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ORIGINAL ARTICLE

# Melatonin induces reactive oxygen species generation and changes in glutathione levels and reduces viability in human pancreatic stellate cells

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## Abstract

In this study, the effects of pharmacological concentrations of melatonin (1 μM–1 mM) on human pancreatic stellate cells (HPSCs) have been examined. Cell type–specific markers and expression of melatonin receptors were analyzed by western blot analysis. Changes in intracellular free $Ca^{2+}$ concentration were followed by fluorimetric analysis of fura-2–loaded cells. Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were determined by fluorescence techniques. Production of reactive oxygen species (ROS) was monitored following 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester and MitoSOX™ Red–derived fluorescence. Cell viability was studied using the AlamarBlue® test. Cultured cells expressed markers typical of stellate cells. However, cell membrane receptors for melatonin could not be detected. Thapsigargin, bradykinin, or melatonin induced changes in intracellular free $Ca^{2+}$ concentration. In the presence of the indole, a decrease in the GSH/GSSG ratio was observed that depended on the concentration of melatonin used. Furthermore, the indole evoked a concentration-dependent increase in ROS production in the mitochondria and in the cytosol. Finally, melatonin decreased HPSC viability in a time and concentration-dependent manner. We conclude that melatonin, at pharmacological concentrations, induces changes in the oxidative state of HPSC. This might regulate cellular viability and could not involve specific plasma membrane receptors.

**Keywords** Melatonin · Calcium · Glutathione · Cell viability · Human pancreatic stellate cells

## Abbreviations

| | |
|---|---|
| $[Ca^{2+}]_c$ | Intracellular free $Ca^{2+}$ concentration |
| CM-$H_2$DCFDA | 5-(and-6)-Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester |
| ER | Endoplasmic reticulum |
| Fura-2-AM | Fura-2-acetoxymethyl ester |
| GSH | Reduced glutathione |
| GSSG | Oxidized glutathione |
| HBSS | Hank's balanced salts |
| HPSCs | Human pancreatic stellate cells |
| $H_2O_2$ | Hydrogen peroxide |
| ROS | Reactive oxygen species |
| RPSCs | Rat pancreatic stellate cells |
| SERCA | Sarcoendoplasmic reticulum $Ca^{2+}$-ATPase |
| Tps | Thapsigargin |
| α-sma | Alpha-smooth muscle actin |

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## Introduction

In the recent years, the interest on the role of pancreatic stellate cells (PSCs) in the physiology and the pathophysiology of the pancreas has increased. Under normal conditions, PSCs remain in a quiescent state but become activated in disease. It has been highlighted that activated PSCs are responsible for the progressive fibrosis and for the accumulation of extracellular matrix that occurs in chronic pancreatitis and in pancreatic cancer [21].

PSCs do not show $Ca^{2+}$ signals in response to the physiological regulators of pancreatic acinar cells such as acetylcholine or cholecystokinin. However, PSCs are capable of signaling to neighbor cells and to bias their physiology [14]. In this line, tumor progression and chemoresistance are enhanced probably due to participation of activated PSCs, being major contributing factors to the release of signaling molecules and the stromal or fibrotic reaction [35]. Therefore, an interesting approach for the treatment of pancreatic illness is to gain understanding of the mechanisms that are involved in the activation and in the regulation of the growth and proliferation of PSCs, in addition to those involved in signalization towards other cell types present in the pancreas.

The interest on the role of melatonin in the regulation of cellular physiology is widespread. Many cellular types express membrane-bound melatonin receptors, which include melatonin type 1 (MT1) and melatonin type 2 (MT2) receptors. Their expression differs among various organs [32]. The presence of MT1 and MT2 receptors in the pancreas has been reported [11]. Additionally, melatonin can bind to cytosolic and to nuclear orphan receptors from the RORα/RZR family [32].

Existing evidences support a major role of melatonin in the regulation pancreatic physiology. The indole regulates enzyme and bicarbonate secretion [17, 30]. Additionally, melatonin exerts antioxidant and anti-inflammatory effects on the pancreas [16, 22]. Interestingly, melatonin induces antitumor effects in the gland [11, 18] and modulates proliferation of rat PSC (RPSCs) [29].

Employment of cultures of PSCs is useful to analyze the effects of different drugs on these cells, mainly to study the processes responsible for cell proliferation. In this regard, application of drugs with anti-inflammatory, antifibrotic, and/or antiproliferative properties could be useful in therapy. Most observations suggest that melatonin exerts protective effects in the healthy pancreas, whereas it induces death of abnormal or transformed cells. Finding out whether the observations obtained in animal cell studies also occur in human cells is of major interest in medicine. However, studies on human tissues are failing due to, specially, the difficulties in their procurement.

Thus, in the present study, we sought to investigate whether melatonin could exert any effect on human PSCs (HPSCs). The interest was based on our former findings which showed that the viability of RPSCs decreased upon treatment with the indoleamine, and that this effect might not involve membrane-bound receptors, which were not detected [29]. In line with our research, we tried to shed more light on the mechanisms involved in the actions of melatonin on the human pancreas and its putative role in the prevention and/or treatment of disease.

## Materials and methods

### Pancreatic tissues and chemicals

Human pancreatic tissues, obtained from three patients, were used in the present study. Samples were provided by Dr. Gerardo Blanco's group (Hepatobiliary-Pancreatic Surgery and Liver Transplant Unit, Infanta Cristina Hospital, Badajoz, Spain), in accordance with the institutional Bioethical Committee. The studies were approved by the Ethics Committee of Clinical Research of Infanta Cristina Hospital and by the Bioethics and Biosecurity Committee of the University of Extremadura. Animals used in the study were obtained from the animal house of the University of Extremadura (Caceres, Spain). Studies were approved by the Bioethics and Biosecurity Committee of the University of Extremadura. Animals were humanely handled and sacrificed in accordance with the institutional bioethical committee. Collagenase CLSPA was obtained from Worthington Biochemical Corporation (Labclinics, Madrid, Spain). Cell lysis reagent for cell lysis and protein solubilization, reduced glutathione, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′*N*′-tetraacetic acid (EGTA), melatonin, *N*-ethylmaleimide, oxidized glutathione, *o*-phthalaldehyde, protease inhibitor cocktail (Complete, EDTA-free), Tween® 20, and thapsigargin were obtained from Sigma Chemicals Co. (Madrid, Spain). AlamarBlue® was purchased from AbD Serotec (BioNova Científica, Madrid, Spain). 5-(and-6)-Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-$H_2$DCFDA), fura-2-acetoxymethyl ester (AM), Hank's balanced salts (HBSS), horse serum, hydrogen peroxide ($H_2O_2$), medium 199, and MitoSOX™ Red were obtained and purchased from Life Technologies (Invitrogen, Barcelona, Spain). Fetal bovine serum (FBS) was purchased from HyClone (Thermo Scientific, Erembodegem, Belgium). Organ preservation solution, Custodiol™ histidine-tryptophan-ketoglutarate (HTK) medium, was purchased from Essential Pharmaceuticals, LLC (CardioLink, Barcelona, Spain). Penicillin/streptomycin was obtained from BioWhittaker (Lonza, Basel, Switzerland). Polystyrene plates for cell culture were purchased from Thermo Scientific (Madrid, Spain). Round cover glasses were purchased from Menzel (VWR International Eurolab S.L., Barcelona, Spain).

Bradford reagent, Tris/glycine/SDS buffer (10×) and Tris/glycine buffer (10×) were from Bio-Rad (Madrid, Spain). SignalFire™ ECL Reagent was obtained from Cell Signaling Technology (C-Viral, Madrid, Spain).

Anti-alpha-actin antibody was purchased from Sigma Chemicals Co. (Madrid, Spain). Antibodies against MEL-1A-R and MEL-1B-R were purchased from Abcam plc (Cambridge, UK). Antibodies against alpha-smooth muscle actin (α-sma), cytokeratin 7, desmin, and vimentin and secondary antibodies (rabbit anti-goat IgG HRP conjugate, goat anti-rabbit IgG HRP conjugate, and goat anti-mouse IgG HRP conjugate) were purchased Thermo Fisher Scientific (Fisher Scientific, Inc., Madrid, Spain). All other analytical grade chemicals used were obtained from Sigma Chemicals Co. (Madrid, Spain).

## Preparation of human pancreatic stellate cell cultures

Cultures of HPSCs were prepared following methods used in our lab for the preparation of cultures of RPSCs [29]. Samples were provided by the surgeon after resection of cancerous tissues. The cancerous part of the pancreas was separated and kept for analysis. The samples that were used in our study derived from the part of the pancreas free of cancerous cells, which had been resected together with the tumor. Once excised, samples were placed immediately into sterile Falcon tubes filled with HTK medium and kept at 4 °C. Upon arrival in the lab, the tissue was placed in sterile HBSS medium and washed twice. Next, the tissue was cut into small pieces (0.5 cm) and injected with a physiological Na-HEPES buffer 1 containing collagenase CLSPA from Worthington (60 units/ml). The composition of the Na-HEPES buffer 1 employed was as follows: 130 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 1.2 mM $KH_2PO_4$, 10 mM glucose, 10 mM HEPES, 0.01% trypsin inhibitor (soybean), and 0.2% bovine serum albumin (pH = 7.4 adjusted with NaOH).

The tissue was placed into a 15-ml tube, oxygenated and incubated for 50 min at 37 °C in a shaking water bath (100 cycles/min). The enzymatic digestion was followed by cutting the tissue into small pieces. The tissue was then centrifuged (30×*g* for 5 min at 4 °C) and resuspended in Na-HEPES buffer 1. For mechanical dissociation of the cells, the suspension was gently pipetted through tips of decreasing diameter. Next, the suspension was centrifuged at 30×*g* for 5 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in culture medium. The culture medium employed consisted of medium 199 supplemented with 4% horse serum, 10% FBS, a mixture of antibiotics (0.1 mg/ml streptomycin, 100 IU penicillin), and 1 mM $NaHCO_3$. This medium was prepared under sterile conditions.

Finally, small aliquots of cell suspension were seeded on different substrates for the experiments: on sterile glass coverslips which were placed in independent dishes (35 mm diameter), in multiwell cell culture polystyrene plates (6–12 wells), or in 10-cm-diameter cell culture polystyrene plates (Thermo Scientific, Madrid, Spain). The cells were grown in culture medium. A humidified incubator with controlled temperature (37 °C) and $CO_2$ (5%) was used. After 8–10 days of culture, 90–95% confluence was reached. For the studies, different batches of cells, which had been obtained from different preparations, were used. The experiments were performed at room temperature (23–25 °C).

## Western blot analysis

Following stimulation, cells were detached, centrifuged, and washed with a standard phosphate-buffered saline (PBS) containing the following: 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, and 2 mM $KH_2PO_4$, with pH adjusted to 7.4. Next, lysis buffer was added to the samples which were sonicated. Quantification of the protein content of the samples was carried out by employing Bradford's method [5]. Protein lysates (15 μg/lane) of each sample were subjected to fractionation by SDS-PAGE, using 10% polyacrylamide gels, and were transferred to nitrocellulose membranes. Next, the membranes were incubated with the specific primary and the corresponding IgG HRP-conjugated secondary antibody. The experiments were carried out by employing different batches of cells, harvested on different days.

## Determination of intracellular free $Ca^{2+}$ concentration

For this set of experiments, cells had been seeded on independent glass coverslips. Culture medium was replaced by Na-HEPES buffer 1, and cells were loaded with fura-2 by incubation during 40 min in the presence of fura-2-AM (4 μM) at room temperature (23–25 °C). For the detection of changes in intracellular free Ca2+ concentration ($[Ca^{2+}]_c$), an image acquisition and analysis system for video microscopy was used [8]. Stimuli were dissolved in a Na-HEPES buffer 2 of the following composition: 140 mM NaCl, 4.7 mM KCl, 1 mM $CaCl_2$, 1.1 mM $MgCl_2$, 10 mM HEPES, and 10 mM glucose (pH was adjusted to 7.4 with NaOH). Stimuli were applied directly to the cells in a perfusion chamber. All fluorescence measurements were made from areas considered individual cells. Results are shown as the ratio of fluorescence emitted by fura-2 (previously normalized to the resting fluorescence).

## Determination of reactive oxygen species generation

For the determination of reactive oxygen species (ROS) generation, cells were detached, resuspended in Na-HEPES buffer 1, and incubated in the presence of CM-$H_2$DCFDA (10 μM) for 40 min at room temperature (23–25 °C). Afterwards, the cells were centrifuged (30×*g* for 5 min) and resuspended in Na-HEPES buffer 2. Different aliquots of dye-

loaded cells were incubated with stimuli (prepared in Na-HEPES buffer 2) during 1 h. Redox state of cells was monitored by measuring cellular fluorescence at 530 nm/590 nm (excitation/emission).

In another set of experiments, cells were loaded with the mitochondrial ROS indicator MitoSOX™ Red. For this purpose, cells were incubated in the presence of 2.5 μM of the dye during 15 min at 37 °C. Following loading with the probe, cells were centrifuged (30×*g* for 5 min) and resuspended in Na-HEPES buffer 2. Afterwards, different aliquots of dye-loaded cells were incubated with stimuli during 1 h. Generation of mitochondrial ROS was determined by measuring cellular fluorescence at 510 nm/580 nm (excitation/emission).

CM-$H_2$DCFDA and MitoSOX™ Red preferentially accumulate in the cytosol and in the mitochondria, respectively [12]. In both experimental protocols, fluorescence was measured by employing a spectrofluorimeter (Tecan Infinite M200, Grödig, Austria). The assays were performed in triplicate. Results are shown as the mean increase of fluorescence expressed in percentage ± SEM (*n*) with respect to control (non-stimulated) cells, where *n* is the number of independent experiments.

### Determination of glutathione levels

Determination of changes in reduced glutathione (GSH) and oxidized glutathione (GSSG) levels was carried out using the method of Hissin and Hilf [15]. Cells were incubated with the different stimuli assayed. Afterwards, the cells were washed twice with PBS, detached, and resuspended with 1 ml of a solution containing 25 mM Tris-HCl buffer (pH = 7.6) and 1% (*w*/*v*) Triton X-100. The samples were next transferred to Eppendorf vials and placed on ice. This step was followed by sonication for 10 min. Samples were then kept at − 20 °C during 5 min. Subsequently, the samples were centrifuged (9000×*g*, at 4 °C for 30 min), and the supernatants were collected and placed in independent vials. Next, proteins in the supernatant were precipitated by the addition of 20% cold trichloroacetic acid. The vials were then centrifuged (10,000×*g* for 10 min at 4 °C) and kept at − 80 °C for later determination of GSH and GSSG. On the moment of use, 50 μl of each sample was incubated with 1 mg/ml of the fluorescent reagent *ortho*-phthalaldehyde (OPT) in a basic (pH 8.0) buffer containing Na phosphate (0.1 M) and EDTA (5 mM). *N*-Ethylmaleimide (40 mM) and NaOH (1 M; volume to reach pH 12) were added to the samples before the determination of GSSG. The reaction mixture was incubated during 45 min at 20 °C. Afterwards, 200 μl of the reaction mixture was transferred to the wells in a black 96-well plate. The presence of GSH or GSSG was determined by measuring fluorescence at 350 nm/420 nm (excitation/emission) using a spectrofluorimeter (Tecan Infinite M200, Grödig, Austria). Standard curves of GSH (3.20–320 μmol/ml) and GSSG (2.5–80 μmol/ml) were used for quantification. The assays were performed in triplicate. A reaction medium without cell extract was employed as control (blank) sample. The total protein concentration in each sample was determined following Bradford's method [5] and was used for normalization. Bovine serum albumin was employed as standard.

GSH and GSSG values (μmol/mg of proteins) were determined, and the ratio GSH/GSSG was calculated. Data are shown as the mean increase in GSH/GSSG ratio expressed in percentage ± SEM (*n*) with respect to control (non-stimulated) cells, where *n* is the number of independent experiments.

### Cell viability assay

For the determination of cell viability, the AlamarBlue® test was employed [31]. Data show the mean reduction of AlamarBlue® expressed in percentage ± SEM (*n*) with respect to control (non-treated) cells, where *n* is the number of independent experiments.

### Statistical analysis

Statistical analysis of data was performed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, and only $P$ values < 0.05 were considered statistically significant. For individual comparisons and statistics between individual treatments, we employed Student's *t* test, and only $P$ values < 0.05 were considered statistically significant.

## Results

### Preparation of HPSC cultures

Pancreatic cells were grown as described in the "Materials and methods" section. On the day of preparation, acinar cells forming clusters could be observed (Fig. 1a, b). Two days later, HPSC could be observed. In some instances, cells started to grow at the periphery of the cell clusters observed on the day of preparation (Fig. 1c, d). At this stage, culture medium was replaced by fresh medium. Five days after, seeding cells attached to the bottom of the plates could be detected that exhibited a flattened shape (Fig. 1e). Cells started to reach confluence approximately on days 8–10 after seeding (Fig. 1f).

### Cell type markers

Proteins such as α-sma, desmin, cytokeratin 7, or vimentin have been signaled as markers for pancreatic stellate cells [3, 21, 33, 35]. In order to ascertain the type of cells growing in

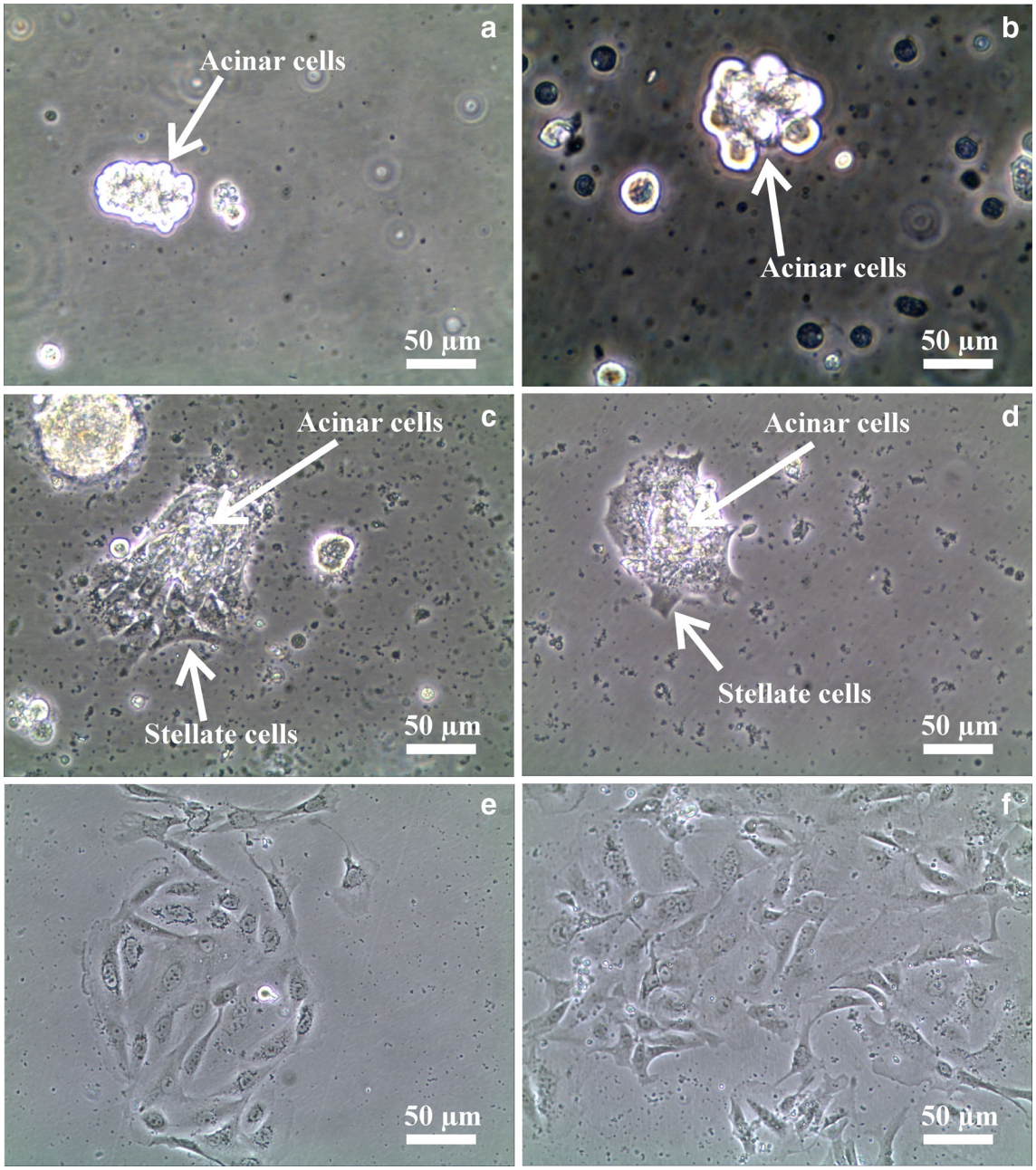


**Fig. 1** Human pancreatic stellate cells growing in culture. Pancreatic cells were prepared as described in the "Materials and methods" section. Acinar cells forming clusters could be observed on the day of preparation (**a**, **b**; white arrows). Two days after seeding, it was possible to detect cells growing at the periphery of the cell clusters observed on the day of preparation (**c**, **d**; white arrows). Five days after seeding, cells exhibiting a flattened shape could be observed (**e**). Cells started to reach confluence approximately 8 days to 10 days after seeding (**f**)

culture, we prepared several samples for western blotting analysis. For comparisons, in addition to the cells obtained from the human pancreas preparation (HPSC), samples from three other types of cells were used as control samples: stellate cells from rat pancreas, which were prepared following the methods used in our laboratory [29] (RPSC); rat pancreatic AR42J cells, which were cultured following the methods used in our laboratory [13] (AR42J); and human glioblastoma U87 cells (U87). The analysis revealed the expression of α-sma (Fig. 2a), cytokeratin 7 (Fig. 2b), desmin (Fig. 2c), and vimentin (Fig. 2d) in RPSCs and in the samples of the cultures prepared from the human pancreas (HPSC). None of the proteins were detected in samples from rat pancreatic AR42J cells, whereas expression of cytokeratin 7 (Fig. 2b) and vimentin (Fig. 2d) was noted in U87 cells. Actin content was detected in all samples (Fig. 2e).

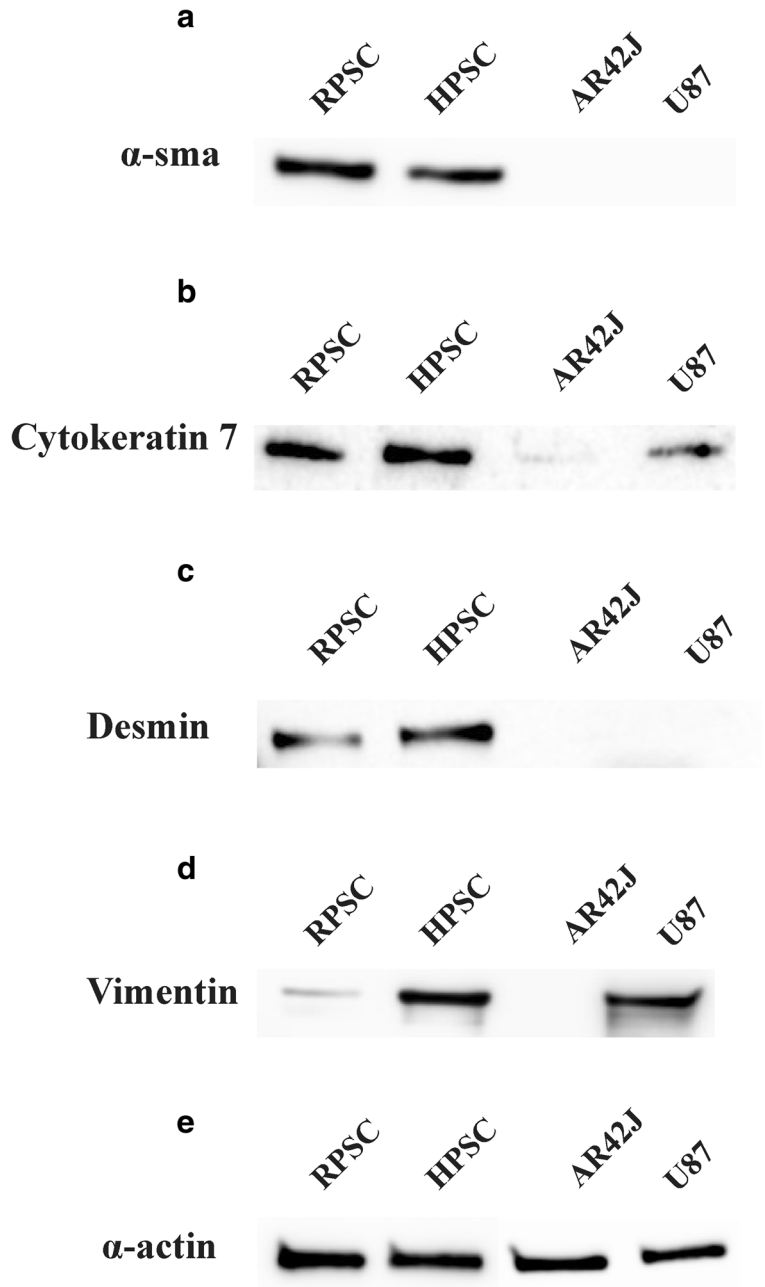


**Fig. 2** Expression of stellate cell markers. Samples from four different types of cells were treated and subjected to western blot analysis: rat pancreatic stellate cells (RPSCs), cells from human pancreas preparation (HPSC), rat pancreatic AR42J cells, and human glioblastoma U87 cells. Incubation with the appropriate antibody revealed the expression of α-sma (**a**), cytokeratin 7 (**b**), desmin (**c**), and vimentin (**d**) in RPSCs and in cells from human pancreas preparation. U87 cells expressed cytokeratin 7 and vimentin, whereas none of the proteins was detected in the samples from rat pancreatic AR42J cells. All samples were tested for actin content (**e**). Determinations were carried out 10 days after seeding of cells. Images are representative of three different preparations

## Expression of melatonin receptors in HPSCs

In a previous work, we showed that RPSCs do not express melatonin 1A (MT1) and melatonin 1B (MT2) receptors [29]. To check the expression of melatonin receptors in HPSCs, immunoblots using specific antibodies for MT1 and MT2 receptors were performed. This maneuver can be used to explore whether melatonin-induced effects involve its plasma membrane receptors. Extracts from different tissues were used as control samples. Neither MT1 nor MT2 receptors could be detected in HPSCs. However, bands falling in the molecular weight of melatonin receptors could be detected in extracts from other cellular types employed as controls (Fig. 3). There were slight differences in the position of the bands after the electrophoresis, which did not coincide exactly in the position between the different samples used. This could be explained by different migration properties depending on the tissue employed. The protein levels of α-actin were employed as controls under the tested conditions. The different densities of the bands observed for actin expression could be explained on the basis of a different content of actin, depending on the cellular type. Stellate cells may express a higher content of actin, and therefore, the density of bands for actin is higher compared with the content of the other cellular types tested. In this line, the absence of bands in lanes corresponding to RPSC and human pancreas preparation (HPSC) cannot be explained

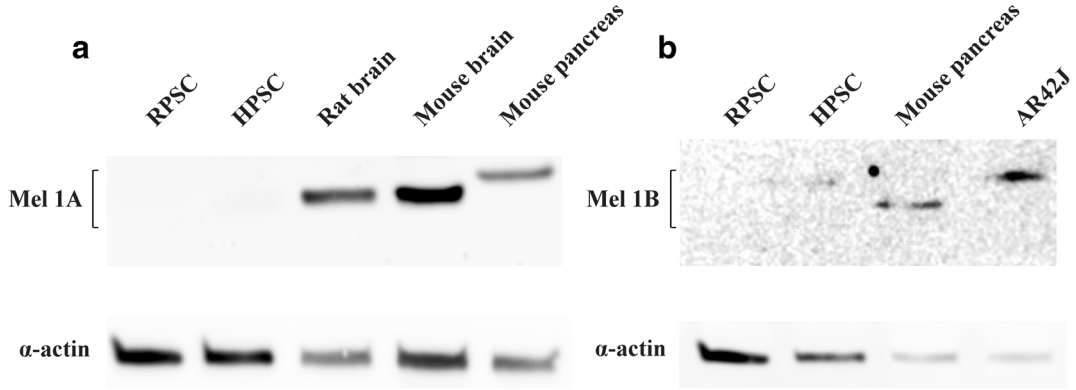


**Fig. 3** Analysis of melatonin receptor expression in HPSCs. Western blot analysis was performed to detect the presence of melatonin receptors. **a** Homogenates prepared from rat pancreatic stellate cells (RPSCs) and cells from human pancreas preparation (HPSC), from rat brain, from mouse brain, and from mouse pancreatic acinar cells (mouse pancreas) were used. Samples were incubated with specific antibodies against melatonin 1A receptors (Mel 1A). **b** Homogenates obtained from cultured RPSC and cells from human pancreas preparation (HPSC), from mouse pancreatic acinar cells (mouse pancreas), and from rat pancreatic AR42J cells were used. Samples were incubated with specific antibodies against melatonin 1B receptors (Mel 1B). Melatonin 1A or 1B receptor expression could not be clearly detected either in RPSC or in HPSC. However, bands falling in the molecular weight of melatonin receptors could be detected in the samples from other cellular types. The protein levels of α-actin were employed as controls under the tested conditions. The different densities of the bands observed could be explained on the basis of a different content of actin, depending on the cellular type. The experiments shown are representative of three others

on the basis of less protein used for the analysis when melatonin receptor antibodies were tested.

## Changes in $[Ca^{2+}]_c$ in HPSCs in response to different stimuli

In order to test the utility of HPSCs prepared following the method described here, we performed a series of experiments in which cells were incubated in the presence of different stimuli with known effects on $[Ca^{2+}]_c$.

It has been shown that bradykinin evokes changes in $[Ca^{2+}]_c$ in pancreatic stellate cells [14]. To test the responsiveness to bradykinin of the cells prepared in our study, we incubated fura-2–loaded HPSCs with 10 nM bradykinin. The compound induced a transient increase in $[Ca^{2+}]_c$ that returned towards an elevated value over the prestimulation level (Fig. 4a; $n = 33$ cells studied in three independent experiments).

It is well known that functional $Ca^{2+}$ stores can be depleted by inhibitors of the sarcoendoplasmic reticulum $Ca^{2+}$-ATPase (SERCA). Thapsigargin (Tps) is a potent selective SERCA inhibitor and is often used to inhibit this pump [24]. When fura-2–loaded HPSCs were incubated with Tps, we observed a transient increase in $[Ca^{2+}]_c$, which later decreased and reached an elevated value over the prestimulation (Fig. 4b; $n = 42$ cells analyzed from four independent experiments).

We have previously shown that melatonin induces changes in $[Ca^{2+}]_c$ in RPSCs [29]. To test whether HPSCs also exhibit $Ca^{2+}$ mobilization in response to melatonin, fura-2–loaded HPSCs were incubated with 100 μM of the indoleamine. Melatonin induced a slow increase in $[Ca^{2+}]_c$ towards an elevated value ($n = 87$ cells studied in five independent experiments; Fig. 4c). The additional inclusion of Tps (1 μM) in the extracellular solution additionally increased $[Ca^{2+}]_c$; however, $Ca^{2+}$ response was smaller compared with that noted when Tps was added alone to the cells (Fig. 4c).

## Effect of melatonin on ROS production

Melatonin, the product of the pineal gland, possesses antioxidant, anti-inflammatory, and antitumor properties in different tissues, in addition to its role as a regulator of biological rhythms [11, 16, 22]. However, a pro-oxidant action has also been suggested [26]. Overwhelming antioxidant defenses by excessive ROS production can impair cellular function and can compromise cell survival. HPSCs, loaded with the ROS-sensitive fluorescent dye CM-$H_2$DCFDA, were incubated during 1 h with melatonin (1 μM, 10 μM, 100 μM, or 1 mM). Melatonin (1 μM) did not induce statistically significant increases in CM-$H_2$DCFDA–derived fluorescence compared with that observed in control (non-treated) cells. However, when the cells were stimulated with 10 μM, 100 μM, or 1 mM melatonin, a concentration-dependent and statistically significant increase in dye-derived fluorescence was detected at each concentration of melatonin tested. As a control, cells were incubated (1 h) in the presence of 100 μM hydrogen peroxide. The oxidant induced a statistically significant increase in CM-$H_2$DCFDA–derived fluorescence, reflecting an increase in oxidation (Fig. 5a).

We additionally tested the effect of melatonin on ROS production by following changes in fluorescence of cells loaded with the mitochondrial ROS indicator MitoSOX™ Red. In the presence of 1 μM melatonin, no significant

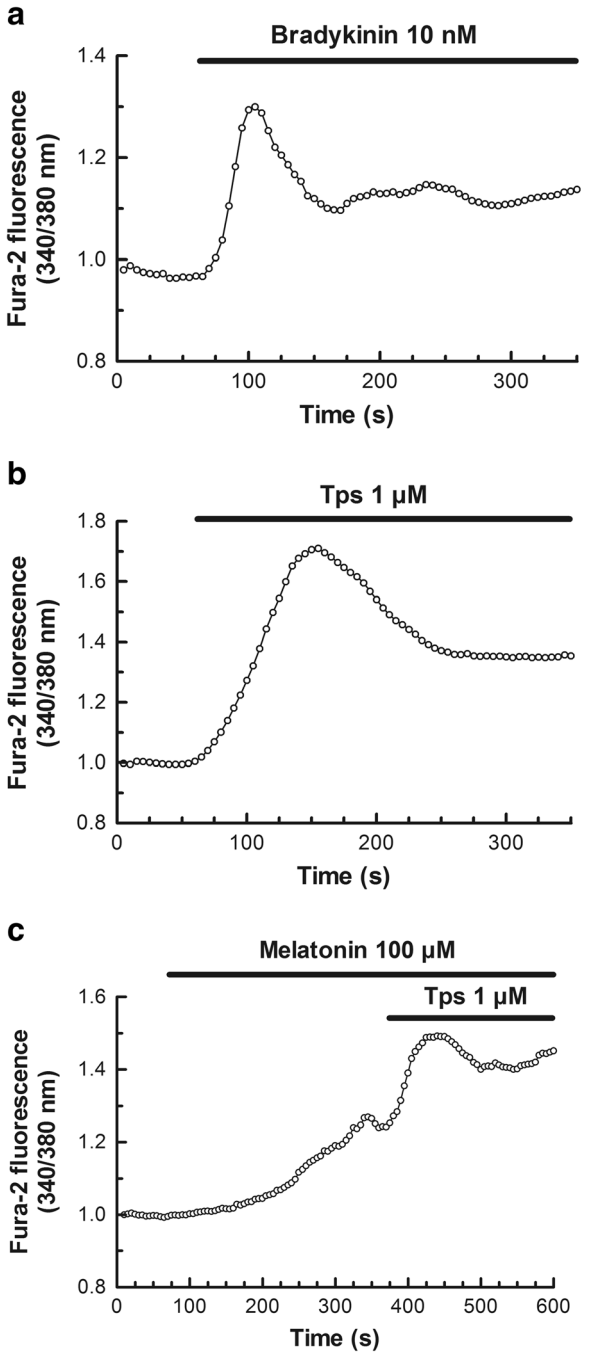


**Fig. 4** Time courses of changes in $[Ca^{2+}]_c$ in cultured HPSCs. Cells growing confluently were stimulated with 10 nM bradykinin (**a**), 1 μM thapsigargin (**b**), or 100 μM melatonin and subsequent addition of 1 μM Tps (**c**). The experiments were carried out in the presence of $Ca^{2+}$ in the extracellular medium. The horizontal bar shows the time during which the desired concentration of stimulus was applied to the cells. The traces show the typical response of one cell for each treatment, taken from 33 to 87 cells studied in three to five independent experiments

changes in ROS production could be observed. However, a concentration-dependent increase in dye-derived fluorescence was detected when the cells were incubated with 10 μM, 100 μM, or 1 mM melatonin. Other batches of cells were incubated (1 h) in the presence of 100 μM hydrogen peroxide. The oxidant induced a statistically significant increase in MitoSOX™ Red–derived fluorescence, reflecting an increase in oxidation (Fig. 5b).

Altogether, these results suggest that melatonin induces changes in the oxidative state of HPSCs.

### Effect of melatonin on glutathione levels

Glutathione is a critical antioxidant defense against oxidative stress [9]. Because melatonin induced ROS production, we were interested in testing the effect of the indoleamine on the glutathione system in HPSCs. For this purpose, different batches of cells were incubated during 1 h in the presence of different concentrations of melatonin (1 μM, 10 μM, 100 μM, or 1 mM), and the effect on the levels of GSH and GSSG was studied. Melatonin induced concentration-dependent changes in the GSH/GSSG ratio, which dropped from the highest level achieved in the presence of 1 μM melatonin towards a value lower than that noted in non-treated (control) cells, which was observed in cells incubated with 1 mM melatonin (Fig. 6).

These results further show that melatonin induces changes in the oxidative state of HPSCs.

### Effects of melatonin on cell viability

The impairment of cellular redox status and the generation of ROS can lead to cell damage and death [2]. At this point, it was of interest to analyze if melatonin, which had induced changes in the oxidative state of HPSC, induced any effect on cell viability. For this purpose, HPSCs were incubated in the absence of stimulus (non-treated cells) or in the presence of 1 μM, 10 μM, 100 μM, and 1 mM melatonin, and cell viability was evaluated at 24 h, 48 h, 72 h, and 96 h of culture. The viability of non-treated cells did not vary during the time of incubation. The viability of cells incubated with melatonin was compared with that of non-treated cells, which was estimated to be 100%.

The viability of HPSCs dropped in time in the presence of any of the concentrations of melatonin used (Fig. 7a–d). A maximal effect was noted at 96 h of incubation. Melatonin 100 μM (Fig. 7c) and 1 mM (Fig. 7d) induced the stronger reduction in cell viability. As a control, separate batches of cells were incubated in the presence of 1 μM thapsigargin (Tps), a cell death inducer [23]. Tps induced a time-dependent decrease in cell viability compared with non-stimulated cells (Fig. 7e).

## Discussion

In a previous study, we have reported data which suggest a role for melatonin on the regulation of viability of

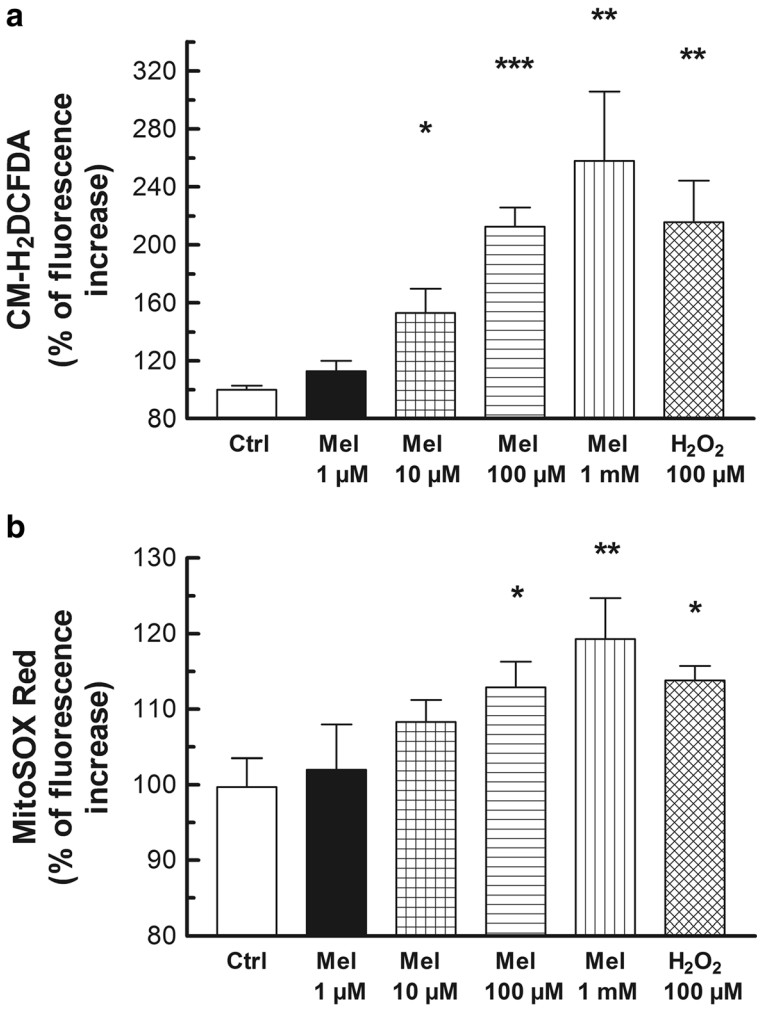


**Fig. 5** Generation of ROS in response to melatonin. **a** Cells were loaded with the redox-sensitive dye CM-$H_2$DCFDA and were challenged with different concentrations of melatonin (1 μM, 10 μM, 100 μM, or 1 mM). As a control, cells were incubated in the presence of 100 μM hydrogen peroxide ($H_2O_2$). **b** Cells were loaded with the mitochondrial superoxide indicator MitoSOX™ Red and were incubated in the presence of melatonin (1 μM, 10 μM, 100 μM, or 1 mM). Separated batches of cells were incubated with 100 μM $H_2O_2$. Bars show the mean increase of dye-derived fluorescence expressed in percentage ± SEM with respect to control (non-stimulated) cells. Results are representative of four independent experiments (Ctrl, control cells; Mel, melatonin; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, vs non-stimulated cells)

RPSCs [29]. Here, we have investigated the effect of the indole on the oxidative state and on the viability of HPSCs. The interest of the present study was aimed at finding out whether the observations obtained in animal cell studies also occur in human cells. This fact represents a major challenge in human medicine, bearing in mind that studies on samples from human tissues are lacking. This is due to, specially, the difficulties in the procurement of samples.

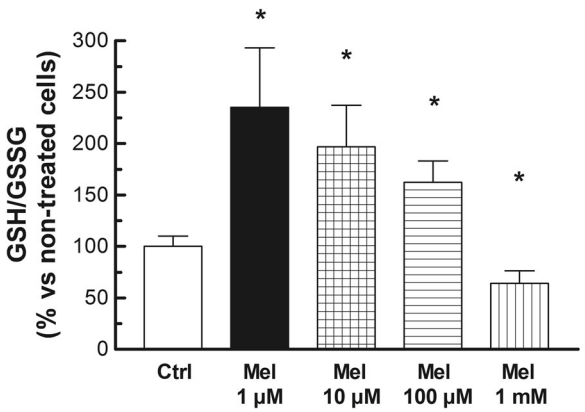


**Fig. 6** Effect of melatonin on glutathione. HPSCs were incubated during 1 h in the presence of melatonin (1 μM, 10 μM, 100 μM, or 1 mM), and the effect on glutathione was analyzed. The bars show the mean increase in GSH/GSSG ratio expressed in percentage ± SEM with respect to control (non-stimulated) cells. Three different cellular preparations were used (Ctrl, control cells; Mel, melatonin; *$P < 0.05$)

Immunoblots with specific antibodies confirmed that the cell type growing in the cultures expressed proteins which have been signaled as markers typical of PSCs [21, 35]. Our results further showed effects of pharmacological concentrations of melatonin on $[Ca^{2+}]_c$, ROS production, glutathione levels, and cell viability. Interestingly, our observations highlight that the effects of the indole formerly reported using animal cells are also induced in human cells and contribute to the transfer of knowledge achieved with animal cells to human medicine. We think that the results here reported are relevant, taking into account that pancreatic stellate cells have been pointed out as responsible for pancreatic fibrosis in inflammation and cancer.

The analysis of intracellular $Ca^{2+}$ signaling is a useful strategy to know how different stimuli control cell physiology

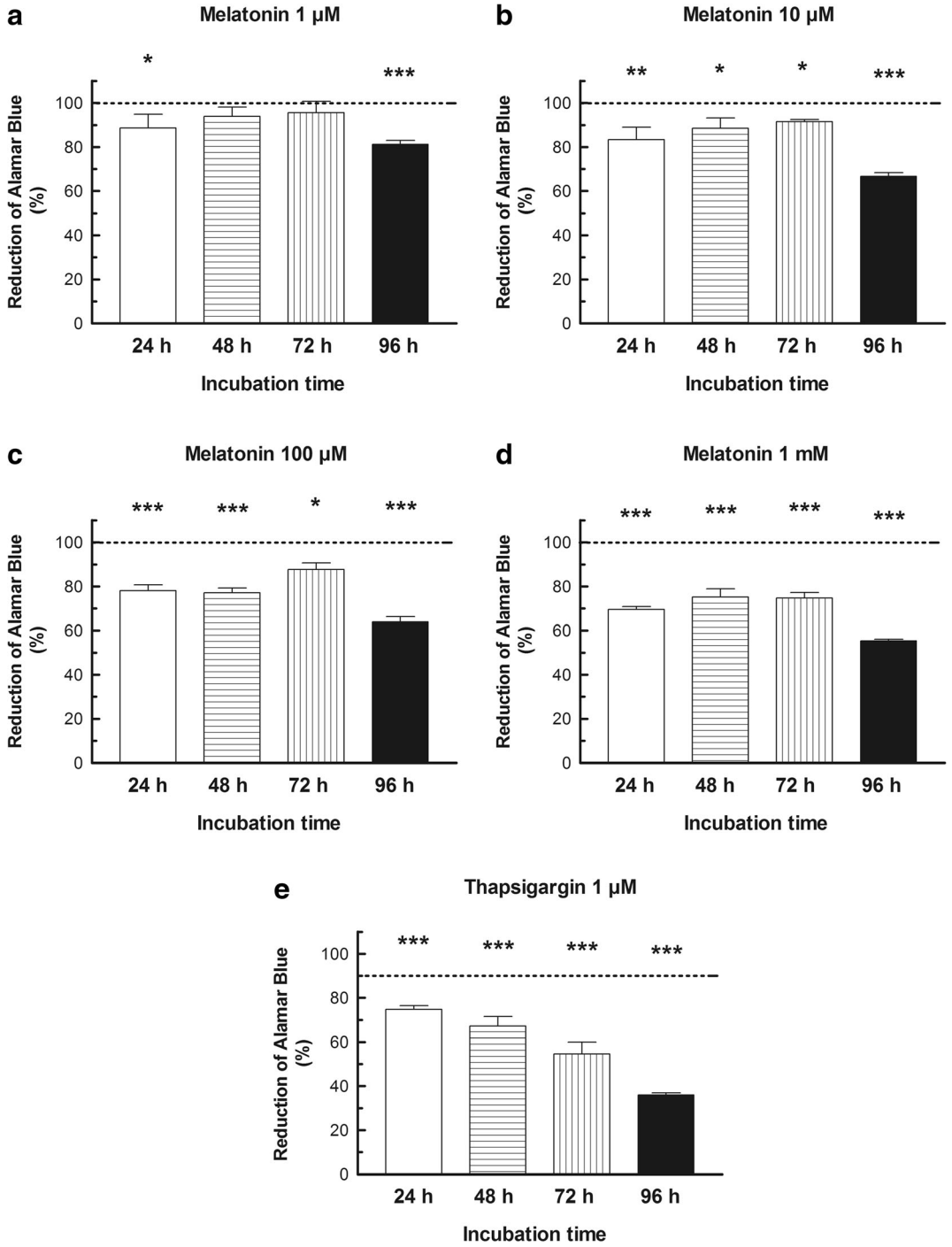


**Fig. 7** Analysis of viability of HPSCs. Cell viability was analyzed by studying the AlamarBlue® reduction by viable cells, as described in the "Materials and methods" section. Cells were incubated in the presence of 1 μM (**a**), 10 μM (**b**), 100 μM (**c**), and 1 mM (**d**) melatonin or 1 μM Tps (**e**). Cell viability was determined at 24 h, 48 h, 72 h, and 96 h of culture and was compared with that of cells in the absence of stimulus (non-treated cells). In each graph, a dotted line represents the viability of cells in the absence of stimulus (non-treated cells). Histograms are representative of three independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, vs non-stimulated cells)

[34]. In a recent work, it has been reported that PSCs do not exhibit $Ca^{2+}$ mobilization in response to activation of cell membrane receptors by the typical pancreatic secretagogues acetylcholine or cholecystokinin. However, this type of cells mobilizes $Ca^{2+}$ when stimulated with bradykinin [14]. Our results show that our cell model displayed $Ca^{2+}$ mobilization when incubated with bradykinin. This observation, together with the expression of α-sma, cytokeratin 7, desmin, and vimentin, confirms that the cells growing in the cultures are stellate cells.

Melatonin effects on cell physiology in different cell types and tissues involve $Ca^{2+}$ signaling [7, 8, 28]. Our results show that incubation of HPSCs with melatonin induced changes in $[Ca^{2+}]_c$. The contribution of $Ca^{2+}$ stored in the endoplasmic reticulum to melatonin-evoked $Ca^{2+}$ mobilization is suggested by the observation that application of Tps, after previous stimulation of cells with melatonin, evoked a smaller $Ca^{2+}$ response compared with that obtained when Tps was applied alone. These results are in agreement with previously reported findings of our lab [8, 29]. The effect of melatonin on $Ca^{2+}$ mobilization that we have noticed might not be mediated through the activation of cell membrane receptors, but rather through an intracellular action of melatonin. The site of action of melatonin is probably the endoplasmic reticulum.

Maintenance of an adequate cellular redox state is of major relevance for cell function and viability [6]. The glutathione system includes GSH and GSSG and the enzymes required for its synthesis and recycling. Glutathione is used in metabolism and in the mechanisms of defense against ROS-induced damage [19]. The increase in oxidative stress and the failure of antioxidant systems, such as the decrease in the GSH content, to control ROS production can lead to cell damage and death.

It has been shown that melatonin exerts a protective role against oxidative stress [4]. However, the indoleamine can also induce a pro-oxidant environment, which has been related with a cytotoxic effect [26]. Although the vast majority of studies proved the antioxidant capacity of melatonin and its derivatives, a few studies using cultured cells found that melatonin promoted the generation of ROS at pharmacological concentrations (micromolar to millimolar range) in several tumor and non-tumor cells; thus, melatonin functioned as a conditional pro-oxidant. This action may highly depend on the concentration of melatonin used. Here, we show that the GSH/GSSG ratio changed in the presence of melatonin in a concentration-dependent manner. In fact, in the presence of 1 μM, 10 μM, or 100 μM melatonin, the GSH/GSSG values that we have observed suggest an antioxidant effect of the indoleamine. However, in the presence of 1 mM melatonin, the GSH/GSSG ratio was lower than that noted in non-treated cells. The drop in the GSH/GSSG ratio that reflects an increase in the oxidized from of glutathione might be related with a pro-oxidant effect of melatonin, which augments with an increasing concentration of the indole. Additionally, the indole induced a concentration-related generation of ROS, which was detected both in the mitochondria and in the cytosol. Interestingly, 1 μM melatonin did not induce statistically significant changes in ROS production. Conversely, 100 μM or 1 mM melatonin induced significant increases of ROS production. These results could be related with the decrease in the GSH/GSSG values that we have observed; i.e., the higher concentration of melatonin that is employed, the higher the oxidation state that is induced. Our results are in agreement with the published observations and suggest that melatonin may induce changes in the oxidative state of HPSCs. Altogether, the observed increase in the oxidative state could set important changes in cell physiology with putative consequences on cell viability.

It has been suggested that transformed epithelia within the pancreas, together with fibroblasts, are essential for growth, proliferation, angiogenesis, and invasion of carcinomas [20]. Furthermore, contribution of stellate cells has been documented [25]. Therefore, modulation of fibrotic tissue growth within tumors might be of relevance in the treatment of cancer [21]. In our former work carried out by employing RPSCs, we showed that cellular viability was modulated in the presence of melatonin [29]. Here, we show that the indoleamine also decreases cell viability of HPSCs. The stronger reduction in cell viability was noted when the cells were treated with 100 μM or 1 mM melatonin, which is the concentration of the indole that has shown higher pro-oxidant effects. In the work of Santofimia-Castaño et al. [29], no detectable changes in cell viability were noted with either 1 μM or 10 μM melatonin. The fact that the same concentrations of melatonin were able to evoke changes in the viability of HPSCs might be explained by different species sensitivity levels to the indole. Our results are in agreement with previously reported observations and suggest a putative role of melatonin to prevent the proliferation of HPSCs. Interestingly, the treatment of tumor cells with melatonin, including pancreatic cancer cells, induces a decrease of cell viability [11, 18]. Therefore, melatonin has evolved as a promising agent with beneficial actions in the treatment of tumors.

Many actions of melatonin are mediated through interaction with cell membrane–bound MT1 and MT2 receptors. Employing specific antibodies has allowed detecting melatonin receptors in pancreatic acinar cells and in pancreatic AR42J cells [11]. However, these receptors could not be detected in RPSCs in a former work carried out in our lab [29]. We could neither observe melatonin receptors in HPSCs. Thus, in a first instance, our observations suggest that the effects of melatonin on the physiology of HPSCs might not be mediated through plasma membrane receptors. It has also been proposed that melatonin binds to a cytosolic receptor (quinone reductase II enzyme, also defined as MT3 receptor) or to nuclear receptors (belonging to the RORα/RZR family) [32]. However, controversies exist regarding these receptors, especially whether melatonin binds to the RORα/RZR family [32]. Moreover, it has been suggested that melatonin actions may involve or not involve plasma membrane receptors [27].

The concentrations of melatonin used in this study could be considered rather pharmacological than physiological, because they are higher than those found normally in the blood. Remarkably, many tissues synthesize melatonin that will be delivered for local use as an autocrine or paracrine agent. Therefore, high values of melatonin (even several orders of magnitude more than the level found in plasma) have been

found in these tissues [1, 10]. Not wrong, blood levels of melatonin cannot always define physiological concentrations in the body, because the local concentrations of the indole have not been determined.

In conclusion, we present results that signal melatonin as a player of an important role in the regulation of HPSC physiology. The indoleamine induces changes in $[Ca^{2+}]_c$ and creates pro-oxidative conditions within the cells that might regulate cell viability. Our observations point towards a putative role of melatonin in the modulation of pancreatic fibrosis. The actions of melatonin might not be mediated through cell membrane receptors and rather seem to be direct actions. The presence of other types of receptors, such as cytosolic or nuclear RORα/RZR receptors, need to be confirmed and, together with the metabolic pathways leading to ROS production, deserve future studies.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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