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ORIGINAL RESEARCH

Effects of melatonin on orofacial pain relief by regulating mitochondrial function in cell viability of peripheral sensory neurons

Yingying You[1], Xianping Yi[2], Hongwen He[3,4,*], Fang Huang[3,4,*]

[1]Department of Stomatology, The Fifth Affiliated Hospital, Sun Yat-sen University, 519000 Zhuhai, Guangdong, China
[2]Department of Pathology, The Fifth Affiliated Hospital, Sun Yat-sen University, 519000 Zhuhai, Guangdong, China
[3]Hospital of Stomatology, Guanghua School of Stomatology, Sun Yat-sen University, 510623 Guangzhou, Guangdong, China
[4]Guangdong Provincial Key Laboratory of Stomatology, 510080 Guangzhou, Guangdong, China

***Correspondence**
hfang@mail.sysu.edu.cn (Fang Huang);
hehw@mail.sysu.edu.cn (Hongwen He)

Abstract

Melatonin (MT) is involved in the pain regulation of peripheral neurons, which is relevant to cell viability. This study aimed to examine the cell proliferation, cell cycle, cell apoptosis and intracellular mitochondrial function of ND7/23 and PC-12 cells treated with different physiological concentrations of MT. Our results showed that MT at concentrations of 10^{-8}, 10^{-10} and 10^{-12} M inhibited cell proliferation and promoted the apoptosis of two cell lines, with the most significant changes observed at a concentration of 10^{-12} M. Further, 10^{-12} M MT promoted mitochondrial respiratory electron transfer and increased mitochondrial function in ND7/23 and PC-12 cells through the non-membrane receptor pathway. Comparatively, 10^{-8} M MT enhanced the mitochondrial effects of ND7/23 cells but showed opposite effects in PC-12 cells. In summary, MT affected cell viability through the non-membrane receptor pathway in a concentration-dependent manner and might be associated with pain regulations.

Keywords

Melatonin; ND7/23; Cell viability; Mitochondrial function

1. Introduction

Orofacial pain (OFP) refers to pain associated with soft and hard tissues of the head, face and neck [1] and is a universal cause for visiting pain clinics [2]. It has a common occurrence in the general population, has profound sociologic effects and can affect the sufferer's quality of life. As the trigeminal system is the primary sensory innervation of the orofacial structures and the cell bodies of first-order sensory neurons are in the trigeminal ganglion (TG) [3], trigeminal neuralgia (TN) accounts for most of the presentations of OFP.

Although animal models are often used to study pain, considering that most signs are objective, indistinct results are often observed due to differences between models or compared with humans. Thus, *in vitro* studies are essential to assess the specific effects of drugs at the cellular level and determine the potential underlying molecular pathways of pain mechanisms. However, due to the low proliferation rate of neurons, primary neuronal cell culture seriously restricts subsequent *in vitro* explorations of cell functions, drug effects and molecular mechanisms. In such cases, immortalized neuron cell line is widely used because it is relatively easy to culture and maintain, and it retains the characteristics of neurons, such as ion channels [4] and neurotransmitter receptors [5], which is conducive to further study of molecular pathways, especially for pain research.

However, there are no cell lines derived from trigeminal neurons. The dorsal root ganglion (DRG) and TG are homologous nerve tissues and the first transfer stations of sensory input of the body. They have many similarities in the expressions of cell surface receptors [5–8], ion channels [4, 9, 10] and responses to nociceptive stimuli such as capsaicin and bradykinin [11]. Hence, the study of DRG-derived cell lines can partly reflect the underlying pain mechanisms of TG neurons.

There are sensory and peptidergic neurons in TG and DRG. The sensory neurons are responsible for the conduction of nociceptive sensation and mechanical proprioception, while peptidergic neurons participate in pain regulation on neuropeptide release. Therefore, a single cell line cannot completely simulate TG neurons. According to neurons' characteristics, secretion, pain receptors' expressions and so on, ND7/23 and PC-12 cells are used as cell models to simulate TG neurons *in vivo*.

ND7/23 cells are derived from the fusion culture of newborn rat DRG neurons and N18TG2 mouse neuroblastoma cells [12, 13], which have the characteristics of peripheral neurons [14]. ND7/23 has a gene expression profile and phenotype related to DRG neurons [15], which has been used to study the response to nociceptive stimulus and downstream signal pathways [13]. PC-12 cells, a classical neurobiological model, are rat pheochromocytoma cells [16] that contain neuronal features, such as neurosecretion (CGRP, Calcitonin Gene Related Peptide [17]), ion channels [18] and neurotransmitter receptors [19], and are widely used as a cell transfection model to study

neuroinflammation [16], neuropeptides and pain [17, 20].

Melatonin (MT) is an endogenous neuroendocrine hormone. It can regulate the circadian rhythm [21] and pain, has antioxidant, anti-inflammatory and free radical scavenging activities, and can protect nerve cells viability. Its secretion is low during the day and high at night [22], and its physiological concentration ranges from 10^{-12} to 10^{-8} M. MT has either "analgesia" or " pain promoting " effects, which may be related to injury-type, duration of damage, site of action and concentration of MT. However, despite numerous research on MT, its pain regulation mechanism remains unclear.

Our previous study showed that the pain pattern of OFP might be related to MT secretion during the day and night [23]. MT can interact with cells through membrane receptors, nuclear receptors and free diffusion. There are two types of membrane receptors in mammals: MT1R and MT2R. Luzindole is a non-selective antagonist of the MT membrane receptor that can simultaneously block the binding of MT to MT1R and MT2R [24].

Cell viability refers to the ability of cells to maintain their physiological functions, such as metabolism and proliferation. It is affected by environmental factors or diseases, including changes in the growth environment, drugs, injury, infection and so on, and has an impact on cell proliferation or apoptosis. Therefore, the detection of cell viability can help determine the effects of drugs on cell proliferation.

The cell cycle refers to the entire process of a cell, from the completion of one cell division to the end of the next. Different intercellular phases have different DNA content, and drugs can interfere with these intercellular phases to affect cell proliferation.

Ectropion of Phosphatidyl serine (PS) refers to a change in cell membrane morphology and is one of the signals of early apoptosis. Changes in the apoptotic state can affect cell viability. Mitochondrial membrane potential (MMP) is synthesized by mitochondria during oxidative respiration. It is stored in the inner membrane of mitochondria as electrochemical potential energy and can cause the asymmetric distribution of electron concentrations. It is the premise of maintaining oxidative phosphorylation and Adenosine Triphosphate (ATP) production of mitochondria to sustain the normal physiological function of cells. Thus, a decrease in MMP is one of the early signals of apoptosis.

Reactive oxygen species (ROS) are small molecules derived from oxygen, formed by combining with the electrons leaked from the respiratory chain. ROS, a marker of mitochondrial function, is usually produced during mitochondrial oxidative phosphorylation and mitochondrial fission. Excessive ROS production occurs when cells are under stress, mitochondria are excessively broken, or when mitochondrial function is enhanced.

Mitochondria play an important part in respiratory activities. Coenzyme I (Nicotinamide Adenine Dinucleotide (NAD^+), reduced from Nicotinamide Adenine Dinucleotide (NADH)) and Coenzyme II (Nicotinamide Adenine Dinucleotide phosphate ($NADP^+$), reduced from Nicotinamide Adenine Dinucleotide phosphate (NADPH)) are electron transporters in redox reactions that participate in the tricarboxylic acid cycle and respiratory chain. Its redox state is an important parameter of mitochondrial function.

Many studies have confirmed that pain is related to mitochondrial dysfunction; however, the role of mitochondrial function in OFP has not been reported, and the mechanism of MT on peripheral pain remains unclear. Therefore, this research detected the changes in cell proliferation and cell and mitochondrial function of peripheral sensory neurons at the physiological concentration of MT to preliminarily study the mechanism of MT in regulating cell viability of peripheral sensory neurons, pave the way for revealing the mechanism of MT regulating OFP, provide a scientific basis for new drug development and explore feasible measures to alleviate or prevent chronic pain by protecting mitochondrial function.

2. Materials and methods

2.1 Cell culture and reagents

ND7/23 cell lines were obtained from the European Collection of Authenticated Cell Culture (London, England, UK). The cells were grown in low glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% GlutaMAX™ (35050-061, Gibco, Grand Island, USA) and 10% fetal bovine serum (FBS) (10100-147, Gibco, Grand Island, USA) in a humidified incubator at 37 ℃ with 5% Carbon dioxide (CO_2). They were then split into a subconfluent culture at a 1:4 ratio and seeded at 2×10^4 cells/cm^2.

PC-12 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in RPMI 1640 medium supplemented with 5% FBS and 10% horse serum (26050-088 Gibco, Grand Island, USA) at the same incubation condition as ND7/23 cells.

All cell culture reagents were obtained from Gibco (Invitrogen, Grand Island, USA). A stock solution of MT (Sigma Aldrich St Louis, Mo, USA) was prepared by dissolving it in dimethyl sulfoxide (DMSO) (D5879, Sigma Aldrich, Saint Louis, USA). The stock solutions were then dissolved in the culture media for cell treatment (MT range, 10^{-12}–10^{-8} M). Luzindole solution was also prepared in DMSO and dissolved at 2 μM in the culture media. The final concentration of DMSO was far below 0.05% (v/v).

2.2 Cell viability assay

Cell viability was assessed with the Cell Counting Kit-8 (CCK-8, Dojindo, Molecular Technologies, Kumamoto, Japan). ND7/23 and PC-12 cells were seeded onto a 96-well plate and allowed to adhere to the walls (10^4/well). The cells were treated with MT (10^{-8} M, 10^{-10} M and 10^{-12} M) for 24 h, 48 h and 72h. Cells cultured in a complete medium were used as control. Each group was provided with 6 parallel wells. Then, 10 μL CCK-8 solution was added to each well and incubated for 1 h, and the optical density was measured using a spectrophotometer (Elx800, Biotek, Winooski, VT, USA) at 450 nm. Three independent experiments were conducted.

2.3 Cell viability assay with Luzindole pretreatment

After the ND7/23 and PC-12 cells had adhered, they were divided into five groups: (1) control group: cultured in a complete medium; (2) MT8 group: cultured with 10^{-8} M MT; (3) L8 group: pretreated with 2 μM Luzindole for 30 min then cultured with 10^{-8} M MT; (4) MT12 group: cultured with 10^{-12} M MT; and (5) L12 group: pretreated with 2 μM Luzindole for 30 min then cultured with 10^{-12} M MT. Six wells were run in parallel for each group. The cells were cultured for 24 h, 48 h, 72 h and 96 h. Then, each well was treated with CCK-8 and assessed using a spectrophotometer. The experiment was repeated three times.

2.4 Cell cycle assay

ND7/23 and PC-12 cells, treated with or without melatonin (10^{-8} M, 10^{-10} M, and 10^{-12} M) for 72 h, were removed from 25T culture flasks. After TrypLE™ (12604-039, Gibco, Grand Island, USA) digesting and centrifugal separation, the cells were fixed in 70% ethanol at 4 ℃ for 18 h in the dark, filtrated and adjusted to 10^6 cells/mL, and re-dissolved in FxCycle (Life Technologies, Carlsbad, USA) containing PI (Propidium Iodide) and RNase for 30 min in the dark at room temperature. The cell cycle was evaluated using flow cytometry (Beckman Coulter, Brea, CA, USA) at 535/617 nm, and results were analyzed using the following formulas:

$$S\ phase\ ratio\,(\%) = \frac{number\ of\ cells\ in\ phase\ S}{total\ cell\ count} \times 100\%$$

$$Proliferation\ Index\,(\%) = \frac{number\ of\ cells\ in\ phase\ S\ and\ G_2}{total\ cell\ count} \times 100\%$$

2.5 Apoptosis assay

Cell grouping and preparation were similarly performed as the cell cycle assay. Briefly, the cells were collected, rinsed and incubated with Annexin V-FITC/propidium (PI) double staining assay (559763, BD, Bergen, NJ, USA) for 15 min at room temperature. Apoptosis was determined by flow cytometry at 488/520 nm, and results were analyzed using FlowJo. The proportions of Annexin V-positive cells were reordered as apoptotic rates.

2.6 Measurement of MMP

ND7/23 and PC-12 cells were divided into five groups: (1) control group: cultured in a complete medium; (2) MT8 group: treated with 10^{-8} M MT; (3) MT10 group: treated with 10^{-10} M MT; (4) MT12 group: treated with 10^{-12} M MT; and, (5) antagonist group: pretreated with 2 μM Luzindole for 30 min then cultured with 10^{-12} M MT. MMP was analyzed using the Mitochondria Membrane Potential Kit (MAK160, Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. After various operations, the cells were collected and incubated with a staining solution in an incubator at 5% CO_2 and 37 ℃ for 30 min in the dark. The fluorescence intensity (λex = 540/λem = 590 nm) was measured using a Multiplus Plate Reader (GloMax, Promega Corporation, Wisconsin, USA).

2.7 NAD(P)$^+$/NAD(P)H assay

Cell grouping and preparation were the same as the measurement of MMP. Intracellular NAD(P)$^+$ and NAD(P)H levels of cells were measured using a NAD/NADH and NADP$^+$/NADPH Quantification Kit, respectively (MAK312, Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's instructions. Briefly, the cells were deproteinized by spin filter and extracted with 400 μL Extraction Buffer by freeze/thawing for 2 cycles (freeze: 20 min on dry ice; thawing: 10 min at room temperature). Intracellular NAD(P)$^+$ was decomposed to NAD(P)H by taking out 200 μL samples and heating them to 60 °C for 30 min. Total NAD(P)t and NAD(P)H were plated in a 96-well plate in the dark. Absorbance was measured at 450 nm using a spectrophotometer. NAD(P)$^+$/NAD(P)H ratio was calculated using the following equation: $Ratio = \frac{NADPt - NAPDH}{NADPH}$.

2.8 Measurement of intracellular reactive oxygen species (ROS)

Cell grouping was the same as the measurement of MMP. ND7/23 and PC-12 cells were prepared as single-cell suspensions using TrypLE™ and resuspended in each complete medium at 5×10^5 cells/mL. The cells were then incubated in 0.5 μM CellROX® Red Reagent (C10422, Invitrogen, Grand Island, NY, USA) at 37 °C for 60 min in the dark and washed with Phosphate Buffer Saline (PBS). Fluorescence intensity (blue: λex = 444/λem = 480 nm, deep red: λex = 644/λem = 665 nm) was determined using a flow cytometer (Beckman Coulter, CA, USA). The mean intensity of red fluorescence was used to represent intracellular ROS levels.

2.9 Statistical analysis

The data was presented as mean ± standard deviation (SD). If the studentized residual of the data exceeded ± 3 times the SD, the data was judged as an outlier. The Kolmogorov-Smirnov test was used to test for normal distribution. Multiple comparisons were analyzed using two-way repeated measures Analysis of Variance (ANOVA) followed by the Bonferroni post hoc test. Statistical analysis was performed using the SPSS 20.0 software (IBM Corp. Armonk, NY, USA). $p < 0.05$ was used to indicate statistical significance.

3. Results

3.1 Melatonin inhibited cell proliferation

Cell viability tests revealed that physiological concentrations of MT briefly inhibited the proliferation of ND7/23 and PC-12 cells (Fig. 1A–B). Compared with control, the proliferation of ND7/23 cells was significantly inhibited on days 2 and 3 ($p < 0.05$). The maximal decrease of proliferation was observed at

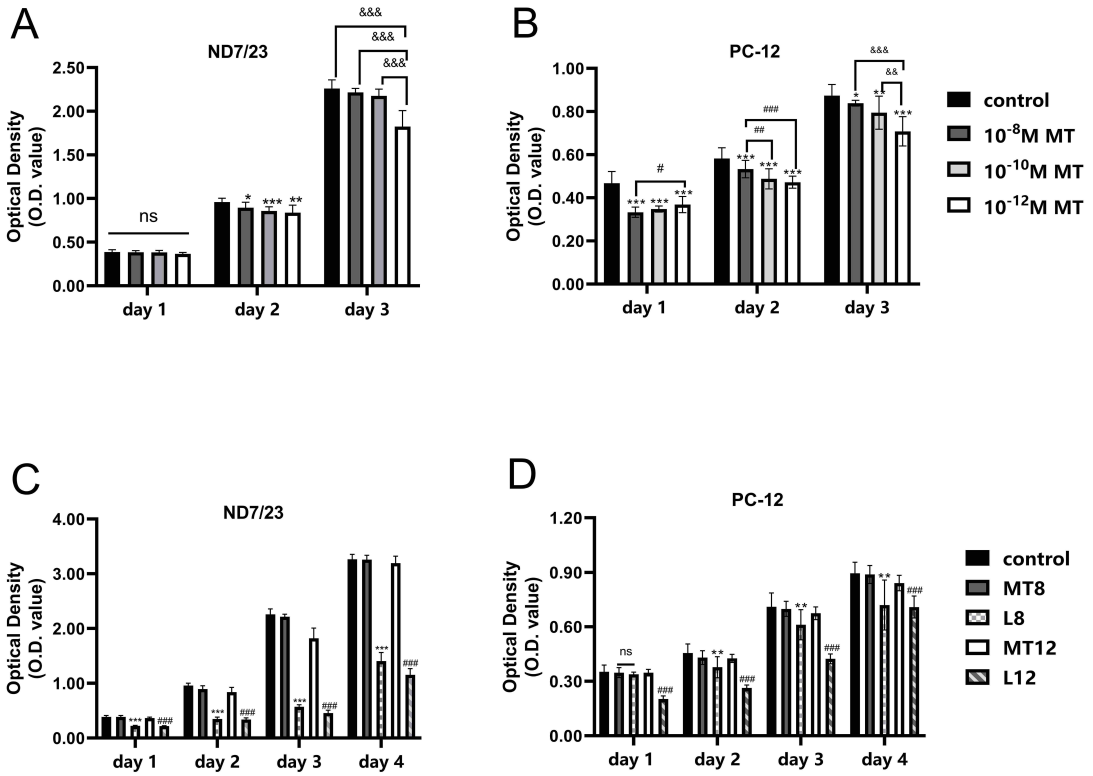


FIGURE 1. Effect of MT on cell viability and cell proliferation with Luzindole pretreatment. Effect of MT on cell viability of ND7/23 cells (A) and PC-12 cells (B). Compared with control, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; compared with 10^{-8} M MT, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; Effect of MT on cell proliferation of ND7/23 cells (C) and PC-12 cells (D) with Luzindole pretreatment. Compared with 10^{-12} M MT, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$.Compared with MT8 group, $^{**}p < 0.01$, $^{***}p < 0.001$; compared with MT12 group, $^{\#\#\#}p < 0.001$. MT: Melatonin.

10^{-12} M MT.

Compared with control, the proliferation of PC-12 cells was significantly suppressed with MT treatment on day 3 ($p < 0.05$). On days 2 and 3, MT was found to be dose-dependent as a lower concentration of MT led to greater inhibition of PC-12 cells ($p < 0.01$), which was opposite to the trend on day 1.

3.2 Luzindole pretreatment promoted the inhibition of cells incubated with MT

Luzindole was used to determine whether 10^{-8} M and 10^{-12} M MT inhibited the proliferation of cells through the membrane pathway. Compared with the MT8 and MT12 group in ND7/23 cells, the values were significantly reduced in L8 and L12 group on days 1 to 4 ($p < 0.001$, Fig. 1C). Further, except for comparison with day 1 between the MT8 and L8 group in PC-12 cells, the values of the L8 and L12 group were significantly lower than the MT8 and MT12 group on days 1 to 4 (MT8 compared with L8, $p < 0.01$ and MT12 compared with L12, $p < 0.001$; Fig. 1D). These results demonstrated that MT could suppress the proliferation of ND7/23 and PC-12 cells via non-membrane receptor pathways.

3.3 Melatonin promoted ND7/23 cell apoptosis

Cell cycle assay showed no statistical differences between each group for the value of S phase and PI ($p > 0.05$), indicating that the physiological concentrations of MT had no effects on the cell cycle of ND7/23 cells (Fig. 2A–B).

The apoptosis rates of the 10^{-10} M and 10^{-12} M MT groups were higher than the control group ($p < 0.001$ and $p < 0.05$, respectively, Fig. 2C), while the 10^{-8} M MT group showed an upward trend without any statistically significant difference ($p > 0.05$, Fig. 2D). Among the MT groups, the apoptosis rate of 10^{-8} M MT was less than that of 10^{-10} M ($p < 0.001$) and 10^{-12} M MT ($p < 0.05$), which indicated that the physiological concentration of MT promoted ND7/23 cell apoptosis, with the 10^{-10} M MT group exhibiting the most pronounced apoptosis effect.

3.4 Melatonin interfered with PC-12 cell cycle and promoted cell apoptosis

Cell cycle assay showed no statistical differences between each group for the value of S phase ($p > 0.05$, Fig. 3A–B), while the value of the proliferation index (PI) of 10^{-8} M MT was lower than the control group ($p < 0.05$). The PI value of 10^{-12} M MT was significantly higher than 10^{-8} M MT ($p < 0.01$) and 10^{-10} M MT ($p < 0.05$), indicating that 10^{-8} M MT could inhibit the proliferation of PC-12 cells, which showed the most significant effect among the MT groups.

The apoptosis rates of 10^{-8} M, 10^{-10} M and 10^{-12} M MT were higher than the control group ($p < 0.01$, $p < 0.001$ and $p < 0.001$, respectively, Fig. 3C–D). Among the MT groups, the apoptosis rate of 10^{-8} M MT was lower than that of 10^{-10} M ($p < 0.05$) and 10^{-12} M MT ($p < 0.001$), indicating that

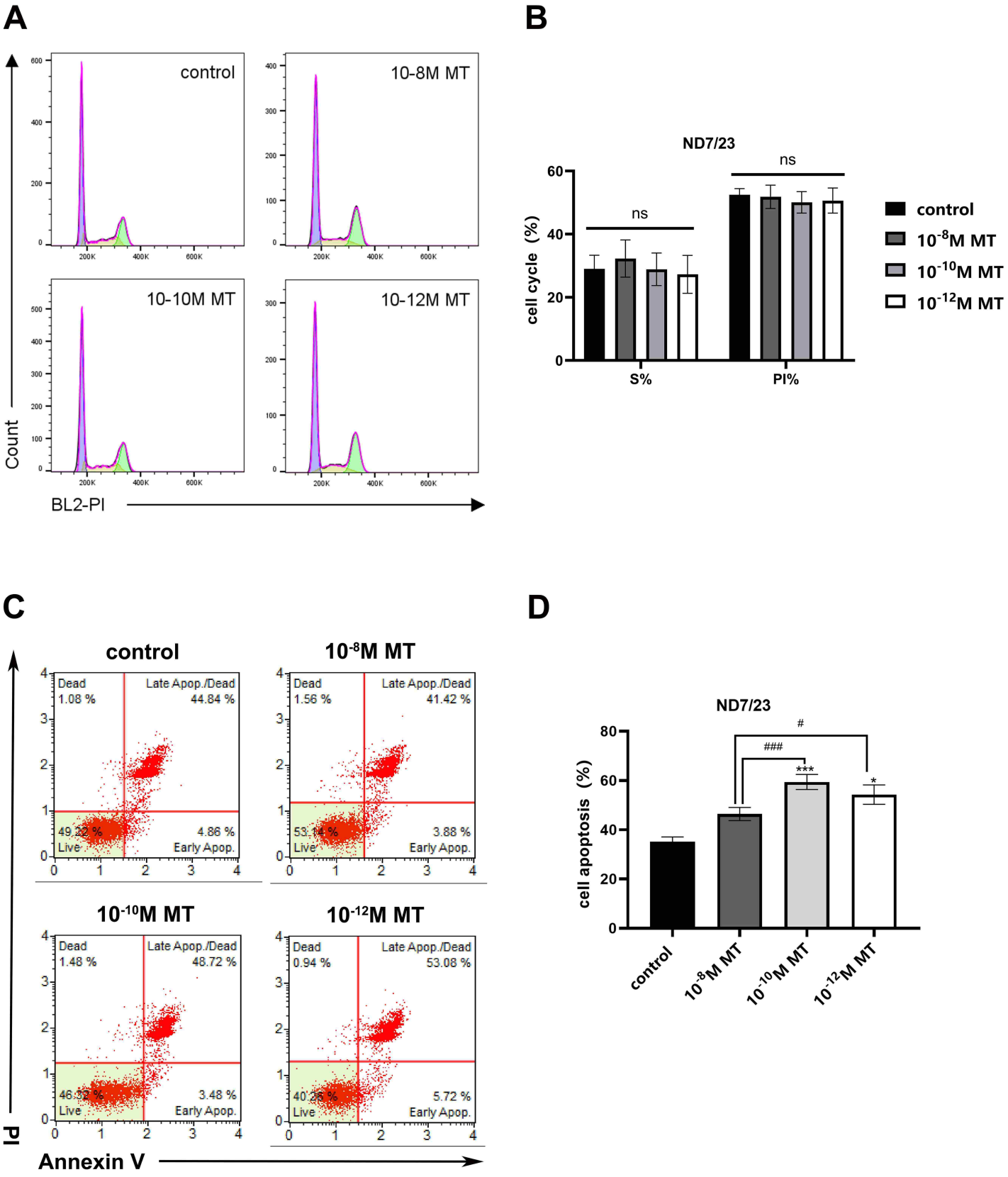


FIGURE 2. Cell cycle assay and cell apoptosis assay of ND7/23 cells with MT treatment. (A) Cell cycle fitting diagram. (B) S phase ratio and PI of each group are shown. The data was determined by FlowJo and presented as mean ± SD (n = 4). The S phase ratio and PI value were not statistically different between each group ($p > 0.05$). (C) Representative dot plots of cell apoptosis. (D) Cell apoptosis ratios (late and early apoptosis) of each group are shown. The data was determined by FlowJo and presented as mean ± SD (n = 4). Compared with control, $^{***}p < 0.001$,$^{*}p < 0.05$; Compared with 10^{-12} M MT, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$. MT: Melatonin; PI: Propidium Iodide.

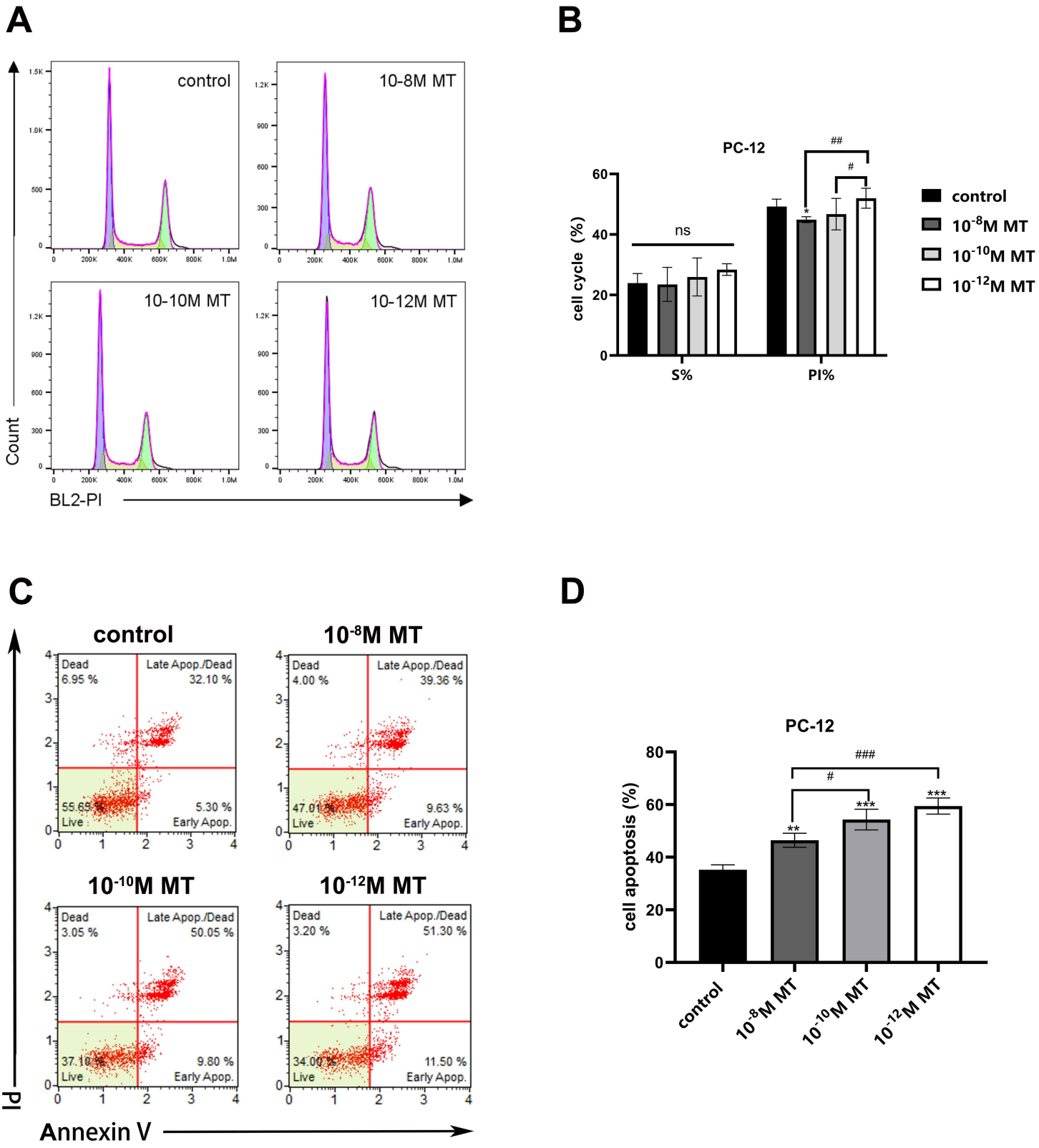


FIGURE 3. Cell cycle assay and cell apoptosis assay of PC-12 cells with MT treatment. (A) Cell cycle fitting diagram. (B) S phase ratio and PI of each group are shown. The data was determined by FlowJo and presented as mean ± SD (n = 6). Compared with control, $^{*}p < 0.05$; Compared with 10^{-12} M MT, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$. (C) Representative dot plots of cell apoptosis. (D) Cell apoptosis ratios (late and early apoptosis) of each group are shown. The data was determined by FlowJo and presented as mean ± SD (n = 6). Compared with control, $^{**}p < 0.01$, $^{***}p < 0.001$; Compared with 10^{-8} M MT, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$. MT: Melatonin; PI: Propidium Iodide.

the physiological concentration of MT promoted PC-12 cell apoptosis, with a lower MT concentration associated with higher apoptosis ratio. Altogether, the results showed that 10^{-8} M MT inhibited cell cycle and promoted cell apoptosis, while 10^{-10} M MT promoted cell apoptosis and 10^{-12} M MT inhibited cell proliferation and promoted the apoptosis of PC-12 cells.

3.5 Luzindole pretreatment up-regulated PC-12 cell ROS level

Each MT group showed a downward trend without any statistically significant ($p > 0.05$, Fig. 4A–B), but the Luzindole group was higher than the MT12 group ($p < 0.05$, Fig. 4B), suggesting that Luzindole pretreatment could up-regulate the ROS levels of PC-12 cells with 10^{-12} M MT via the non-member receptor pathway.

3.6 Luzindole pretreatment up-regulated ND7/23 cell ROS level

The results showed that ROS levels were significantly elevated by 10^{-12} M MT ($p < 0.05$) and Luzindole ($p < 0.01$), compared with control (Fig. 4C–D). In addition, the data of Luzindole was significantly different from those of 10^{-8} M and 10^{-10} M MT ($p < 0.05$), indicating that 10^{-12} M MT could up-regulate the ROS level of ND7/23 cells via the non-member receptor pathway.

3.7 10^{-12} M MT down-regulated the ratio of NAD(P)$^+$/NAD(P)H of ND7/23 cells and was enhanced with Luzindole pretreatment

NAD(P)H and NAD(P)$^+$ are classic molecules involved in mitochondrial respiratory function and are the major electron donors for the electron transport chain. The NAD(P)$^+$/NAD(P)H ratio is a modulator of oxidative phosphorylation, which can significantly influence mitochondrial functions [25]. Although a reduction in the NADP$^+$/NADPH ratio was observed in the MT groups ($p < 0.001$, Fig. 5A), only 10^{-12} M MT showed significant down-regulation in NAD$^+$/NADH ratio ($p < 0.001$, Fig. 5B). Further, Luzindole significantly promoted this down-regulation effect compared with 10^{-12} M MT ($p < 0.001$, Fig. 5B). These results demonstrated that 10^{-12} M MT could promote mitochondrial electron transfer and oxidate the phosphorylation of ND7/23 cells via the non-membrane receptor pathways.

3.8 MT up-regulated the NADP$^+$/NADPH ratio but down-regulated the NAD$^+$/NADH ratio of PC-12 cells

NAD(P)H and NAD(P)$^+$ are classic molecules involved in mitochondrial respiratory function and are the major electron donors for the electron transport chain. The NAD(P)$^+$/NAD(P)H ratio is the modulator of oxidative phosphorylation and can significantly influence mitochondrial functions [25]. The NADP$^+$/NADPH ratio of MT was increased compared with control ($p < 0.001$, Fig. 5C) but could be weakened by Luzindole ($p < 0.001$, Fig. 5C). In addition, 10^{-10} M and 10^{-12} M MT down-regulated the ratio of NAD$^+$/NADH compared with control ($p < 0.001$, Fig. 5D) and was enhanced by Luzindole ($p < 0.05$, Fig. 5D), while the opposite effect was observed with 10^{-8} M MT ($p < 0.001$, Fig. 5D). These results demonstrated that MT could affect the mitochondrial respiratory chain of PC-12 cells via non-membrane receptor pathways. In addition, 10^{-10} M and 10^{-12} M MT could down-regulate NAD$^+$/NADH to promote election chain transfer and oxidative phosphorylation.

3.9 Impact of physiological concentration of MT on MMP in ND7/23 versus PC-12 cells

There was no significant difference in MMP between the control group and each of the MT groups in ND7/23 cells ($p > 0.05$, Fig. 6A). However, a mild increase in MMP was observed with Luzindole, which was statistically different from control ($p < 0.05$, Fig. 6B). In PC-12 cells, a slight reduction in MMP with 10^{-8} M MT ($p < 0.05$, Fig. 6B) and an up-regulation with Luzindole ($p < 0.05$, Fig. 6B) were observed compared with control. These results demonstrated that 10^{-12} M MT could affect the MMP of ND7/23 and PC-12 cells through the membrane and non-membrane receptor pathways.

4. Discussion

4.1 Cell line models of TG neurons

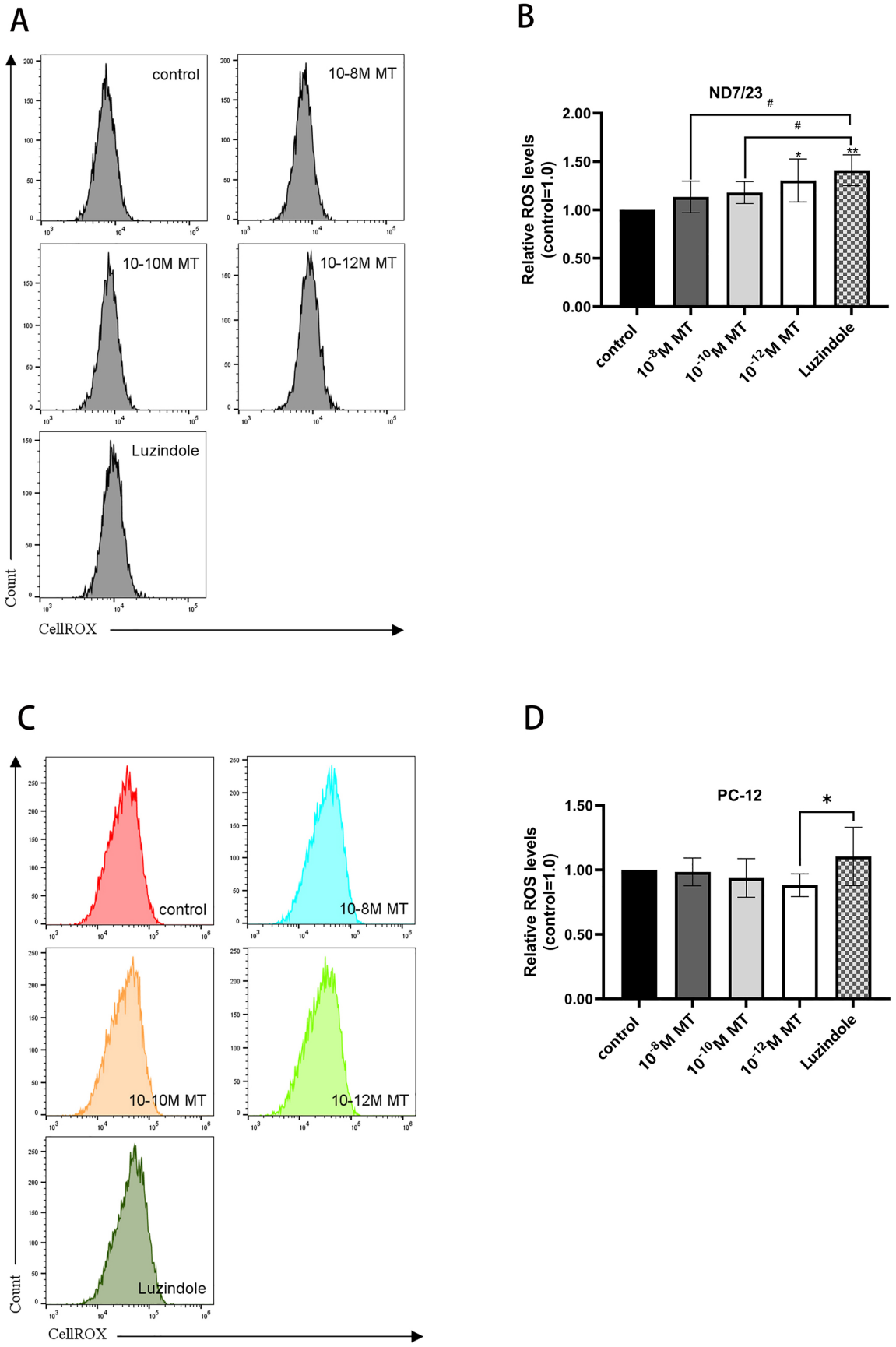
The *in vitro* study of cell viability is mainly based on chemical-induced effects in immortalized cell lines and can be used to determine the impacts on cells or relevant molecular pathways [26].

PC-12 cells are used in neuroscience research because they can exhibit the features of neurons [27, 28]. ND7/23 cells are known as models of small nociceptive neurons that produce myelinated C fibers, express the genes and ion channels of peripheral sensory neurons (*e.g.*, NaV1), secrete neuropeptides, and contain pain neurotransmitter receptors [12].

The primary sensory system requires the integrated function of multiple cell types [29]. In this study, ND7/23 and PC-12 cell lines were selected to explore the functional changes of peripheral neurons and to simulate the cellular functions of peripheral sensory neurons.

4.2 Melatonin is involved in pain regulation

Multiple studies have shown that MT regulates acute, chronic, inflammatory, and neuropathic pain. Intrathecal injection of MT can inhibit the synaptic enhancement effects of C fiber in a dose-dependent manner [30]. MT inhibits glial cell activity and the release of inflammatory cytokines to reduce pain response [31]. It also plays an “analgesic” of “pain promoting” effects in pain regulation [32], depending on the types of stimulation, duration of injury and doses of MT. It was shown that high concentrations of MT had a free radical scavenging function to eliminate ROS and reduce pain responses [33]. The production of ROS is one of the main functions of intracellular mitochondria [34]. Thus, mitochondrial dysfunction is considered to be closely related to pain regulation [35]. When MT enters the cell, it can penetrate the mitochondrial



F I G U R E 4. Flow cytometry analysis of mitochondrial ROS in ND7/23 cells and PC-12 cells with Luzindole pretreatment. (A) Representative histogram of CellROX® Red fluorescence intensities. (B) Quantitative evaluation of ROS in ND7/23 cells. (C) Representative histogram of CellROX® Red fluorescence intensities. (D) Quantitative evaluation of ROS in PC-12 cells. Data are presented as mean ± SD and determined by FlowJo (n = 4). Compared with control, $^{*}p < 0.05$, $^{**}p < 0.01$; Compared with Luzindole, $^{\#}p < 0.05$. MT: Melatonin; ROS: Reactive oxygen species.

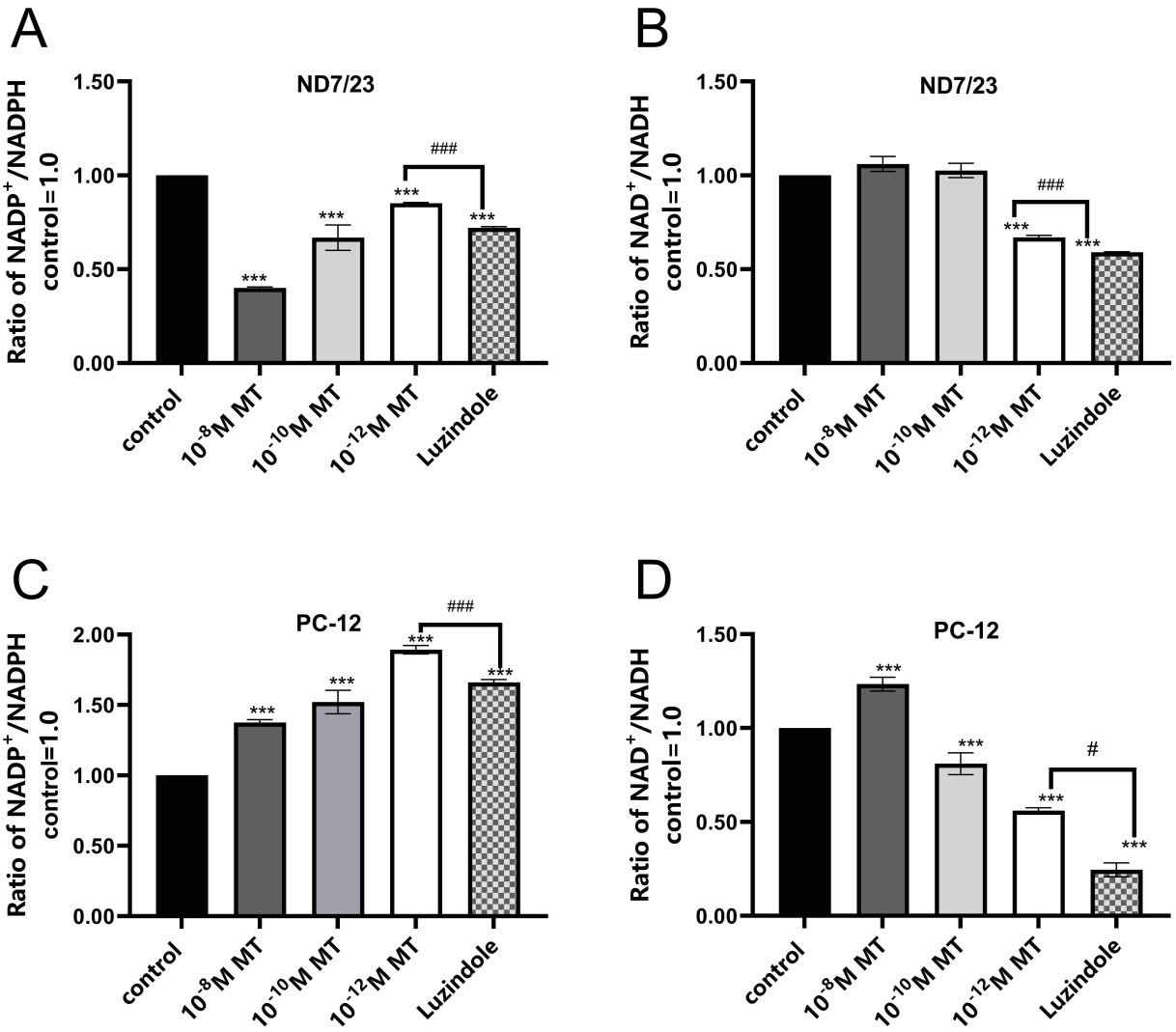


FIGURE 5. Effects of MT and Luzindole on respiratory chain of ND7/23 cells and PC-12 cells. (A) $NADP^+$/NADPH ratio of ND7/23 cells. (B) NAD^+/NADH ratio of ND7/23 cells. (C) $NADP^+$/NADPH ratio of PC-12 cells. (D) NAD^+/NADH ratio of PC-12 cells. Data are presented as mean ± SD (n = 4) and determined using a spectrophotometer. Compared with control, $^{***}p < 0.001$; Compared with MT12, $^{###}p < 0.001$; Compared with Luzindole, $^{\#}p < 0.05$, $^{###}p < 0.001$. MT: Melatonin; NAD: Nicotinamide Adenine Dinucleotid; NADH: reduced from Nicotinamide Adenine Dinucleotide; NADP: Nicotinamide Adenine Dinucleotide phosphate; NADPH: reduced from Nicotinamide Adenine Dinucleotide phosphate.

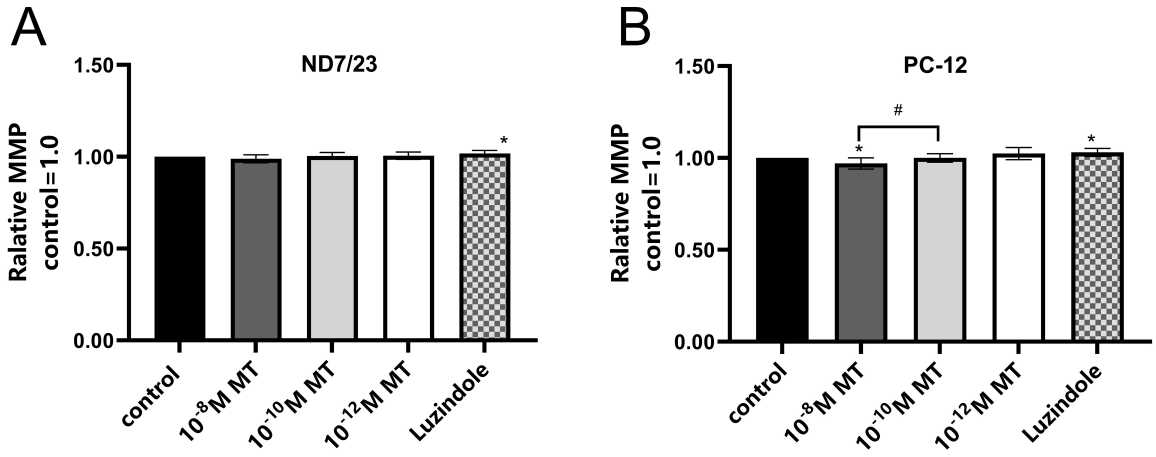


FIGURE 6. Effects of MT and Luzindole on MMP in ND7/23 and PC-12 cells. (A) Relative MMP of ND7/23 cells. (B) Relative MMP of PC-12 cells. Data are presented as mean ± SD (n = 4) and determined using a spectrophotometer. Compared with control, $^{*}p < 0.05$; Compared with MT8 group, $^{\#}p < 0.05$. MT: Melatonin; MMP: Mitochondrial membrane potential.

membrane and accumulate at high concentrations. It can stabilize the internal mitochondrial membrane and improve the activity of the electron transport chain [36]. In this study, the effects of MT on peripheral sensory neuronal cell viability and mitochondrial function indirectly showed its regulatory role in pain.

4.3 MT inhibits cell proliferation in peripheral sensory neurons with diurnal variations

It is generally recognized that 10^{-10}–10^{-8} M is the physiological concentration of MT, and 10^{-6}–10^{-4} M is the pharmacological concentration [22]. In this study, we explored the differences in MT effects on cell viability under physiological conditions. Since the concentration of MT in tissue fluid might be lower than in blood, cells may directly interact with MT *in vitro,* and the minimum concentration used in this study was lower than 10^{-10} M. Three gradient concentrations of 10^{-12}, 10^{-10} and 10^{-8} M were therefore determined. In addition, 10^{-8} M MT corresponded to nighttime concentration, while 10^{-10} and 10^{-12} M MT corresponded to daytime concentrations.

Cell proliferation is an important feature of cell viability. There are two main factors affecting cell proliferation: (1) external factors existing in cell environments, *i.e.*, various growth factors, hormones and so on, and (2) internal factors, including the combination of cyclin-dependent kinase (CDK) with cyclin to regulate cell cycle orderly, as well as the regulation or synergistic effects of certain genes and their products on cell proliferation. MT is an indole neuroendocrine hormone that regulates the proliferation of various types of cells [37]. Thus, different concentrations of MT have different effects on different types and states of cells [38].

In this study, the physiological concentration of MT was shown to inhibit cell proliferation in ND7/23 and PC-12 cells. As culture days increased, a lower MT concentration led to a more obvious inhibition of cell proliferation in both cells. The cell viability of 10^{-12} M MT was significantly lower than with 10^{-8} M, which might be related to differences in MT secretion. We also observed that cell viability might be significantly reduced during the day (10^{-12} M) compared with nighttime (10^{-8} M). When cell viability was reduced in PC-12 cells, the secretion of neuropeptides decreased, nerve conduction slowed down, and the expression of pain weakened, manifesting an "analgesic" effect. Our results showed that the inhibition of 10^{-12} M MT was significant compared with 10^{-8} M, indicating the "analgesic" effect of MT during the day might be more pronounced than at night and that patients might be more sensitive to pain during the day than at night.

Studies have shown that MT has neuroprotective functions [39], but the mechanism of action remains undetermined. Scholars have shown that 10^{-8} M and 5×10^{-8} M of MT inhibited the proliferation of PC-12 cells, while 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M of MT had opposite effects [40] and Might be activated by the MEK/ERK and PI3K/AKT signaling pathways. The anti-proliferative effects of 10^{-8} M MT were concordant with our results. It was proved again that different concentrations of MT might play different roles in pharmacological and physiological concentrations through different pathways. However, the specific mechanism is not yet clear and is believed to be related to the diversification of MT.

Different physiological concentrations of MT also had anti-proliferative effects on ND7/23 cells and were dose-dependent on days 2 and 3, whereby a lower MT concentration was associated with the more obvious inhibitory effects and was consistent with observations with PC-12 cells. Thus, it could be speculated that cell viability in MT daytime concentration (10^{-12} M) was significantly lower than in nighttime concentration (10^{-8} M). Further, a decreased cell viability in ND7/23 cells could indicate slowed stimulative reaction, weakened pain conduction and diminished pain expression, manifesting an "analgesic" effect. Our results showed that the anti-proliferative effects of 10^{-12} M MT were significant compared with 10^{-8} M MT, indicating that the "analgesic" effects of MT during the day could be more pronounced than at night, causing greater pain sensitivity at night. It can be seen that the physiological concentration of MT inhibited the two cell activities. However, the inhibitory effects of daytime (10^{-12} M) were more obvious than that of nighttime (10^{-8} M), and the related neuropeptide secretion and cell pain conduction were weakened, suggesting that the "analgesic" effect during the day could be more significant than at night.

It was reported that 10^{-3}–10^{-2} M of MT could inhibit the proliferation of HepG2 cells (hepatocarcinoma cell line) and induce apoptosis in a time- and dose-dependent manner [41]. Also, 10^{-3} M MT demonstrated anti-proliferative effects on the endothelial cells of human umbilical veins [37], 10^{-9} M MT inhibited the proliferation of MCF-7 breast cancer cells [42], and 10^{-5} M MT led to a significant increase in the proliferation of PC-12 cells [40]. Activation or inhibition of cell proliferation affects cell viability, thereby enhancing or weakening the corresponding functions of cells. Neurons are highly differentiated cells that cannot be cultured; thus, immortalized cells instead of primary neurons are used for *in vitro* studies, including pharmacological research.

4.4 Luzindole pretreatment promotes MT to inhibit peripheral neuron viability

MT interacts with cells through various pathways, including membrane receptors (MT1R and MT2R), nuclear receptors (RZR/ROR) [43] and free-spreading and mitochondrial receptors; therefore, different concentrations of MT might have different effects on different cells via different pathways.

Luzindole is a non-selective antagonist of MT membrane receptors, which can simultaneously block the binding of MT to MT1R and MT2R on cell membranes, affecting the interaction between MT and cells [44]. In this study, pretreatment with 2 μM Luzindole for 30 min demonstrated a linear growth trend in ND7/23 and PC-12 cells, while the cell viability was significantly lower than that of control and the MT group, indicating that Luzindole pretreatment could enhance the anti-proliferative effects of MT, which was consistent with Liu's research [40]. If this inhibition is achieved via the membrane receptor pathway, MT cannot act on cells after Luzindole pretreatment, implying no inhibition and that PC-12 and ND7/23 cells should be able to proliferate normally

(absorbance similar to the control group). If this inhibition is achieved entirely via a non-membrane receptor pathway, MT should not be affected after Luzindole pretreatment, and the effect should be consistent with MT alone (absorbance similar to the MT group). However, the opposite result was obtained after Luzindole pretreatment. The physiological concentration of MT showed a more obvious anti-proliferative effect than MT alone, suggesting that the anti-proliferative effects of physiological concentrations of MT occurred through membrane and non-membrane receptor pathways in PC-12 and ND7/23 cells, with the latter pathway being more dominant than the former. After Luzindole pretreatment, more MT entered the cell through the non-membrane receptor pathway, enhancing the anti-proliferative effects and reducing cell viability.

4.5 MT delayed cell cycle and promoted cell apoptosis

Our results showed a reduction of PI in PC-12 cells with 10^{-8} M MT treatment, implying that MT delayed cell cycle. However, 10^{-12} M MT showed an increase in the ratio of PI compared with 10^{-8} M MT, which cannot be judged as promoting proliferation. In addition, it showed that more PC-12 cells entered to S and G2 phases with 10^{-12} M MT treatment, but no mitosis occurred, leading to proliferation block and reduction in cell viability.

Physiological concentrations of MT had no significant effects on the cell cycle of ND7/23. Meanwhile, MT had anti-proliferative effects, inferring that the inhibition may not occur by a change in cell cycle but could be caused by comprehensive effects such as apoptosis, oxidative stress, *etc*. Further, 10^{-4} and 10^{-3} M MT delayed the cell cycle of H9c2 cells (embryonic rat cardiomyocyte line) in the G_1 phase and simulated apoptosis in a dose-dependent manner to inhibit the cell growth of H9c2 cells [45]. Treatment of ELT3 cells (uterine leiomyoma cell line) with 2×10^{-3} M MT shortened the G_0/G_1 phase, prolonged the sub-G_1 phase and inhibited cell growth [46].

Apoptosis is the physiological process of removing non-functional cells [47]. It was found that different concentrations of MT had a pro-apoptotic effect on PC-12 cells, which was dose-dependent. A lower MT concentration led to a higher ratio of apoptotic cells and lower cell viability, which was observed by the promotion of apoptosis with 10^{-10} and 10^{-12} M MT in ND7/23 cells, indicating that the anti-proliferative effects of the physiological concentration of MT might be related to its pro-apoptotic effects on PC-12 and ND7/23 cells. It was shown that pretreatment with 5×10^{-5} M and 10^{-4} M MT could reduce hydrogen peroxide (H_2O_2)-induced apoptosis of ARPE-19 cells (retinal pigment cells) [48]. Scholars have also found that both high (10^{-5} M) and low doses (10^{-9} M) of MT played an important role in regulating granular cell apoptosis, cell cycle and antioxidant activities.

The comprehensive effects of physiological concentrations of MT on cell cycle and apoptosis are relevant to the secretion characteristics of MT. Daytime concentration (10^{-12} M) inhibits cell viability by delaying cell cycle and promoting apoptosis of PC-12 cells, while nighttime concentration (10^{-8} M) inhibits cell viability by inhibiting proliferation and promoting apoptosis of PC-12 cells. Thus, inhibition of cell viability may reduce the secretion of pain-related neuropeptides. A higher proportion of apoptosis was caused by daytime concentrations than nighttime, whereby neuropeptides at daytime are lesser than at night, making the daytime "analgesic" effect more significant than at night in PC-12 cells. MT in daytime concentration inhibits cell viability by promoting apoptosis of ND7/23 cells; however, the effects of nighttime concentration were not obvious. When the cell viability worsens, neuronal pain conduction might be weakened, leading to the "analgesic" effect. A higher apoptosis ratio in daytime concentration could lead to a greater "analgesic" effect than in nighttime.

4.6 MT enhanced the mitochondrial oxidative phosphorylation function of peripheral sensory neurons

MT at a concentration of 10^{-12} M mainly down-regulated the ratio of NAD^+/NADH and ROS, increased MMP and promoted mitochondrial respiratory chain electron transport in two cells. 10^{-12} M MT enhanced oxidative phosphorylation and reduced mitochondrial damage to PC-12 cells. In general, the reduction of oxidative stress may reduce pain, resulting in an "analgesic" effect, manifesting as a more obvious downregulation of NAD^+/NADH during the day (10^{-12} M MT) than at night (10^{-8} M MT) and more significant "analgesic" effect during the day than at night. Further, 10^{-12} M MT mainly down-regulated the ratio of NAD^+/NADH and $NADP^+$/NADPH, increased MMP and promoted mitochondrial respiratory chain electron transport, which enhanced oxidative phosphorylation and reduced mitochondrial damage to ND7/23 cells, suggesting a more obvious down-regulation of NAD^+/NADH during the day (10^{-12} M MT) than at night (10^{-8} M MT), and more significant "analgesic" effect during the day than at night. The pretreatment of Luzindole promoted the downregulation of NAD^+/NADH, inferring that 10^{-12} M MT may interact with PC-12 and ND7/23 cells mainly through the non-membrane receptor pathway.

5. Conclusions

In summary, our study demonstrated that melatonin at physiological concentration inhibited the proliferation of ND7/23 and PC-12 cells and promoted their cell apoptosis. MT at a concentration of 10^{-12} M showed the most significant inhibition, possibly through the non-membrane receptor pathway, promoted mitochondrial respiratory electron transfer, and increased antioxidant activity and mitochondrial function in PC-12 and ND7/23 cells through the non-membrane receptor pathway. Also, 10^{-8} M MT inhibited mitochondrial respiratory electron transfer of PC-12 cells but promoted that of ND7/23 cells, resulting in increased mitochondrial function through the non-membrane receptor pathway.

This study might provide an in-depth insight into the circadian mechanism of MT in regulating peripheral sensation. Nevertheless, further studies are still needed to define the specific molecular mechanism of mitochondrial dynamics of MT on pain regulation.

AUTHOR CONTRIBUTIONS

YYY—performed the experiments, analyzed the data, authored and reviewed the paper, approved the final draft; XPY—performed the experiments, authored and reviewed the paper, and approved the final draft; HWH—conceived and designed the experiments, authored and reviewed the paper, and approved the final draft; FH—authored and reviewed the paper, and approved the final draft.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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