



Melatonin finely tunes proliferation and senescence in hematopoietic stem cells

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ABSTRACT

Human hematopoietic stem/progenitor cells (HSPCs) are pluripotent cells that gradually lose their self-renewal and regenerative potential, to give rise to mature cells of the hematopoietic system by differentiation. HSPC infusion is used to restore hematopoietic function in patients with a variety of onco-hematologic and immune-mediated disorders. The functionality of these cells is therefore of great importance to ensure the homeostasis of the hematopoietic system. Melatonin plays an important role as immunomodulatory and oncostatic hormone. In the present manuscript, we aimed at evaluating the activity of melatonin in modulating HSPC senescence, in the attempt to improve their hemopoietic regenerative potential. We exposed HSPCs to melatonin, in different conditions, and then analyzed the expression of genes regulating cell cycle and cell senescence. Moreover, we assessed cell senescence by β -galactosidase and telomerase activity. Our results showed the ability of melatonin to counteract HSPC senescence, thus paving the way for enhanced efficiency in their clinical application.

1. Introduction

Human hematopoietic stem/progenitor cells (HSPCs) have been described in animals and in humans as pluripotent cells, able to differentiate into a series of multipotent progenitors, being mainly involved in hematopoietic reconstitution after transplantation (Seita and Weissman, 2010; Bertolini et al., 1998). As pluripotent cells, they are capable of self-renewal, and production of mature blood cells, including erythrocytes, leukocytes, platelets, and lymphocytes (Ogawa, 1993). CD34 is a key marker in defining hematopoietic stem cells (Pei, 1999; Krause et al., 1994), routinely used to isolate HSPCs for clinical application in bone marrow transplantation (AbuSamra et al., 2017). In physiological conditions, HSPCs' location is almost exclusively restricted to the bone marrow, where they are distributed in highly organized

three-dimensional microenvironmental niches (Li and Calvi, 2017; Zhang et al., 2019). The release of these cells into the circulation and their subsequent migration are essential for their development (Bonig, Papayannopoulou, 2012). Cell mobilization by granulocyte-colony stimulating factor (G-CSF) or cytokines, including stem cell factor (SCF) and FMS-like tyrosine kinase 3 (FLT3), as well as the c-kit ligand, is critical in clinical practice, in particular following autologous transplantation (Eid et al., 2015; Damon and Damon, 2021). HSPC aging is associated to a reduced human blood system function, resulting in worsening of myeloproliferative diseases, and even leukemia (Dong et al., 2021, 2018). Senescent cells show reduced immunomodulatory properties and impaired function, losing the ability to maintain tissue homeostasis, especially when transplanted (Gnani et al., 2019; Chen et al., 2021). Levels of reactive oxygen species (ROS) increase with

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senescence, contributing to the onset of a stage of inflammation and premature depletion of self-renewal potential (Davalli et al., 2016; Lam, 2016). Stress-induced premature senescence (SIPS) occurs following exposure to a strong stressor event, as H_2O_2 , hyperoxia, or UV (Toussaint et al., 2000). This event is characterized by increased senescence-associated beta-galactosidase (SA- β -Gal) activity, and proliferation arrest mediated by p16, p19, p21, and p53 (Kumari, Jat, 2021; Herranz, Gil, 2018). Moreover, oncogene-induced senescence (OIS) is an antiproliferative response against cancer cells, resulting from an activating mutation of oncogenes or inactivation of tumor suppressor genes (Ogrunc et al., 2014). Myc expression is deregulated in a wide range of human cancers and is often associated with increased cell cycle progression and cellular transformation, also contributing to oxidative stress resistance (Miller et al., 2012; Benassi et al., 2006).

Increased ROS production induces DNA damages and is implicated in the pathogenesis and progression of a wide variety of onco-hematologic disorders (Hole et al., 2011; Guida et al., 2014; Shao et al., 2010). Several natural molecules are used as antioxidants to counteract senescence and ROS production in vitro as well as in vivo (Abruzzo et al., 2020). Extracts from plants can prevent premature senescence in stem cells and fibroblast exposed to oxidative stress (Cruciani, 2019; Cruciani et al., 2020; Cruciani et al., 2019). Melatonin is a hormone produced by the pineal gland and many extra-pineal tissues, including skin, lymphoid tissue and bone marrow (Acuña-Castroviejo, 2014; Markus and Sousa, 2021; Odinkov and Hamblin, 2018; Slominski, 2017a,b), and its production can be altered by aging (Popović et al., 2018). Melatonin acts as free radical scavenger antioxidant and anti-senescence molecule, following stimulation of anti-oxidative responses including Nrf-2 and P53 pathways (Janjetovic et al., 2017; Hacısevki, Baba, 2018) and by G-protein-coupled receptors (GPCRs) or calmodulin (Jockers et al., 2016). Indeed, it has been demonstrated that melatonin protects against premature radiation-induced senescence, as exposure to UVB, by inducing cell cycle arrest through increasing of p53 expression and ROS scavenging (Ma et al., 2021; Kleszczynski, Fischer, 2012). Moreover, melatonin protects MSCs against replicative senescence acting on mitochondrial dysfunction (Lee et al., 2020). Maintaining HSPC hemopoiesis is critical management of clonal disorders affecting the hematopoietic niche (Li et al., 2020). Within this context, in the present study we aimed at evaluating the ability of melatonin to regulate senescence patterning and oxidative stress induced by H_2O_2 , in HSPCs isolated from patients with onco-hematologic disorders, in the attempt to improve the functional integrity of hematopoietic system. For these reasons, we exposed HSPCs to melatonin before and after H_2O_2 -oxidative stress treatment. We then evaluated cell proliferation, the gene expression of TERT, encoding a major catalytic core of telomerase, c-Myc, p16, p19, p21, p53, telomerase activity, and analyzed mTOR signaling and beta-galactosidase activity. We finally detected the antioxidant activity of melatonin by measuring nitric oxide production and

catalase activity (Fig. 1).

2. Results

2.1. Melatonin modulates the expression of cell cycle regulators

Tripan blue exclusion test of viability and BrdU Cell Proliferation Assay were used to evaluate cell viability and proliferation in HSPCs cultured according to the different described condition. After plating, cells were cultured for 7 days, and then collected and counted by an automatic cell counting (Table 1). Figs. 2 and 3 shows that melatonin was able to significant counteract HSPC hyperproliferation, restoring the normal cell cycle progression of these cells. These data were further inferred by gene expression analysis of the main cell cycle regulators.

Fig. 4 shows that the expression of c-Myc, a proto-oncogene that plays a role in cell cycle progression and cellular transformation (Miller et al., 2012), was significantly increased in H_2O_2 -stressed HSPCs (yellow bar) as compared to control untreated cells (grey bar). This effect could not be observed when cells were treated with melatonin after H_2O_2 exposure (orange bar). Moreover the same figure shows that melatonin can prevent the overexpression of c-Myc (blue bar). These results suggest that melatonin is capable of counteracting the survival of cancer cells triggering growth arrest.

At the same observational time in culture (7 days), melatonin exerted a significant upregulation in the gene expression of p16, p19, p21 and p53, a set of major cell cycle inhibitors and oncosuppressor genes (Fig. 5). p19, p21 and p53 mRNA (Fig. 5, panels B and D, respectively) showed a remarkable upregulation in H_2O_2 -stressed HSPCs exposed to melatonin (orange bar) or pre-treated with melatonin and then exposed to H_2O_2 (blue bar), as compared to control untreated cells. Moreover, p16 gene expression was also slightly enhanced under the same experimental conditions (Fig. 5, panel A, orange bar). HSPCs exposed to H_2O_2 showed no significant difference in the expression of all the cell cycle inhibitors analyzed (Fig. 5, yellow bar). This antiproliferative effect of H_2O_2 has been already described by other authors in MCF7 breast cancer cells Mahalingaiah, Singh (2014). The gene expression analysis of p21 and p53 were further inferred by western blot analysis (Fig. 5).

2.2. Melatonin modulates TERT transcription and telomerase activity in HSPCs

In cells cultured in the presence of melatonin alone, the expression of TERT gene, was superimposable to that of control untreated cells (Fig. 6, green bar). Here we found that H_2O_2 -stressed HSPCs (Fig. 6, yellow bar) show a substantial increase in TERT gene expression. On the other hand, both pre-treatment or post-treatment with melatonin (blue and orange bars respectively) significantly counteract the stimulatory effect elicited by H_2O_2 on its transcription.

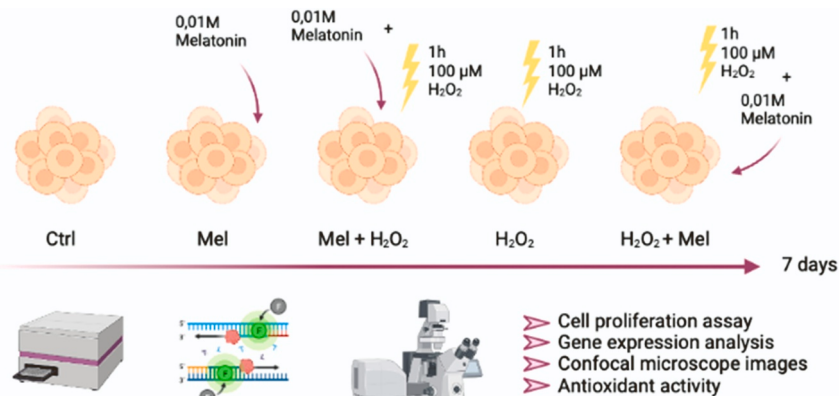


Fig. 1. HSPC treatment and experimental design. Created with BioRender.com.

Table 1

HSPC concentration (cells/ml) in the different culturing conditions.

	Ctrl	Mel	Mel+H ₂ O ₂	H ₂ O ₂	H ₂ O ₂ +Mel
Total cell Concentration (cells/ml)	9.55×10^5	7.25×10^5	1.79×10^6	2.47×10^6	9.21×10^5
Live cell concentration (cells/ml)	7.6×10^5	9.70×10^4	2.42×10^5	1.58×10^6	4.85×10^5
Dead cell concentration (cells/ml)	1.95×10^5	6.30×10^5	1.55×10^6	8.9×10^5	4.36×10^5
Viability	79.58 %	13.30 %	13.5 %	63.9 %	52.6 %

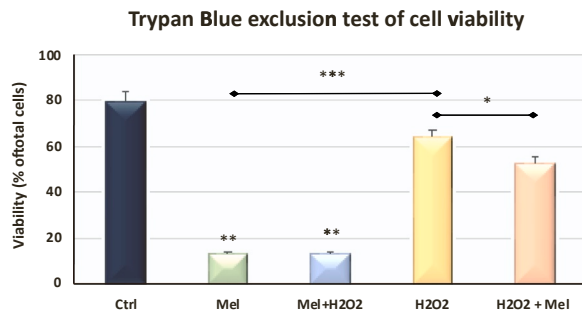


Fig. 2. Viability of HSPCs cultured for 7 days in the presence melatonin with or without the pre-treatment with H₂O₂ (orange and green bar respectively) and in HSPCs pre-treated with melatonin and then exposed to H₂O₂ (blue bar), as compared to control untreated cells (grey bar). Yellow bar represents H₂O₂-stressed HSPCs. After plating (5×10^5 cells/plate), cells were collected and counted using an automatic cell counter. The percentage of vital cells was calculated as the number of positive cells divided by the total number of counted cells (mean $\pm$ SE; n = 6) control (* p $\leq$ 0.05; ** p $\leq$ 0.01; *** p $\leq$ 0.001). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

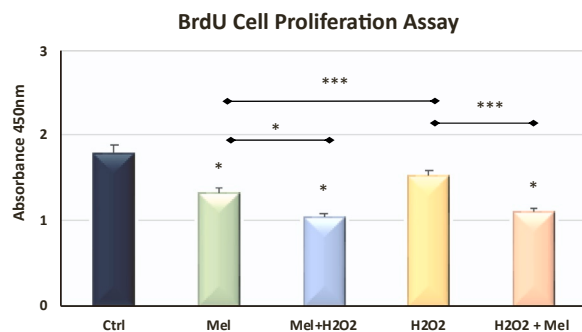


Fig. 3. HSPC proliferation after 7 days in the presence of melatonin with or without the pre-treatment with H₂O₂ (orange and green bar respectively) and in HSPCs pre-treated with melatonin and then exposed to H₂O₂ (blue bar), as compared to control untreated cells (grey bar). Yellow bar represents H₂O₂-stressed HSPCs. Cell proliferation is expressed in OD units as compared to control untreated cells. Data are expressed as mean $\pm$ SD (n = 6) referring to the control (* p $\leq$ 0.05; ** p $\leq$ 0.01; *** p $\leq$ 0.001). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

Fig. 7 shows that melatonin not only remarkably decreased telomerase activity by itself, but it also downregulated the same activity in H₂O₂ exposed HSPC to a level below that detected in control untreated cells.

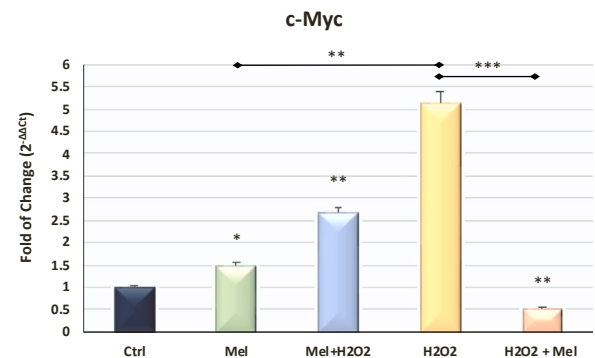


Fig. 4. The expression of c-Myc was evaluated in HSPCs exposed to melatonin with or without the pre-treatment with H₂O₂ (orange and green bar respectively) and in HSPCs pre-treated with melatonin and then exposed to H₂O₂ (blue bar), all cultured for 7 days, as compared to control untreated cells (grey bar). Yellow bar represents H₂O₂-stressed HSPCs. The mRNA levels for each gene were normalized to HPRT1 and expressed as fold of change ($2^{-\Delta\Delta C_t}$) of the mRNA levels observed in control untreated cells defined as 1 (mean $\pm$ SD; n = 6). Data are expressed as mean $\pm$ SD referred to the control (* p $\leq$ 0.05; ** p $\leq$ 0.01; *** p $\leq$ 0.001). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

2.3. Melatonin regulates the expression of mTOR

Immunofluorescence analysis showed that melatonin is able to modulate the expression of mTOR, clearly evident in the cytoplasm of control untreated cells and in H₂O₂-stressed HSPCs (Fig. 8). Its expression was significantly inhibited when HSPCs were cultured in the presence of melatonin, and in H₂O₂-stressed HSPCs exposed to melatonin or pre-treated with melatonin and then exposed to H₂O₂ (Fig. 8).

2.4. Melatonin can counteract H₂O₂-induced cell senescence

Fig. 9 shows the results from senescence-associated β -galactosidase (SA- β Gal) staining assay in H₂O₂-stressed HSPCs treated in the absence or presence of melatonin after 7 days in culture. Control untreated HSPCs showed a high number of positively stained blue cells (Fig. 9), an observation consistent with previous evidence for enhanced senescent traits in malignant cells (Lee and Lee, 2019). In H₂O₂-stressed cells, a reduced number of positively stained HSPCs was observed in comparison to untreated cells (Fig. 9). A different situation was detected when HSPCs were cultured in the presence of melatonin, and in H₂O₂-stressed HSPCs exposed to melatonin or pre-treated with melatonin and then exposed to H₂O₂. Indeed, the number of positive blue cells after the different treatments with melatonin and H₂O₂, was significantly decreased, as compared to control untreated cells (Fig. 9).

2.5. Melatonin modulates nitrite secretion and catalase activity

Cells exposed to melatonin alone (green bar) or to melatonin before

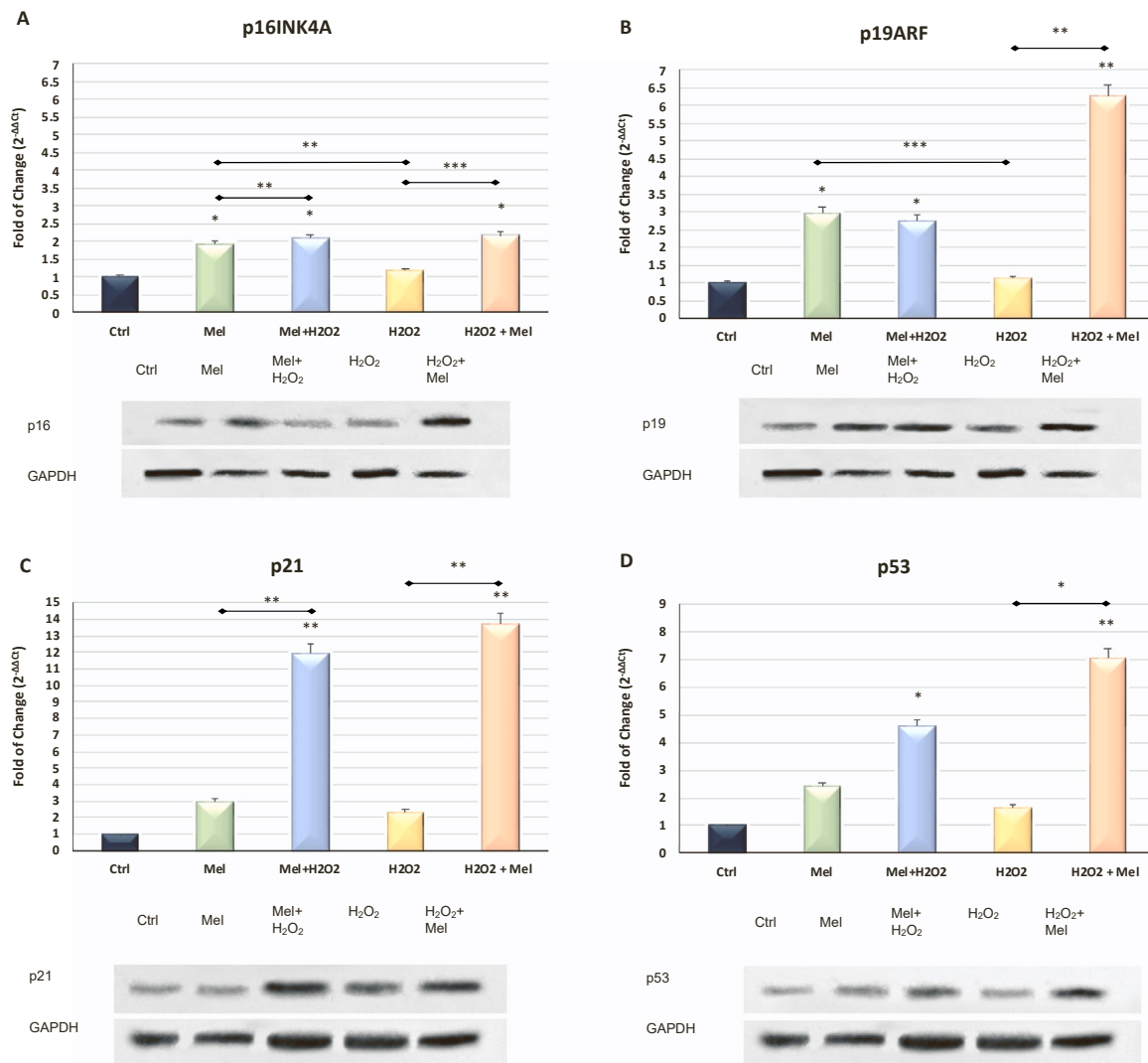


Fig. 5. The expression of p16 (Panel A), p19 (Panel B), p21 (Panel C) and p53 (Panel D) was evaluated in HSPCs exposed to melatonin with or without the pre-treatment with H₂O₂ (orange and green bar respectively) and in HSPCs pre-treated with melatonin and then exposed to H₂O₂ (blue bar), all cultured for 7 days, as compared to control untreated cells (grey bar). The mRNA levels for each gene were normalized to HPRT1 and expressed as fold of change (2^{-ΔΔCt}) of the mRNA levels observed in control untreated cells defined as 1 (mean ± SD; n = 6). Data are expressed as mean ± SD referred to the control (* p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

H₂O₂ exposure (blue bar) or to melatonin after H₂O₂ treatment (orange bar), showed a reduced production of nitric oxide (NO) (Fig. 10) and a better antioxidant activity. Indeed, Fig. 10 shows that melatonin significantly increased catalase activity, eliciting H₂O₂ degradation in oxygen and water (Fig. 11).

3. Discussion

HSPCs are stem cells residing in the bone marrow and used in the treatment of many malignant diseases, as leukemia and lymphoma, to restore the patient's hematopoietic system (Hatzimichael and Tuthill, 2010). Any alterations in HSPC homeostasis and cross talk with their niche may contribute to the pathogenesis of onco-hematologic disorders affecting the hematopoietic cascade (Calvi and Link, 2015). For instance, myelodysplastic syndromes (MDS)-HSPCs exhibit reduced long-term functional capability, undergoing replicative senescence, and promoting inflammation and cancer progression (Mattiucci et al., 2018). Several factors, both intrinsic and extrinsic in nature, contribute to the regulation of the balance between self-renewal and differentiation in stem cells (Kosan and Godmann, 2016). Within this context, melatonin is a molecule with pleiotropic effects, including free radical

detoxification and antioxidant actions, bone formation and protection, immune regulation and oncogenic effects (Panzer and Viljoen, 2022; Tordjman et al., 2017; Bastani et al., 2021). The molecular mechanisms by which melatonin exerts its action are still unclear, but may involve different transduction pathways, one inhibiting adenylyl cyclase and the other regulating phospholipid metabolism and intracellular Ca²⁺ concentration (Vanecek, 1998). In addition, melatonin acts through receptor-dependent and receptor-independent mechanisms, with many of its effects mediated by its metabolites (Slominski et al., 2017). For example, 6-hydroxymelatonin [6(OH)M], N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and 5-methoxytryptamine (5-MT), exhibited selective antiproliferative effects on human primary epidermal keratinocytes, fibroblasts, and melanocytes in vitro (Kim et al., 2013). Melatonin acts directly, by blocking the formation and growth of metastatic cells, or indirectly by modifying the cellular microenvironment. Another possible mechanism of action seems to be related to extracellular vesicles (EVs), due to their role in modulating immune responses, restoring tissue homeostasis, and promoting the healing processes (Feng et al., 2021). Nocturnal melatonin is a key element in regulating the retention of HSPCs in the bone marrow niche via GPCR receptors, preventing their proliferation and

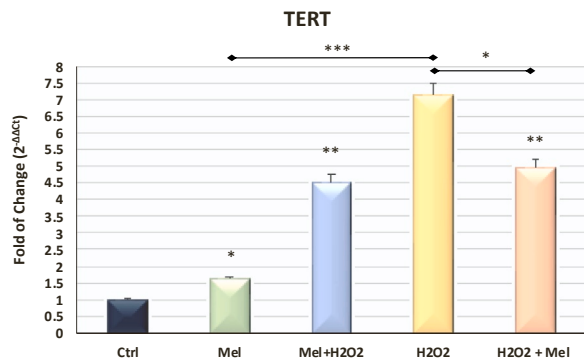


Fig. 6. The expression of TERT was evaluated in HSPCs exposed to melatonin with or without the pre-treatment with H_2O_2 (orange and green bar respectively) and in HSPCs pre-treated with melatonin and then exposed to H_2O_2 (blue bar), all cultured for 7 days, as compared to control untreated cells (grey bar). Yellow bar represents H_2O_2 -stressed HSPCs. The mRNA levels for each gene were normalized to HPRT1 and expressed as fold of change ($2^{-\Delta\Delta C_t}$) of the mRNA levels observed in control untreated cells defined as 1 (mean $\pm$ SD; $n = 6$). Data are expressed as mean $\pm$ SD referred to the control (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

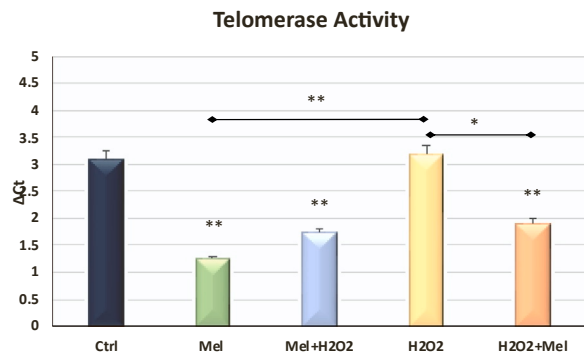


Fig. 7. Effect of melatonin on telomerase activity in HSPCs exposed to melatonin with or without the pre-treatment with H_2O_2 (orange and green bar respectively) and in HSPCs pre-treated with melatonin and then exposed to H_2O_2 (blue bar), all cultured for 7 days, as compared to control untreated cells (grey bar). Yellow bar represents H_2O_2 -stressed HSPCs (mean $\pm$ S.E.; $n = 6$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

translocation of cells from the marrow to the blood (Golan et al., 2018; Golan and Lapidot, 2019). In addition, melatonin regulates the expression of tumor suppressor genes through epigenetic modifications and the induction of tumor cell apoptosis (Bondy and Campbell, 2018; Ferreira et al., 2020). In this regard, trypan blue assay, BrdU assay and microscope images show that melatonin exerts an antiproliferative effect on cells, regulating their typical hyperproliferation and influencing cell size and shape. We then analyzed the expression of the pro-oncogene c-Myc and the main cell cycle regulators, p16, p19, p21 and p53. In addition to its function in regulating cell cycle and transformation, c-Myc is also involved in the apoptotic response (Prendergast, 1999). c-Myc expression is highest in HSCs and decreases during myeloid differentiation (Krygier et al., 2020). Overexpression of c-Myc induces apoptosis in several cell types exposed to growth factor deprivation or hypoxia. Moreover, there is a close correlation between c-Myc and p53 that is dependent on ARF upregulation (Chen et al., 2009; Weber et al., 2002). p19ARF is highly upregulated during human terminal erythropoiesis (Han, Liu, 2017). High levels of c-Myc activate p53 function in ARF-expressing cells. p53 is a tumor suppressor gene, that is induced by DNA damage and other stresses, as well as p21 (He et al., 2005; Zindy

et al., 1998). p21 is considered another tumor suppressor, whose reduced expression has been observed in many solid tumors, usually associated with poor survival (Zhao et al., 2013). p16INK4 and p19 are also two important proteins that act as oncosuppressors, through a tight regulation of p53 (Ai et al., 2003; Ceballos et al., 2022). c-Myc might regulate p19ARF which in turn induces p53 expression, triggering apoptosis (Cleveland and Sherr, 2004). Our results show that melatonin finely tunes both growth stimulatory and inhibitory boosts, by modulating c-Myc gene transcription, as well as the expression of cell cycle arrest-related genes, respectively. c-Myc is a proto-oncogene that plays a role in cell cycle progression and cellular transformation and can contribute to oxidative stress resistance in tumor cells (Miller et al., 2012; Benassi et al., 2006). The finding that in the presence of H_2O_2 c-Myc, as well as p19, p21 and p53 gene transcription, were conversely regulated by melatonin suggests that this molecule was likely modulating a delicate interplay in the course of an oxidative stress, inhibiting cell growth (due to H_2O_2 -induced transcriptional activation of c-Myc), and triggering cell cycle inhibitory players (Fig. 12). The latter response may be viewed in the context of a pleiotropic action of melatonin, attempting at limiting dangerous self-sustained enduring mechanisms in a malignant cell type, as the currently observed anti-senescence response to an oxidative stress. While oxidative stress is usually linked with telomere shortening and decreased telomerase activity (Gav-ia-García et al., 2021), in cancer cells oxidative stress has been reported to enhance the malignant potential by telomerase activation (Nishikawa et al., 2009). This view is further supported by the capability of melatonin to concomitantly activate cell cycle arrest genes, while counteracting the upregulation of TERT transcription and telomerase activity, also decreasing SA- β Gal expression, in H_2O_2 exposed HSPCs. Telomerase activity and hTERT expression have been found to be increased in in some onco-hematologic diseases (Briatore et al., 2009; Nowak et al., 2006). The current observations support the ability of melatonin to maintain cell cycle control and proliferation of HSPCs in such conditions. Moreover, our results confirmed that melatonin exerts antioxidant activity, modulating nitrites production, having a role in the tumor aggressivity, and significantly increasing catalase activity. These effects are closely related to the ability of melatonin to induce tumor cell growth arrest (García-Navarro et al., 2007; Doskey et al., 2016; González et al., 2021). Finally, we analyzed the expression of mTOR, a key regulating factor in hematopoiesis (Malik et al., 2018; Fernandes et al., 2021). An abnormal activation of mTOR induces cell growth and metastasis, influencing autophagy and apoptosis (Zou et al., 2020). Furthermore, hyperactivation of the mTOR pathway affects HSC function inducing leukemia (Kalaitzidis et al., 2012). Confocal microscopy analysis showed that melatonin significantly decreased the expression of mTOR, which, in contrast, was clearly evident in the cytoplasm of control untreated cells and in that of H_2O_2 -stressed HPSCs. In conclusion, our results demonstrate that melatonin effectively counteract HPSC senescence and hyperproliferation, providing a potential therapeutic platform which could be ideally exploited in onco-hematologic disorders affecting the hematopoietic system.

4. Materials and methods

4.1. Ethics statement

This study was performed according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of the Centre for developmental biology and reprogramming (CEDEBIOR) of University of Sassari, Italy. Protocol code N.01/2021, date of approval 18 January 2021.

4.2. Cell isolation and culturing

HPCs and HSCs were isolated from adult female and male patients with onco-hematologic disorders, after achieving complete remission

mTOR expression

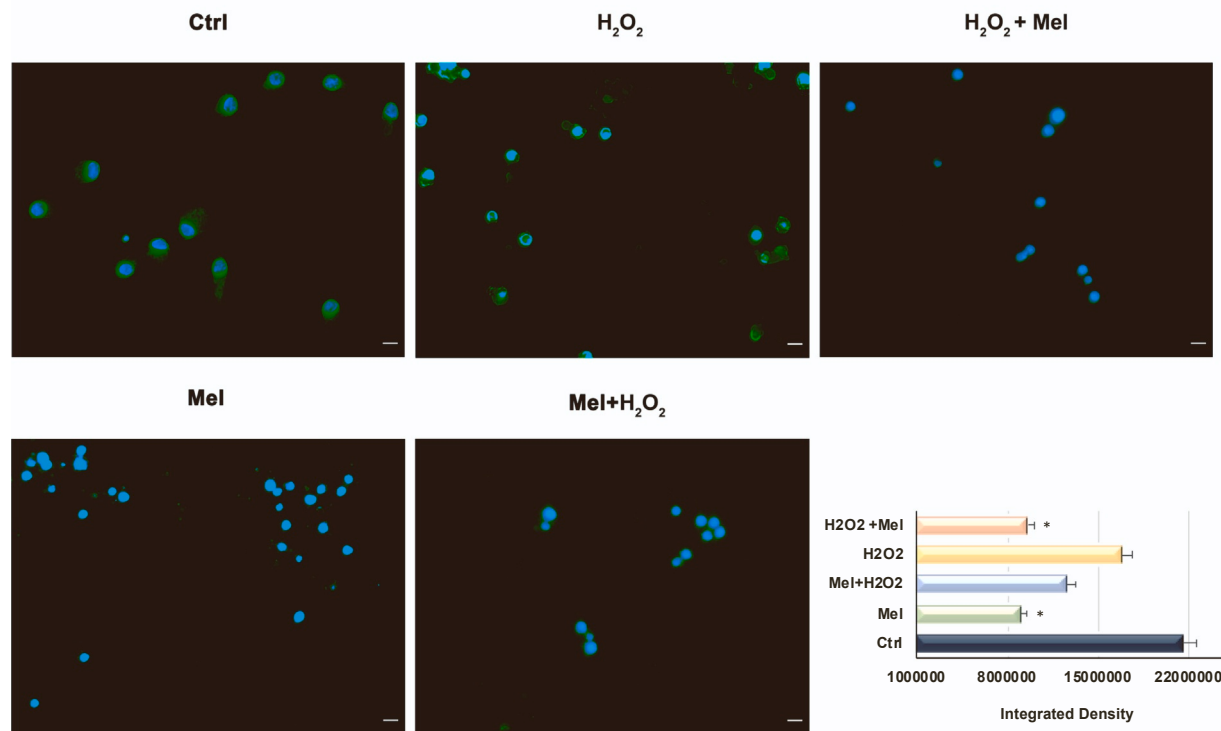


Fig. 8. Analysis of mTOR expression after 7 days in culture. Immunofluorescence analysis of the expression of mTOR was assessed in untreated control HSPCs (Ctrl), and in HSPCs exposed to melatonin with or without the pre-treatment with H_2O_2 and in HSPCs pre-treated with melatonin and then exposed to H_2O_2 (blue bar), after 7 days in culture. H_2O_2 -senescent HSPCs were exposed to H_2O_2 in the growing medium. The figures are representative of different independent experiments. Nuclei are labelled with 4,6-diamidino-2-phenylindole (DAPI, blue). Magnification 40x. Scale bars: 40 μ m. The fluorescence intensity for each treatment was calculated using an image software analysis (ImageJ). Data are expressed as mean $\pm$ SD referring to the control (n = 6; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

and in the context of peripheral stem-cell harvesting finalized to autologous stem cell transplantation (n = 6; age = 50 ± 15 years). All patients were affected by multiple myeloma, an onco-hematologic disorder not directly affecting HSCs and involving the most differentiated stages of B lymphopoiesis. HSPCs were collected after achieving complete remission (i.e., negative immunofixation on the serum and urine and disappearance of any soft tissue plasmacytomas and < 5 % plasma cells in bone marrow) (Durie et al., 2022). Cell collection was performed in the context of stem cell harvesting finalized to autologous stem cell transplantation which is usually applied to further maintain the condition of complete remission. As stem cell harvesting follows a long term chemioimmunotherapy treatment, we may hypothesize that even in patients in complete remission the abnormal gene expression pattern observed in HSCs may mirror the pharmacological pressure exerted by such treatments on the hematopoietic compartment.

The samples were immediately processed using Ficoll-Paque PLUS density gradient media (GE Healthcare Life Sciences, United Kingdom) according to the manufacturer's protocol and cells were then immunoselected for CD34 + expression. The cells obtained were then cultured and seeded in tissue culture dishes measuring 100 mm at a concentration of 8×10^5 – 1×10^6 cells/plate in a medium composed of Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific, USA), 10 % fetal bovine serum (FBS, Thermo Fisher Scientific, USA), 200 mM L-glutamine (Euroclone, Milano, Italy) and 200 U/ml penicillin–0.1 mg/ml streptomycin (Euroclone, Milano, Italy). Cells used as untreated control were cultured for 7 days in the growing medium alone (Ctrl). A group of cells was incubated for 1 h with 100 μ M H_2O_2 in basic growing medium (H_2O_2) and then put in culture for 7 days in the growing medium alone, after removal of H_2O_2 . A group of cells was pre-treated for

1 h with 100 μ M H_2O_2 and then cultured for 7 days in the growing medium supplemented with 0,01 M Melatonin (H_2O_2 + Mel), after removal of H_2O_2 . Another group of HSPCs was cultured for 7 days in the growing medium supplemented with 0,01 M Melatonin (Mel). A group of cells was pre-treated for 7 days with 0,01 M Melatonin and then, after removal of melatonin, exposed for 1 h to 100 μ M H_2O_2 (Mel+ H_2O_2).

4.3. Trypan Blue exclusion test of cell viability

The dye exclusion test was used to evaluate the number of viable cells cultured in the above-described condition. For each experimental condition, 5.0×10^5 cells/plate were seeded and counted at the end of 7 days in culture. The cell suspension was collected and mixed with 0.4 % dye solution (Thermo Fisher Scientific, USA) in a proportion of 1:1 and then counted using a LUNA-II™ Automated Cell Counter, to obtain information about the cell viability and cell size distributions. The percentage of vital cells was calculated as the number of positive cells divided by the total number of counted cells (mean $\pm$ SE; n = 6).

4.4. BrdU cell proliferation assay

The BrdU Cell Proliferation Assay (Roche, Sigma-Aldrich, Darmstadt, Germany) was used for the quantification of cell proliferation, based on measurement of the incorporation of 5-bromo-2'-deoxyuridine during DNA synthesis. Cells were seeded at a concentration of 5.0×10^5 cells/plate and cultured under the above-described condition for 7 days. Cells were then fixed and incubated with 1X detection antibody solution and then with 1X HRP-conjugated secondary antibody solution, according to the manufacturer's protocol. Cell proliferation was measured by

SA β -gal Activity

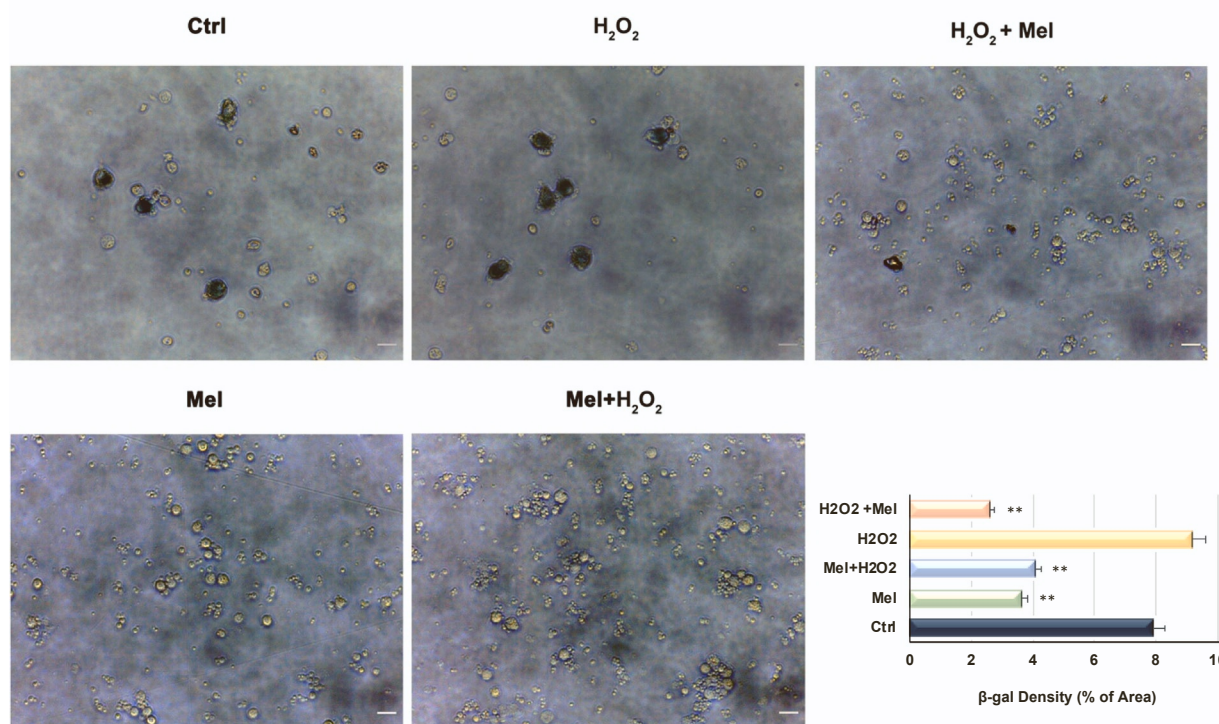


Fig. 9. Senescence-associated β -galactosidase activity. β -galactosidase was evaluated in HSPCs cultured in the presence of melatonin with or without pre-treatment with H_2O_2 . Senescent HSPCs were exposed to H_2O_2 in the growing medium. All cells were compared to control untreated HSPCs (Ctrl). Scale bar = 100 μ m. Magnification 40x. The percentage of SA- β -Gal-positive cells for each treatment was calculated as the number of positive cells divided by the total number of cells counted using an image software analysis (ImageJ). Data are expressed as mean $\pm$ SD referring to the control (n = 6; * p $\leq$ 0.05; ** p $\leq$ 0.01; *** p $\leq$ 0.001). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

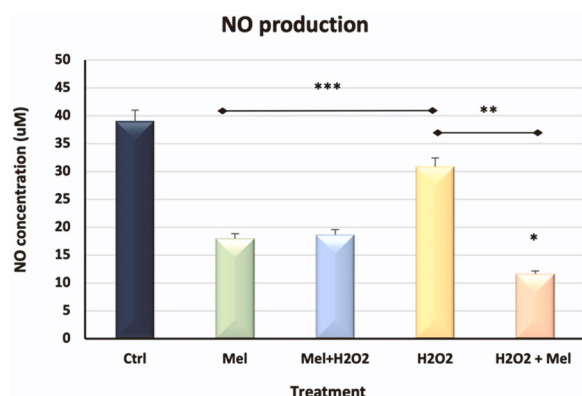


Fig. 10. Nitric oxide production. Nitrite concentration was evaluated in HSPCs exposed to melatonin with or without the pre-treatment with H_2O_2 (orange and green bar respectively) and in HSPCs pre-treated with melatonin and then exposed to H_2O_2 (blue bar), after 7 days in culture, as compared to control untreated cells (grey bars). Yellow bar represents H_2O_2 -stressed HSPCs. The absorbance of the various samples was measured at 548 nm. The concentration was read corresponding to the absorbance of the experimental samples from the standard curve. Data are expressed as mean $\pm$ SD referring to the control (mean $\pm$ S.E.; n = 6; * p $\leq$ 0.05; ** p $\leq$ 0.01; *** p $\leq$ 0.001).

spectrophotometric reading at 370 nm (Akribis Scientific, Common Farm, Frog Ln, Knutsford WA16 0JG, Great Britain) and expressed in OD units. Data are expressed as mean $\pm$ SD (n = 6), referring to the control untreated cells.

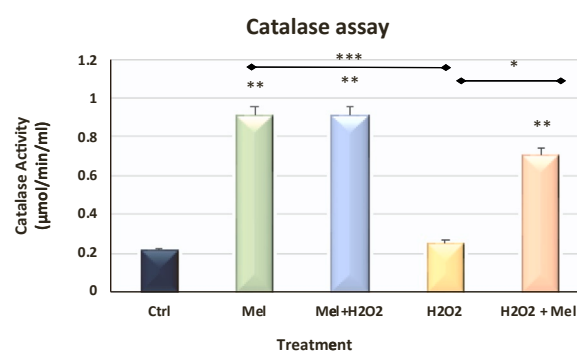


Fig. 11. Catalase activity. The activity of catalase was evaluated in HSPCs exposed to melatonin with or without the pre-treatment with H_2O_2 (orange and green bar respectively) and in HSPCs pre-treated with melatonin and then exposed to H_2O_2 (blue bar), after 7 days in culture, as compared to control untreated cells (grey bars). Yellow bar represents H_2O_2 -stressed HSPCs. The absorbance of the various samples was measured at 520 nm. The experiments were performed two times with three technical replicates for each treatment. Data are expressed as mean $\pm$ SD referring to the control (mean $\pm$ S.E.; n = 6; * p $\leq$ 0.05; ** p $\leq$ 0.01; *** p $\leq$ 0.001).

4.5. Gene expression analysis of real-time PCR

For each experimental condition, 5.0×10^5 cells/plate were used. RNA extraction for all samples was performed from HSPCs at the end of 7 days in culture in the above-described conditions, using the ChargeSwitch total RNA cell kits (Thermo Fisher Scientific, USA) after 7 days

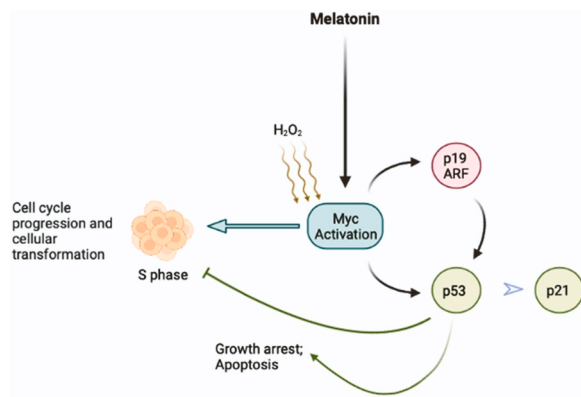


Fig. 12. p19-ARF-p53 induction following c-Myc activation, which can force proliferation or trigger apoptosis. Created with BioRender.com.

in culture. Approximately 1 µg of total isolated mRNA was amplified with real-time PCR using a CFX-96 Thermal Cycler (Bio-Rad) (Applied Biosystems). Quantitative polymerase chain reaction was performed in triplicate according to the protocol specified in the Luna® Universal One-Step RT-qPCR Kit (New England Biolabs), according to the manufacturer's protocol. The standard qRT-PCR conditions were 55 °C for 10 min, 95 °C for 1 min, and then cycled at 95 °C for 10 s, 60 °C for 30 s, for 40–45 cycles. The total volume of each reaction was 20 µl, composed of Luna Universal One-Step Reaction Mix (2X), Luna WarmStart® RT Enzyme Mix (20X), 0.4 µM of each primer, and the total RNA template. The target Ct values were normalized on HPRT1, considered as a reference gene, while the mRNA levels of the HSPCs cultured in the presence or absence of melatonin with or without the pre-treatment with H₂O₂ were expressed as fold of change ($2^{-\Delta\Delta C_t}$) relative to the mRNA levels observed in the control untreated HSPCs. Each experiment included a distilled water control. The qRT-PCR analysis was performed for the following genes: Cellular myelocytomatosis oncogene (c-Myc); tumor suppressor protein p16 (p16INK4A); RNA silencing suppressor p19 ARF (p19ARF), cyclin-dependent kinase inhibitor 1 (p21), tumor suppressor protein p53 (p53) and telomerase reverse transcriptase (TERT). All primers were obtained from Invitrogen and are presented in Table 2.

4.6. Telomerase activity detection

For each experimental condition, 5.0×10^5 cells/plate were used. To evaluate the telomerase activity, the TRAPeze® Kit RT Telomerase Detection Kit (Millipore, MA, USA) was used. It directly measures the fluorescence emission of telomerase activity through real-time PCR. The HSPCs were cultured for 7 days in the above-described conditions. All samples underwent the procedure in triplicate using a CFX-96 Thermal Cycler (Bio-Rad), accordingly with the manufacturer's instructions. Briefly, the cells were lysed with a CHAPS lysis buffer included in the kit; the protein concentrations were determined using Nanodrop (Thermo Fisher Scientific, USA). The master mix was composed of 5X TRAPeze® RT reaction mix, Taq polymerase (5 units/µl), nuclease free water, and samples, for a final volume of 20 µl. The PCR amplification conditions

Table 2

Primer sequences.

Primer	Forward	Reverse
HPRT1	AGCCCTGGCGTCGTGATTA	TGGCTCCCATCTCCTTCA
c-Myc	TGAGGAGACACGCCAC	CAACATCGATTCTCTCATCTTC
p16INK4	CAACGCACCGCTAGTTACGG	AACCTCGTCTCCAGAGTCGC
p19ARF	GCCITCGGCTGACTGGCTGG	TCGTCTCCAGAGTCGCCCC
p21	CAAAGGCCGCTCTACATCTT	AGGAACCTCTCATTACCCGA
p53	TGGCCTTGAAACACCTTTT	AACACCAACCCACAGCCAA
TERT	GACGTGAAGATGAGCGTG	GACGACGTACACATCATC

were 30 °C for 30 min, 95 °C for 2 min, and then cycled at 94 °C for 15 s, 59 °C for 60 s, and 45 °C for 10 s for 45 cycles. Each sample included a negative and a positive control. The telomerase activity of each sample was normalized to the Ct of the standard curve generated from the control reaction mix included in the kit. Telomerase activity was calculated by comparing the average Ct values from the samples against the standard curve generated by the TSR8 control template (mean ± SE; n = 6; **p < 0,01).

4.7. Immunostaining

HSPCs (2.0×10^4 cells/well in 8-well chamber slides) were cultured for 7 days in the above-described conditions and fixed with 4 % of paraformaldehyde (Sigma Aldrich Chemie GmbH, Germany) for 30 min at room temperature. After permeabilization by 0.1 % Triton X-100 (Thermo Fisher Scientific, Grand Island, NY, USA)-PBS, cells were washed in PBS three times for 5 min. After washing, HSPCs were incubated with 3 % Bovine Serum Albumin (BSA)—0.1 % Triton X-100 in PBS (Thermo Fisher Scientific, Grand Island, NY, USA) for 30 min and then exposed overnight at 4 °C to the primary anti-rabbit polyclonal antibodies directed against mTOR (Abcam, United Kingdom). Finally, cells were washed two times in PBS for 5 min and stained at 37 °C for 1 h in the dark with the fluorescence-conjugated goat anti rabbit IgG secondary antibody (Life Technologies, USA). Nuclei were labelled with 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, Grand Island, NY, USA). All microscopy analyses were performed with a confocal microscope (TCS SP5, Leica, Nussloch, Germany).

4.8. SA-β-Gal Staining

To identify senescent cells in culture, “The Senescence Cells Histochemical Staining Kit” (Sigma-Aldrich, Germany) was used. HSPCs (1.0×10^4 cell/well in 24-well plate) were cultured in 6-well plate for 7 days in the above described conditions. At the end of 7 days, cells were then fixed and processed according to the manufacturer's instructions. For evaluation of SA-β-Gal activity, cells were then observed by light microscopy. The number of positively blue-stained cells was calculated as the percentage of total number of cells.

4.9. Nitric oxide (NO) determination

Nitrites determination was performed using the Griess Reagent kit for Nitrite Determination (Thermo Fisher Scientific, Grand Island, NY, USA) in HSPCs cultured in the above describe conditions. HSPCs (5.0×10^3 /well in 96-well plate) were cultured for 7 days and then incubated with Griess reagent accordingly with the manufacturer's instructions. A photometric reference was prepared using the standard nitrite solution. The absorbance of each samples was measured in triplicate by spectrophotometric reading at 548 nm (Akribis Scientific, Common Farm, Frog Ln, Knutsford WA16 0JG, Great Britain). Nitrite determination was calculated corresponding to the absorbance of experimental samples from the standard curve.

4.10. Antioxidant activity

The catalase antioxidant activity was evaluated by Catalase Assay Kit (Sigma-Aldrich, Saint Louis, MO, USA), evaluating the activity of this enzyme in HSPCs cultured under the above described conditions. HSPCs (5.0×10^3 /well in 96-well plate) were cultured for 7 days and then incubated with the reagents present in the kit at room temperature for 15 min to evaluate color development. The absorbance of each samples was measured in triplicate by spectrophotometric reading at 520 nm (Akribis Scientific, Common Farm, Frog Ln, Knutsford WA16 0JG, Great Britain). The activity of catalase was calculated on the number of micromoles present in each sample, referring of a standar curve, and compared with the activity of the untreated control.

4.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0 software (GraphPad, San Diego, CA, USA). For this study Wilcoxon signed-rank test was used to evaluate the congruity of the observed set, while Kruskal-Wallis rank sum was used to assess the values found in the different observation topics, assuming a p value < 0.05 as statistically significant. ANOVA test followed by a post hoc test was used to compare the effects of melatonin and hydrogen peroxide. Then, each experimental group was compared to its own control by independent Mann-Whitney U test non-parametric data. All the experiments were performed two times with three technical replicates for each sample ($n = 6$).

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CRediT authorship contribution statement

Conceptualization, S.C. and M.M.; Methodology, S.C.; G.G.; R.P.; A.R. P.B.; Validation, C.V.; C.F. and M.M.; Formal analysis, S.C.; Investigation, S.C. and M.M.; Resources, M.M.; Data curation, S.C.; Writing—original draft preparation, S.C.; Writing—review and editing, C.V.; C.F. and M.M.; Visualization, F.F. and C.F.; Supervision, M.M. All authors have read and agreed to the published version of the manuscript.

Institutional review board statement

This study was performed according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of the Centre for developmental biology and reprogramming (CEDEBIOR) of University of Sassari, Italy. Protocol code N.01/2021, date of approval 18 January 2021.

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest

The authors declare no conflict of interest.

Data Availability

All the data is contained in the manuscript and supplementary material.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ejcb.2022.151251](https://doi.org/10.1016/j.ejcb.2022.151251).

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