA 1-6 [41], and lung and laryngeal cancer cell lines [42]. Additionally, melatonin has been reported to intensify the cytotoxicity of doxorubicin in normal nontumor cells, as human keratinocytes [42], while in human peripheral blood mononuclear cells [43] or OSEN human normal ovarian epithelial cells [32], melatonin showed protective effects against cisplatin-induced cytotoxicity. In contrast, there is also one report indicating that melatonin does not interfere with the cytotoxic effect of cytarabine, daunorubicin, and etoposide in leukemia cell lines, such as Jurkat, MOLT-4, Daudi, HL-60, CMK, and K562 cells [28].

Annexin V assays showed that the potentiating effect of melatonin and chemotherapy on cytotoxic activity correlated with the induction of apoptosis in rat pancreatic carcinoma AR42J cells. Apoptosis or programmed cell death is an essential physiological process that plays a critical role in development and tissue homeostasis. However, apoptosis is also involved in a wide range of pathological conditions. Apoptotic defects are a common event in oncogenesis and contribute to drug resistance [44]. Various chemotherapeutic drugs are known to trigger intrinsic apoptotic pathway and/or increase the susceptibility of mitochondria to apoptotic signals [17, 18, 45]. Similarly, melatonin has been previously shown to induce apoptosis in various cancer cells [6, 23–27]. Our data revealed that treatment with 5-fluorouracil, cisplatin, doxorubicin, as well as melatonin significantly increased the number of apoptotic AR42J cells and induced mitochondrial membrane depolarization, except for the treatment with 5-fluorouracil that exerted a negligible effect on mitochondrial membrane potential. Importantly, treatments for 12 and 24 hr with each chemotherapeutic agent in combination with melatonin further augmented chemo-

therapy-induced apoptosis as detected by the increase in the number of annexin V-positive cells. Furthermore, combined treatment of chemotherapeutic drugs with melatonin increased the mitochondrial membrane depolarization compared to the single treatments, except for AR42J cells incubated for 24 hr with cisplatin or doxorubicin in the presence of melatonin.

An increase in intracellular ROS production is one of the mechanisms through which the intrinsic pathway of apoptosis may be triggered, and it is usually followed by a loss of mitochondrial membrane potential [19]. In this sense, our group and others have found that melatonin is able to increase intracellular ROS production in tumor leukocytes, as detected by increases in fluorescence of oxidation-sensitive intracellular probes [37, 38]. Here, we have found a significant increase in intracellular ROS generation after 24 hr of treatment with 5-fluorouracil, cisplatin, and doxorubicin, but not with melatonin alone. However, when combining each of the three chemotherapeutic agents with melatonin, we found a significant and marked rise in intracellular ROS production in all cases. Taken together, our findings suggest that the mitochondria-mediated intrinsic apoptosis pathway is involved in the potentiating effects of melatonin on the antitumoral action exerted by different chemotherapies against rat pancreatic tumor AR42J cells.

In summary, our data demonstrate that in vitro melatonin promotes cytotoxicity and apoptosis via activation of intrinsic apoptosis pathway in chemotherapy-treated rat pancreatic AR42J cells. Therefore, melatonin could be potentially useful as a combination chemotherapeutic agent to improve the therapeutic effect of chemotherapy on pancreatic carcinoma, which is consistent with previous studies that show that melatonin improves the efficiency and reduces the toxicity of standard anticancer chemotherapies [14–16, 35, 46]. Nonetheless, in vivo animal models and pharmacological doses of melatonin should be used in further studies to corroborate the findings herein.

Acknowledgements

This work was supported by MICINN-FEDER grant BFU2010-15049. J. Espino is beneficiary of a grant by Ministerio de Educación (AP2009-0753). A.C. Uguz was supported by an academic fellowship from The Council of Higher Education of Turkey. The authors thank Servicio de Apoyo a la Investigación de la UEX (SAIUEX) for its excellent technical assistance.

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