

Effects of melatonin on HIF-1 α and VEGF expression and on the invasive properties of hepatocarcinoma cells

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Abstract. Liver cancer is the sixth most commonly occurring cancer globally, and the main histological type is hepatocellular carcinoma. This type of neoplasia has a poor prognosis due to a high rate of recurrence and intrahepatic metastasis, which are closely associated with the angiogenic process. Vascular endothelial growth factor (VEGF), which is under the control of hypoxia inducible factor-1 α (HIF-1 α), stimulates the proliferation of endothelial cells and increases cell permeability, promoting the growth, spread and metastasis of tumors. Melatonin, the main hormone secreted by the pineal gland, may have a significant role in tumor suppression and has demonstrated antiangiogenic and antimetastatic effects. The aim of the present study was to analyze the cell viability, migration and invasion, as well as the expression of proangiogenic proteins VEGF and HIF-1 α , in HepG2 hepatocarcinoma cells, following treatment with melatonin. Cells were cultured and cell viability was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The expression of proangiogenic proteins VEGF and HIF-1 α , under conditions of normoxia and hypoxia, was verified using immunocytochemistry and quantified by densitometry. The analysis of the processes of cell migration and invasion was

performed in a Boyden chamber. The MTT assay revealed a reduction in cell viability (P=0.018) following treatment with 1 mM melatonin for 24 h. The expression of proangiogenic proteins VEGF and HIF-1 α was reduced in cells treated with 1 mM melatonin for 24 h in normoxic (P<0.001) and hypoxic (P<0.001) conditions, compared with the control group and with induced hypoxia alone. The rate of cell migration and invasion was additionally reduced in cells treated with 1 mM melatonin for 48 h when compared with the control group (P=0.496). The results of the present study suggest that melatonin may have an antiproliferative, antiangiogenic and antimetastatic role in hepatocarcinoma cells and may present a novel therapeutic option for the treatment of liver cancer.

Introduction

Hepatocellular carcinoma (HCC) is the most commonly observed type of liver cancer, accounting for 80% of cases (1). Regarding incidence, liver cancer is the sixth most common cancer and the third most common cause of cancer-associated mortality (1). The choices for treatment, particularly for advanced HCC, are limited and the prognosis is poor (2). This is due to the fact that the tumors are typically diagnosed at a late stage (2).

HCC tumors are characterized by neovascularization and a high propensity for venous invasion (3). Increased neovascularization is a major clinicopathological feature of hepatocellular carcinoma, and tumor microvessel density is closely associated with disease progression and patient prognosis (3). Vascular endothelial growth factor (VEGF) is one of the most potent proangiogenic agents, which specifically stimulates the proliferation of endothelial cells and increases cell permeability (4). This factor is involved in angiogenesis in multiple tumor types, promoting growth, spread and tumor metastasis (5). Previous studies have correlated increased VEGF expression with poor prognosis in HCC (6,7).

The expression of VEGF in tumor cells is under the control of hypoxia inducible factor-1 α (HIF-1 α), a transcription factor that is induced in hypoxic conditions, which consists of two subunits: HIF-1 α and HIF-1 β (8). Increased expression of

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Abbreviations: AU, arbitrary units; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; HIF-1 α , hypoxia-inducible factor 1 α ; MOD, mean optical density; VEGF, vascular endothelial growth factor

Key words: liver cancer, HepG2, vascular endothelial growth factor, hypoxia-inducible factor 1 α , invasion assay, melatonin

HIF-1 α and/or VEGF results in activation of angiogenesis, which is associated with malignant progression and increased invasive and metastatic potential of tumor cells (9-11), representing an adverse prognostic factor in the treatment of cancer (10,12,13).

Tumor recurrence in hepatocarcinoma may occur as metastasis and >90% of HCC-associated deaths are the result of secondary local or distant disease (14). Metastasis and invasion are strongly dependent on the ability of tumor cells to migrate (15). In general, the earliest occurrence of metastasis of primary liver cancer is the invasion of the hepatic portal vein, the branches of which then form a tumor thrombus, which facilitates intrahepatic metastatic proliferation (16). Extrahepatic metastatic sites include lung (55%), lymph nodes (53%), bone (28%), adrenal glands (11%), peritoneum and/or omentum (11%) and brain (2%) (17).

As hepatocarcinoma presents a high rate of mortality and morbidity, there is an increasing interest in the identification of novel therapeutic agents which are able to interact with specific molecular markers, primarily associated with angiogenesis and invasion, and leading to inhibition of tumor progression (16).

Melatonin, a hormone naturally produced and secreted in the pineal gland, has been demonstrated to be effective in tumor inhibition in *in vitro* and *in vivo* studies (18,19). This hormone exerts oncostatic activity through a variety of mechanisms, including antiproliferative actions, anticancer immunity stimulation, modulation of expression of oncogenes, antiinflammatory, antioxidant, antiangiogenic and antimetastatic effects (20).

In vitro studies have reported that melatonin is able to inhibit the expression of HIF-1 α in prostate cancer cells (21) and glioblastoma (22), as well as VEGF expression in breast (23) and pancreatic cancer cells (5), as well as glioblastoma (22). Carbajo-Pescador *et al.* (24) reported that melatonin exerted antiangiogenic activity in HepG2 cells by interfering with the transcriptional activation of the VEGF gene by HIF-1 α and signal transducer and activator of transcription 3.

Experimental results have additionally indicated that melatonin may be capable of reducing migration and invasiveness in glioblastoma (22,25), lung (26) and breast cancer cells (27-29). However, much remains to be elucidated regarding the antimetastatic properties of melatonin in HCC cells.

As angiogenesis and metastasis are two fundamental processes in tumor progression and are associated with patient prognosis and survival, the present study aimed to evaluate the expression of the proangiogenic proteins HIF-1 α and VEGF and the invasion ability, a parameter of metastatic potential, in a hepatocarcinoma cell line following treatment with melatonin.

Materials and methods

Cell culture. In the present study the HepG2 cell line was used, which was kindly provided by Dr Bruno Cogliati from the Department of Pathology, College of Veterinary Medicine, University of São Paulo (São Paulo, Brazil) and were originally purchased from the American Type Culture Collection (Manassas, VA, USA). The HepG2 cell line is derived from the liver tissue of a 15-year-old Caucasian male with differentiated hepatocellular carcinoma.

Cells were maintained at 37°C in an atmosphere of 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; Cultilab Materiais Cultura Células, São Paulo, Brazil), supplemented with 10% fetal bovine serum (FBS; Cultilab Materiais Cultura Células), 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cells were passaged every 7 days.

Test of cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The individual wells of a 96-well plate (Sarstedt, Nümbrecht, Germany) were inoculated with 100 μ l DMEM containing 5x10⁴ cells. Cells were incubated in DMEM with various concentrations of melatonin (Sigma-Aldrich; 1 and 100 nM, 10 μ M and 1 mM) for 24 h. Melatonin was diluted in 50 μ l ethanol (0.05%; Dinâmica, São Paulo, Brazil). In control cells, the equivalent amount of ethanol was added as vehicle. Following 24 h of treatment with melatonin, the culture medium was discarded and 100 μ l fresh DMEM was added to each well. Subsequently, 10 μ l of MTT solution at 12 mM (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was added to each well and the plate was incubated at 37°C for an additional 4 h. To solubilize the formazan crystals, the cells were incubated with dimethyl sulfoxide (Sigma-Aldrich).

The plate was incubated at 37°C for 10 min and the samples were homogenized prior to assessment of the absorbance using an ELISA reader (Multiskan™ FC Microplate Photometer; Thermo Fisher Scientific, Inc.) at a wavelength of 540 nm. DMEM was used as background and its absorbance value was subtracted from that of the samples to avoid interference in the results. Cell viability (%) was calculated for all groups compared to the control sample. All experiments were performed in triplicate.

Treatment of cells in vitro. Cells were divided into the following groups: Control cells (untreated), hypoxic cells (treated with 100 μ M CoCl₂), hypoxic cells treated with 1 mM melatonin and normal cells treated with 1 mM melatonin for 24 h (30). These treatment conditions were used for immunocytochemistry assay.

Immunocytochemistry. For immunocytochemistry techniques, 0.5x10⁶ cells in culture were transferred to a slide with coupled flask (Thermo Fisher Scientific, Inc.) and incubated at 37°C for 24 h. Following this period, the culture medium was removed and the cells were washed with phosphate-buffered saline (PBS). To fix the cells, the flask was incubated with 1 ml of 4% formaldehyde (Sigma-Aldrich) for 20 min.

Subsequently, the flasks were uncoupled from the slides and incubated with 10% hydrogen peroxide for 30 min to block endogenous peroxidase activity. Antigen retrieval was performed in a pressure cooker (ARNO, São Paulo, SP, Brazil) at 95°C with citrate buffer (pH 6; Dinâmica) for 30 min. Following cooling, the slides were immersed in a solution of bovine serum albumin and incubated for 4°C overnight with primary antibodies for HIF-1 α (mouse monoclonal anti-human; dilution, 1:50, catalog no., sc-53546; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and VEGF (rabbit polyclonal anti-human; dilution, 1:500, catalog no., sc-152; Santa Cruz Biotechnology, Inc.). Subsequently, the samples were washed with PBS for 15 min and incubated with the

Starr Trek Universal HRP Detection System (catalog no., STUHRP700 H, L10; Biocare Medical, Inc., Concord, CA, USA), which consisted of biotinylated secondary antibody (anti-mouse, anti-rabbit and anti-goat immunoglobulins) that was incubated with the samples for 20 min at room temperature, and streptavidin-peroxidase complex that was incubated at room temperature with the samples for 10 min, followed by washing with PBS for 15 min. Subsequently, diaminobenzidine-tetrahydrochloride-dihydrate (Covance Laboratories, Dedham, MA, USA) was applied as a chromogen for 5 min at 22°C. The slides were counterstained with Harris's hematoxylin (CARLO ERBA Reagents, Milan, Italy) for 40 sec.

Evaluation of immunocytochemical staining by optical densitometry. Various fields were examined on each slide, particularly areas delimited with brown staining. The slides were photographed, and protein expression was quantified using Image J software (imagej.nih.gov/ij/) at magnification, x40 on a Nikon Eclipse E200 microscope (Nikon Corp., Tokyo, Japan). For each slide, 3 images were captured (Eurekam; Bel Photonics, Piracicaba, Brazil). A total of 20 regions of interest were randomly selected in each image. The intensity was determined from a total of 60 regions of interest marked on each slide. The values were obtained as arbitrary units (AU), and the mean optical density (MOD) revealed the intensity of staining in specifically immunoreactive areas (31).

Invasion assay. The invasiveness of HepG2 cells was assessed using Transwell chambers with an 8- μ m pore size membrane (Corning Inc., Corning, NY, USA). In the upper compartment of the chamber, $\sim 2.5 \times 10^4$ cells/insert were added into DMEM without serum, while to the lower compartment 750 μ l of DMEM (with 5 and 10% FBS for negative and positive controls, respectively) was added with 1 mM melatonin.

The cells were incubated at 37°C in an atmosphere of 5% CO₂ for 48 h. Following incubation, the Transwell membranes were washed and impermeabilized. The invading cells were stained with hematoxylin and the non-migrating cells were removed from the upper surface of the Transwell membrane using a cotton swab. Cell counting was performed under an inverted optic microscope by placing the cell invasion assay insert over a plate containing 50% glycerol (Sigma-Aldrich).

The invasiveness of HepG2 cells was assessed in 24-well plates with 8- μ m pore size Matrigel-coated membranes (BD Biosciences, Billerica, MA, USA). Cell invasion (%) of each group was calculated from the mean values of cells that migrated and invaded the Matrigel membrane in duplicate. The positive control group was assumed to represent 100%. The result was calculated from the difference compared to the positive control group.

Statistical analysis. The results were submitted to analysis of normal distribution using column statistics and the Gaussian distribution test. The means of optical densitometric analysis associated with the expression of VEGF and HIF-1 α proteins were compared in various groups, using analysis of variance, followed by the Bonferroni correction. Differences between the groups in the invasion assay were evaluated by Student's *t*-test.

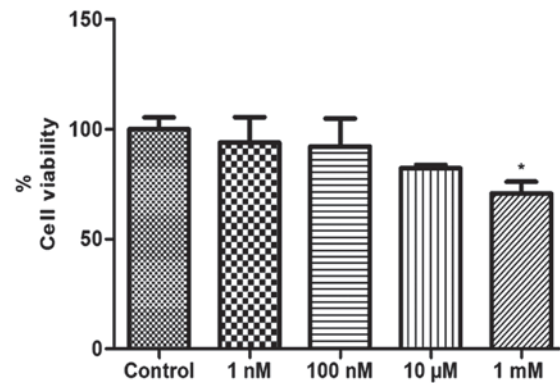


Figure 1. Analysis of cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in liver cancer cells following 24 h of treatment with melatonin (1 nM, 100 nM, 10 μ M and 1 mM). Data are expressed as a percentage of the control group, and presented as the mean \pm standard deviation. *P<0.05 compared with the control.

All values are expressed as the mean \pm standard deviation. P<0.05 was considered to indicate a statistically significant difference. All analyses were performed using GraphPad Prism 4 software version 5.01 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Test of cell viability by MTT assay. The number of viable cells was evaluated by MTT assay following treatment with melatonin (1 and 100 nM, 10 μ M and 1 mM) for 24 h. The results demonstrated that treatment with concentrations of 1 nM, 100 nM and 10 μ M melatonin did not alter the viability of the HepG2 cells following 24 h of treatment (P=0.6550; P=0.5977; and P=0.0837, respectively; Fig. 1). Only 1 mM melatonin, the pharmacological concentration, significantly decreased the viability of HepG2 cells relative to the control cells (P=0.0180; Fig. 1). In the literature, concentrations above 1 μ M are described as pharmacological, and physiological concentrations are considered those below 1 nM. In general, when used for therapeutic purposes, endogenous substances are administered at concentrations much higher than their physiological secretion, allowing their clinical use (32,33).

Immunocytochemistry. Immunocytochemistry followed by quantification of immunostaining by optical densitometry was used to detect VEGF and HIF-1 α protein expression. The results were obtained in AU and demonstrated the value of the MOD \pm standard error.

The HepG2 cells demonstrated an increase in immunostaining of HIF-1 α in cells with induced hypoxia, when compared with the control group (P<0.001). In cells with induced hypoxia treated with melatonin and cells treated only with melatonin, there was a decrease in immunostaining of HIF-1 α when compared with the control group (P<0.001) and group with induced hypoxia alone (P<0.001; Fig. 2A-E).

For VEGF, there was a statistically significant increase in immunostaining in the group with induced hypoxia when compared with the control group (P=0.0014). As observed for HIF-1 α expression, there was a statistically significant reduction in the immunostaining of VEGF in cells with induced

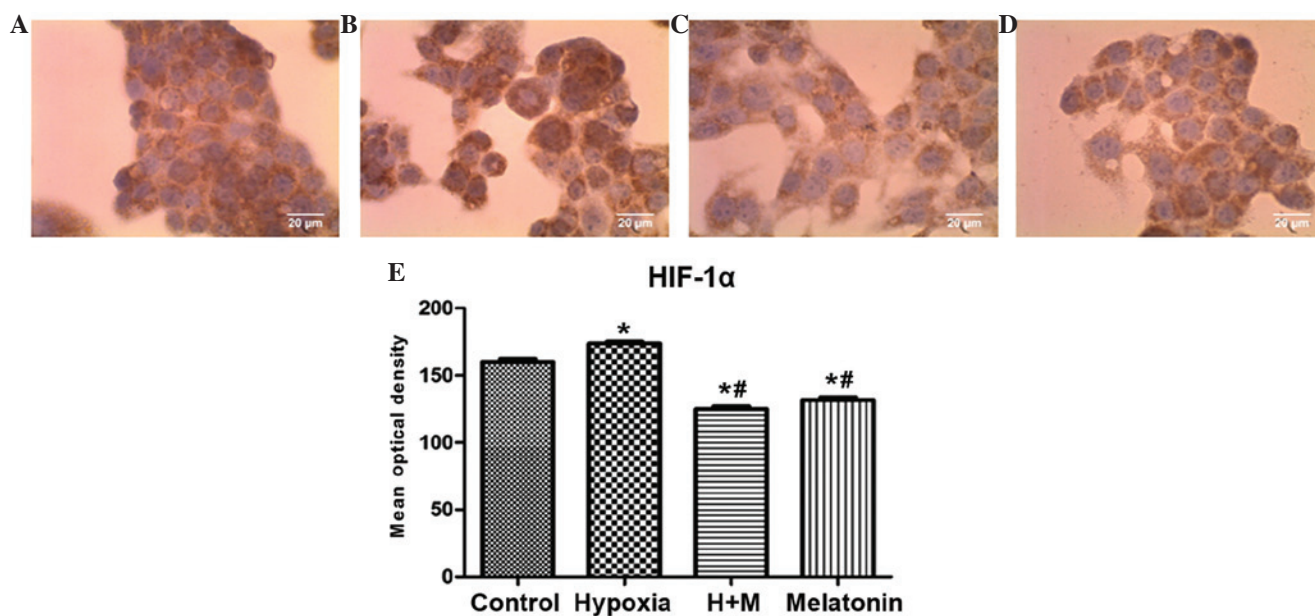


Figure 2. Immunocytochemistry of the protein HIF-1 α , showing HepG2 cells (magnification, x40) following 24 h of treatment with CoCl₂ (100 μ M) for induction of hypoxia and/or melatonin (1 mM). (A) Untreated cells (control group). (B) Cells with induced hypoxia. (C) Cells with induced hypoxia treated with melatonin. (D) Cells treated with melatonin alone. (E) Statistical analysis of protein expression of HIF-1 α in the experimental groups. Data are presented as the mean optical density \pm standard error, and are shown in arbitrary units. *P<0.05 compared with the control group. #P<0.05 compared with cells treated with CoCl₂ alone. H + M, hypoxia and melatonin; M, melatonin; HIF-1 α , hypoxia-inducible factor 1 α .

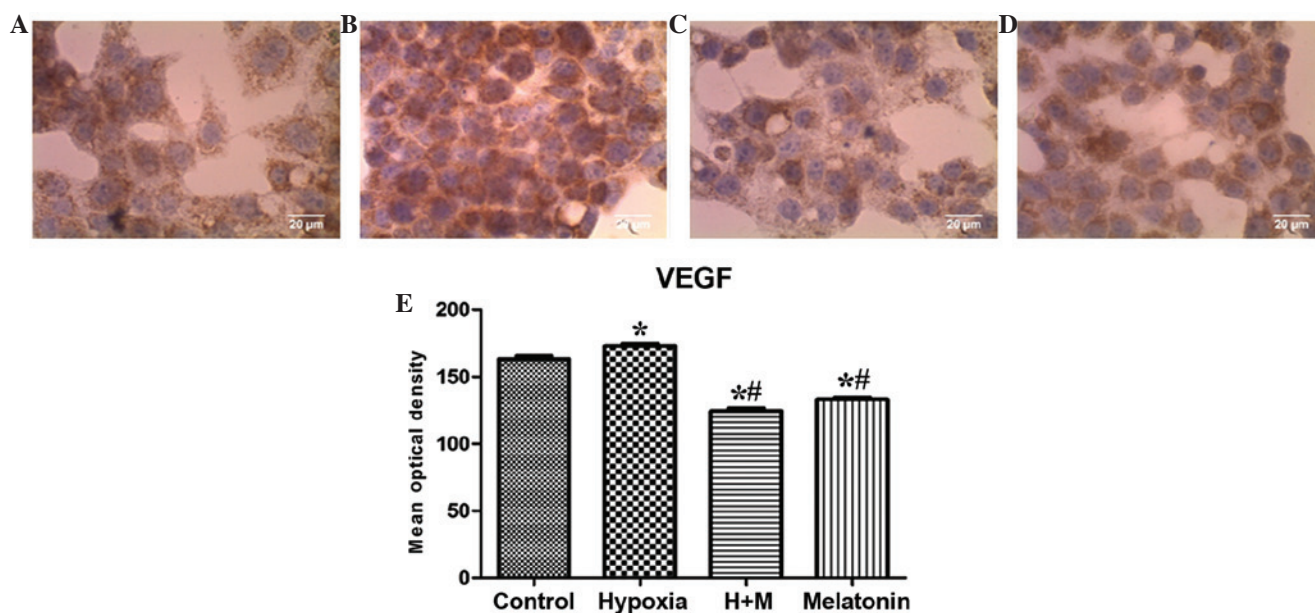


Figure 3. Immunocytochemistry of the protein VEGF, showing HepG2 cells (magnification, x40) following 24 h of treatment with CoCl₂ (100 μ M) for induction of hypoxia and/or melatonin (1 mM). (A) Untreated cells (control group). (B) Cells with induced hypoxia. (C) Cells with induced hypoxia treated with melatonin. (D) Cells treated with melatonin alone. (E) Statistical analysis of protein expression of VEGF in the experimental groups. Data are presented as the mean optical density \pm standard error, and are shown in arbitrary units. *P<0.05 compared with the control group. #P<0.05 compared with cells treated with CoCl₂ alone. H+M, hypoxia and melatonin; M, melatonin; VEGF, vascular endothelial growth factor.

hypoxia treated with melatonin and in the cells treated only with melatonin when compared with the control group (P<0.001) and group with induced hypoxia alone (P<0.001; Fig. 3A-E).

Migration and invasion assay. A Transwell chamber assay was performed to investigate the invasion ability of HepG2 cells treated with 1 mM melatonin for 48 h. Treatment was able to

reduce the migration and invasion capability of HepG2 cells by 59.5% when compared with the positive control (P=0.0496; Fig. 4A-B).

Discussion

The main aim of the present study was to evaluate the effects of melatonin treatment on the expression of proteins associated

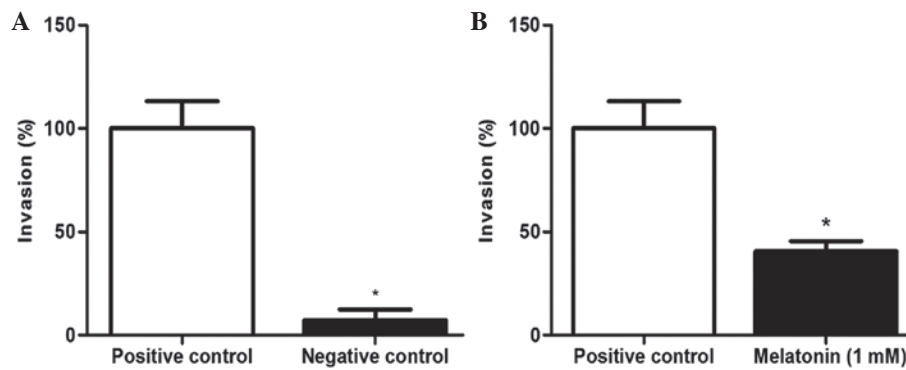


Figure 4. Migration and invasion rate of the HepG2 cell line following 48 h of treatment with 1 mM melatonin. (A) Comparison between positive and negative control. (B) Comparison between the control with 10% fetal bovine serum without treatment and cells treated with 1 mM melatonin. Data are presented as the mean \pm standard deviation. * $P < 0.05$.

with angiogenesis and on the invasion ability in hepatocellular carcinoma cells, as these two processes are associated with disease progression and patient prognosis.

In the present study, melatonin decreased viability in the HepG2 cell line. The inhibition of cell proliferation following treatment with melatonin has additionally been observed in other studies, using hepatocarcinoma (34), breast cancer (35) and gastric adenocarcinoma (36) cells. However, the exact mechanism via which melatonin inhibits cell growth in *in vitro* and *in vivo* models remains to be fully elucidated, although a number of mechanisms have been proposed. These include induction of apoptosis, changes in the lipid metabolism and an increase in the activity of natural killer cells, as well as stimulation of production of cytokines, including interleukin (IL)-2, IL-6, IL-12 and interferon (37).

Previous studies have additionally demonstrated the antiangiogenic action of melatonin, through decreasing the expression of HIF-1 α in colon (13) and liver cancer cells (24), and glioblastoma (22), as well as decreasing the expression of VEGF in breast (23), pancreatic (5) and liver cancer cells (24).

The results of the present study demonstrate that melatonin treatment in conditions of normoxia and hypoxia inhibited the expression of proangiogenic proteins HIF-1 α and VEGF when compared with the control group and with the group with induced hypoxia alone. Carbajo-Pescador *et al* (24) and Park *et al* (13) observed similar results in hepatocellular carcinoma and colon cancer, respectively.

According to Park *et al* (13), the effects of melatonin treatment on the expression of HIF-1 α and VEGF are primarily observed in hypoxic conditions. This is potentially due to the fact that cell normoxia leads to ubiquitination of HIF-1 α and subsequent proteasomal degradation (30). Nevertheless, under hypoxic conditions, HIF-1 α is stabilized and is able to translocate to the nucleus and induce the expression of multiple genes, including VEGF (30). In addition, although VEGF expression may be directed by growth factors and ILs, it appears that hypoxia, and consequently the expression of HIF-1 α , are the principal regulators of VEGF production (38).

However, the present study additionally observed decreased expression of VEGF and HIF-1 α protein in cells treated with melatonin under normoxic conditions when compared with the control. In the work of Carbajo-Pescador *et al* (24) the expression of VEGF was decreased in normoxic HepG2 cells treated

with melatonin compared with the control. However, the expression of HIF-1 α increased in cells treated with melatonin in normoxic conditions (24).

According to Dai *et al* (30) the effects of the pharmacological concentration of melatonin on VEGF and HIF-1 α expression in normoxic conditions is unclear, which indicates that the regulatory mechanisms of HIF-1 α and VEGF in the absence or presence of CoCl₂ are different, and additional transcription factors may be involved.

The decrease in the expression of HIF-1 α and VEGF proteins in HepG2 cells following melatonin treatment in the present study is of particular interest as increased expression of HIF-1 α (39) and VEGF (7,39) correlates with unfavorable prognostic features, including large tumor size and reduced survival in patients with hepatocellular carcinoma.

The regulation of cell invasion is crucial for maintaining cellular homeostasis, and the loss of this regulation is a basic characteristic of cancer cells. The ability of tumor cells to migrate is an important prerequisite for tumor dissemination and metastasis (15,39). A number of studies have additionally indicated that melatonin may have antimetastatic properties and may be able to reduce migration and invasiveness in certain types of cancer, including glioblastoma (22,25), lung (26) and breast cancer cells (27-29). According to Ortíz-López *et al* (28) melatonin inhibits the migration process and cell invasion in breast cancer cells via the rho-associated-1 protein, by modulating the dynamic cytoskeleton. In addition, Mao *et al* (29) reported that melatonin exerts an inhibitory effect on breast cancer cell invasion via downregulation of the p38 signaling pathway, and inhibition of matrix metalloproteinase (MMP)-2 and MMP-9 expression and activity.

The results of the present study indicate that treatment with 1 mM melatonin may prevent invasiveness in HepG2 cells and this may be associated with the antimetastatic properties of melatonin. However, the mechanism by which melatonin inhibits the invasiveness of HepG2 cells must be further investigated, as few studies have been performed. According to Ordoñez *et al* (40) melatonin reduces the invasiveness of the HepG2 hepatocarcinoma cell line *in vitro* by a molecular mechanism that involves increased expression of TIMP metalloproteinase inhibitor 1 (TIMP-1) and decreased expression and activity of MMP-9, via inhibition of nuclear factor κ -B. Additionally, in a study performed by Mishra *et al* (41), it was

observed that melatonin treatment exerted a hepatoprotective role by downregulation of MMP-9 and upregulation of tissue inhibitor of TIMP-1 expression in liver tissue.

In conclusion, the present study demonstrates that treatment with the pharmacological concentration of melatonin is capable of inhibiting the expression of proangiogenic proteins HIF-1 α and VEGF and invasiveness *in vitro* in HepG2 cells. *In vivo* studies are required to support the potential application of melatonin in the treatment of liver cancer.

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