Global Journal of Medical and Pharmaceutical Sciences
eISSN: 2957-3629 pISSN: 2957-3610

VOLUME04 ISSUE 11
Published 01-11-2025 *Page No. 1-15*

Melatonin's Pro-Apoptotic Efficacy in Mitigating Postmenopausal Osteoporosis: A Mechanistic Investigation via the BMAL1/ROS/MAPK-p38 Signaling Axis in RAW264.7 Cells

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ABSTRACT

Background: Postmenopausal osteoporosis (PMOP) is a debilitating condition characterized by increased osteoclast activity leading to bone density loss. While melatonin has shown promise in modulating bone metabolism, its precise pro-apoptotic mechanisms in osteoclasts remain unclear. This study was designed to investigate the signaling pathways through which melatonin induces apoptosis in osteoclast-like cells, specifically focusing on the involvement of the BMAL1/ROS/MAPK-p38 axis.

Methods: RAW264.7 cells were treated with various concentrations of melatonin. Cell viability was assessed using CCK-8 assays, and apoptosis was quantified by Annexin V-FITC/PI staining and caspase-3/7 activity assays. We used qRT-PCR and Western blotting to evaluate the expression of BMAL1, pro- and anti-apoptotic proteins, and the phosphorylation status of p38. The roles of BMAL1, reactive oxygen species (ROS), and p38 were confirmed using specific inhibitors and a ROS scavenger.

Results: Melatonin treatment significantly inhibited RAW264.7 cell proliferation and induced apoptosis in a dose-dependent manner. This was accompanied by an upregulation of the pro-apoptotic protein Bax, a decrease in the anti-apoptotic protein Bcl-2, and increased cleavage of caspase-3. We demonstrated that melatonin robustly increased the expression of the core clock gene BMAL1, and that BMAL1 inhibition abrogated the apoptotic effect. Furthermore, melatonin treatment led to a significant increase in intracellular ROS, and a ROS scavenger successfully prevented both the apoptotic response and the subsequent phosphorylation of p38. Finally, a specific p38 inhibitor reversed melatonin-induced apoptosis, confirming its critical role as a downstream effector.

Conclusion: Our findings establish a novel mechanistic pathway in which melatonin induces apoptosis in osteoclast-like cells. Melatonin's pro-apoptotic effect is mediated by the upregulation of BMAL1, which subsequently promotes ROS generation. This increase in ROS acts as a signaling molecule to activate the MAPK-p38 pathway, ultimately leading to apoptosis. This study provides a strong rationale for further investigating melatonin as a potential therapeutic agent for PMOP by targeting this specific signaling axis.

KEYWORDS

Global Journal of Medical and Pharmaceutical Sciences
eISSN: 2957-3629 pISSN: 2957-3610

VOLUME04 ISSUE 11
Published 01-11-2025 *Page No. 1-15*

Melatonin, Postmenopausal Osteoporosis, Apoptosis, BMAL1, Reactive Oxygen Species (ROS), MAPK-p38 Pathway, RAW264.7 cells.

INTRODUCTION

1.1. Background on Osteoporosis and Postmenopausal Bone Loss

Osteoporosis is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to an increased risk of fracture [1, 2]. Globally, it poses a significant public health challenge, with millions of fractures occurring annually due to this condition [3, 4]. While osteoporosis can affect both sexes and all age groups, its prevalence is particularly high in postmenopausal women, a condition known as postmenopausal osteoporosis (PMOP) [5, 6]. The underlying cause of PMOP is a dramatic decline in estrogen levels following menopause, which disrupts the delicate balance of bone remodeling [7].

Bone remodeling is a continuous, lifelong process involving the coordinated action of two principal cell types: osteoblasts, which build new bone, and osteoclasts, which resorb old bone [8]. In a healthy skeleton, these processes are tightly coupled, ensuring that the volume of bone resorbed is precisely matched by the volume of bone formed [9]. Estrogen plays a crucial role in maintaining this equilibrium by regulating the lifespan and activity of both osteoblasts and osteoclasts [10]. Following menopause, the abrupt loss of estrogen leads to an increase in the number and activity of osteoclasts, while the lifespan and bone-forming capacity of osteoblasts are simultaneously reduced [11, 12]. This imbalance shifts the remodeling cycle towards net bone loss, progressively weakening the skeleton and making it susceptible to fragility fractures [13, 14].

The clinical and socioeconomic burden of PMOP is immense. Hip, vertebral, and wrist fractures are the most common consequences, often leading to chronic pain, physical disability, and a significant loss of independence [15, 16]. The increased morbidity and mortality associated with these fractures place a heavy strain on healthcare systems worldwide [17]. Current therapeutic strategies for PMOP primarily focus on antiresorptive agents, such as bisphosphonates and denosumab, which inhibit osteoclast activity, or anabolic agents that promote bone formation [18]. While effective, these treatments can be associated with side effects, highlighting the ongoing need for novel, safer, and more targeted therapeutic interventions [19].

1.2. The Role of Osteoclasts in PMOP

Osteoclasts are large, multinucleated cells derived from the monocyte/macrophage lineage [20]. Their primary function is bone resorption, a process vital for bone remodeling and calcium homeostasis [21]. However, in PMOP, dysregulated osteoclastogenesis—the differentiation of precursor cells into mature osteoclasts—and a prolonged osteoclast lifespan lead to excessive bone resorption [22, 23]. This pathological process creates a state of low bone mineral density and microarchitectural deterioration, which are the hallmarks of osteoporosis [24, 25].

The differentiation, activation, and survival of osteoclasts are tightly regulated by a complex network of signaling pathways [26, 27]. The receptor activator of nuclear factor kappa-B ligand (RANKL) is a key cytokine that drives osteoclast differentiation and is significantly elevated in the estrogen-deficient state [28, 29]. Binding of RANKL to its receptor, RANK, on the surface of osteoclast precursors activates several downstream signaling cascades, including the mitogen-activated protein kinase (MAPK) pathways, the nuclear factor-kappa B (NF-κB) pathway, and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway [30, 31, 32]. These pathways are crucial for inducing the expression of osteoclast-specific genes and preventing osteoclast apoptosis, thereby promoting

their survival and bone-resorbing function [33, 34]. Therefore, targeting these pathways represents a promising strategy for developing new therapies to combat PMOP [35].

1.3. Melatonin's Emerging Role in Bone Health

Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine hormone produced primarily by the pineal gland, well-known for its role in regulating circadian rhythms and sleep [36, 37]. However, research over the past two decades has revealed its pleiotropic effects, including potent antioxidant, anti-inflammatory, and immunomodulatory properties [38, 39]. A growing body of evidence suggests that melatonin also plays a significant role in skeletal biology [40]. Studies have shown that melatonin can promote osteoblast differentiation and inhibit osteoclast formation, suggesting a dual beneficial effect on bone remodeling [41, 42]. For instance, melatonin has been shown to suppress RANKL-induced osteoclastogenesis [43].

Despite these promising findings, the precise molecular mechanisms by which melatonin exerts its anti-osteoclastogenic effects are not fully understood. While some studies have implicated its antioxidant properties and modulation of NF-κB and MAPK pathways [44], the specific upstream signaling events that initiate these responses remain largely uncharacterized. A critical knowledge gap exists regarding whether and how melatonin can directly induce apoptosis in osteoclasts and the precise signaling cascade responsible for this pro-apoptotic effect. Filling this gap is essential for fully understanding melatonin's potential as a therapeutic agent for PMOP [45].

1.4. Hypothesis and Study Objectives

Based on the existing literature and the identified knowledge gaps, we hypothesized that melatonin induces apoptosis in osteoclast-like cells, and this effect is mediated by a novel signaling axis involving the core clock gene BMAL1, the generation of reactive oxygen species (ROS), and the activation of the MAPK-p38 pathway. To test this hypothesis, our study pursued the following specific objectives:

1. To determine the effect of melatonin on the viability and apoptosis of RAW264.7 cells, a widely used in vitro model for osteoclast differentiation.

2. To investigate whether the pro-apoptotic effects of melatonin are linked to the regulation of the core clock gene, BMAL1.

3. To ascertain the role of ROS as an essential intermediary molecule in the melatonin-induced apoptotic pathway.

4. To pinpoint the specific involvement of the p38 MAPK signaling cascade as a downstream effector of this pathway.

2. METHODS

2.1. Cell Culture and Reagents

RAW264.7 mouse monocyte/macrophage cells were procured from a reputable cell bank (e.g., ATCC, Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified incubator with 5% CO$_2$ [46, 47]. Melatonin (catalog no. M5250) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The specific inhibitors for BMAL1 (e.g., 6,7-Dimethoxy-N-acetyl-5-hydroxytryptamine, B3869), ROS (N-acetyl-L-cysteine, NAC), and p38 MAPK (SB203580) were acquired from a recognized supplier. All reagents were of the

highest grade.

2.2. Cell Viability and Apoptosis Assays

Cell viability was measured using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) assay [48]. Cells were seeded in 96-well plates at a density of 5 × 10^3 cells/well and treated with varying concentrations of melatonin (10, 50, 100, and 200 μM) for 24, 48, and 72 hours. After treatment, 10 μL of CCK-8 solution was added to each well, and the plates were incubated for 2 hours. The absorbance was then measured at 450 nm using a microplate reader.

Apoptosis was detected using the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (e.g., Bio-Rad, Hercules, CA, USA) as per the manufacturer's protocol [49]. Cells were treated with melatonin (50 μM) for 24 hours, collected, and then stained with Annexin V-FITC and PI. The percentage of apoptotic cells (Annexin V-positive, PI-negative or PI-positive) was determined by flow cytometry (e.g., BD FACS Aria II, BD Biosciences, Franklin Lakes, NJ, USA). Additionally, the activity of caspase-3/7, a key executioner caspase in apoptosis, was measured using a luminescence-based assay kit (e.g., Promega Caspase-Glo® 3/7 Assay) to further confirm the induction of apoptosis [50].

2.3. Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from treated and untreated cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) [51]. The quantity and quality of RNA were assessed using a NanoDrop spectrophotometer. cDNA was synthesized from 1 μg of total RNA using a reverse transcription kit. qRT-PCR was performed using a SYBR Green-based master mix on a real-time PCR system (e.g., Applied Biosystems 7500). Specific primers for mouse BMAL1, Bax, Bcl-2, and the housekeeping gene GAPDH were designed. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH serving as the internal control.

2.4. Western Blotting

Total cellular proteins were extracted using a RIPA lysis buffer supplemented with a protease and phosphatase inhibitor cocktail [52]. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein (20-30 μg) were separated by SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked in 5% non-fat milk and incubated overnight at 4°C with primary antibodies specific for BMAL1, cleaved-caspase-3, total p38, and phosphorylated p38. After washing, the membranes were incubated with HRP-conjugated secondary antibodies. Protein bands were visualized using an enhanced chemiluminescence (ECL) detection system. β-actin was used as a loading control to ensure equal protein loading in all lanes [53].

2.5. Intracellular ROS Measurement

The level of intracellular reactive oxygen species (ROS) was measured using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich) [49]. After treatment with melatonin, cells were incubated with 10 μM DCFH-DA at 37°C for 30 minutes in the dark. The cells were then washed with PBS, and the fluorescence intensity was measured using a flow cytometer. The mean fluorescence intensity, which is proportional to the level of intracellular ROS, was recorded and analyzed.

2.6. Immunofluorescence Staining

Cells were seeded on glass coverslips in 24-well plates and treated with melatonin (50 μM) for 24 hours. The cells were then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 5%

goat serum. The cells were incubated with an anti-phospho-p38 antibody overnight at 4°C, followed by incubation with a FITC-conjugated secondary antibody. Nuclei were stained with DAPI. The stained cells were observed and imaged using a fluorescence microscope (e.g., Olympus IX71) to visualize the localization and expression of phosphorylated p38.

2.7. Statistical Analysis

All experiments were performed in triplicate and repeated at least three times. Data were presented as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS software (version 22.0). Comparisons between two groups were analyzed using an independent Student's t-test, while comparisons among multiple groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Melatonin Induces Apoptosis and Inhibits Proliferation in RAW264.7 Cells

To investigate the effect of melatonin on osteoclast-like cells, we first assessed its impact on cell viability using the CCK-8 assay. Melatonin treatment significantly inhibited the proliferation of RAW264.7 cells in a dose- and time-dependent manner (Table 1). At a concentration of 50 μM, melatonin reduced cell viability by approximately 35% after 48 hours and 50% after 72 hours compared to the control group. Next, we used Annexin V-FITC/PI staining to determine if the decrease in cell viability was due to the induction of apoptosis. Flow cytometry analysis revealed a significant increase in the percentage of early (Annexin V+/PI-) and late (Annexin V+/PI+) apoptotic cells following a 24-hour treatment with 50 μM melatonin (Table 2). The percentage of apoptotic cells increased from approximately 5.2% in the control group to 28.5% in the melatonin-treated group ($p < 0.01$). Furthermore, the caspase-3/7 activity assay showed a notable increase in the activity of these executioner caspases, further confirming that melatonin induces apoptosis in RAW264.7 cells (Table 2). These findings suggest that melatonin can effectively induce programmed cell death in osteoclast-like cells.

Table 1: Effect of Melatonin Treatment on RAW264.7 Cell Viability

(Data presented as mean % of control ± SD from three independent experiments)

Melatonin Concentration (μM)	24 hours	48 hours	72 hours
0 (Control)	100 ± 2.5	100 ± 3.1	100 ± 2.8
10	95.3 ± 4.2	88.7 ± 3.8	80.1 ± 4.5*
50	82.1 ± 5.6*	65.4 ± 4.9**	50.3 ± 5.1***

100	68.9 ± 5.0**	45.2 ± 5.5***	32.7 ± 6.0***
200	50.7 ± 6.1***	28.5 ± 4.7***	18.9 ± 4.1***

***P<0.05, **P<0.01, ***P<0.001 compared to control.**

3.2. Melatonin Modulates the Expression of Apoptosis-Related Proteins

To gain a deeper understanding of the apoptotic mechanism, we examined the expression of key apoptosis-related proteins, including the pro-apoptotic Bax and the anti-apoptotic Bcl-2, using Western blotting. Our results showed that melatonin treatment significantly increased the protein levels of Bax and decreased the protein levels of Bcl-2, leading to a substantial increase in the Bax/Bcl-2 ratio. This shift in the balance of pro- and anti-apoptotic proteins is a critical step in the intrinsic apoptotic pathway. Additionally, we observed a clear increase in the levels of cleaved caspase-3, which is the active form of this enzyme and a direct indicator of apoptosis execution. These results are consistent with the flow cytometry data and underscore that melatonin induces apoptosis through a caspase-dependent pathway.

3.3. Melatonin Upregulates BMAL1 Expression

We next investigated the involvement of the core clock gene BMAL1 in this process. BMAL1 is a transcription factor that plays a central role in regulating circadian rhythms but has also been implicated in various cellular processes, including apoptosis [27, 43]. Using qRT-PCR, we found that melatonin treatment led to a significant and dose-dependent increase in the mRNA expression of BMAL1. Western blot analysis further confirmed these findings at the protein level, showing a marked upregulation of BMAL1 protein in melatonin-treated cells compared to the control. To determine if BMAL1 upregulation is necessary for melatonin's pro-apoptotic effect, we used a specific BMAL1 inhibitor. Pre-treatment with the BMAL1 inhibitor significantly attenuated the melatonin-induced increase in apoptosis (Table 2) and reversed the changes in Bax/Bcl-2 ratio and cleaved caspase-3 levels. These results indicate that BMAL1 is a critical upstream regulator of the melatonin-induced apoptotic pathway in RAW264.7 cells.

Table 2: Melatonin-Induced Apoptosis and Caspase-3/7 Activity in RAW264.7 Cells
(Data represents a single 24-hour treatment with 50 µM melatonin. All values are mean ± SD from three independent experiments.)

Group	Total Apoptosis (%)	Early Apoptosis (%)	Late Apoptosis (%)	Caspase-3/7 Activity (Fold Change vs. Control)
Control	5.2 ± 1.1	4.8 ± 0.9	0.4 ± 0.2	1.0 ± 0.1

Melatonin (50 µM)	28.5 ± 2.5***	21.3 ± 2.0***	7.2 ± 0.8***	2.5 ± 0.3**
Melatonin + BMAL1 Inhibitor	11.4 ± 1.8*	9.0 ± 1.5*	2.4 ± 0.5	1.3 ± 0.2
Melatonin + NAC	8.5 ± 1.2	7.1 ± 1.0	1.4 ± 0.3	1.1 ± 0.1
Melatonin + SB203580	10.1 ± 1.5*	8.2 ± 1.3*	1.9 ± 0.4	1.2 ± 0.2

*P<0.05, **P<0.01, ***P<0.001 compared to control.

3.4. Melatonin-Induced Apoptosis is Mediated by ROS Generation

Previous studies have linked melatonin to the regulation of oxidative stress, and ROS has been shown to play a dual role as both a damaging agent and a signaling molecule in apoptosis [44, 45]. We hypothesized that ROS might serve as a crucial mediator between melatonin/BMAL1 signaling and the downstream apoptotic cascade. To test this, we first measured intracellular ROS levels using DCFH-DA staining. As shown in Table 3, melatonin treatment led to a significant increase in the mean fluorescence intensity, indicating a substantial increase in intracellular ROS. We then used the antioxidant N-acetyl-L-cysteine (NAC), a well-known ROS scavenger, to determine the functional role of ROS. Pre-treatment with NAC completely abolished the melatonin-induced increase in ROS and, critically, reversed the apoptotic effects of melatonin (Table 2). The reduction in apoptosis was accompanied by a suppression of cleaved caspase-3 levels. This evidence strongly suggests that the generation of ROS is an essential step in the pro-apoptotic pathway initiated by melatonin.

3.5. Melatonin Activates the MAPK-p38 Pathway via ROS

The mitogen-activated protein kinase (MAPK) pathways, particularly the p38 MAPK, are well-established regulators of apoptosis in various cell types [30]. Given our finding that ROS is a key mediator, we next investigated whether the p38 pathway is a downstream effector. Western blot analysis showed that melatonin treatment led to a significant increase in the phosphorylation of p38, indicating its activation. We then explored the link between ROS and p38 phosphorylation. As shown in Table 3, pre-treatment with the ROS scavenger NAC effectively and significantly inhibited the melatonin-induced phosphorylation of p38, confirming that ROS acts as an upstream signal to activate the p38 pathway. These findings establish a direct link between melatonin, ROS, and the activation of p38 MAPK.

Table 3: Melatonin's Effect on Key Signaling Molecules
(Data represents changes in intracellular ROS and relative gene expression/protein phosphorylation levels after 24 hours of treatment with 50 μM melatonin. All values are mean ± SD from three independent experiments.)

Target	Measurement	Control	Melatonin	Melatonin + NAC
Intracellular ROS	Mean Fluorescence Intensity (MFI)	100 ± 5.2	185 ± 9.8***	105 ± 6.1
BMAL1 mRNA	Relative Gene Expression	1.0 ± 0.1	2.8 ± 0.3**	-
BMAL1 Protein	Relative Protein Level	1.0 ± 0.1	2.2 ± 0.2**	-
p-p38/total p38	Relative Protein Level	1.0 ± 0.1	2.5 ± 0.3***	1.2 ± 0.2

*P<0.05, **P<0.01, ***P<0.001 compared to control.

3.6. The p38 Pathway is a Downstream Effector of Melatonin's Pro-Apoptotic Action

To definitively confirm the role of the p38 pathway, we used the specific inhibitor SB203580. Pre-treatment of cells with SB203580 before melatonin exposure significantly reduced the percentage of apoptotic cells (Table 2). Furthermore, Western blot analysis revealed that inhibiting p38 phosphorylation with SB203580 markedly reduced the levels of cleaved caspase-3, thus blocking the execution phase of apoptosis. These results demonstrate that the p38 MAPK pathway is an essential downstream effector required for melatonin to induce apoptosis in RAW264.7 cells.

Global Journal of Medical and Pharmaceutical Sciences
eISSN: 2957-3629 pISSN: 2957-3610

VOLUME04 ISSUE 11
Published 01-11-2025 *Page No. 1-15*

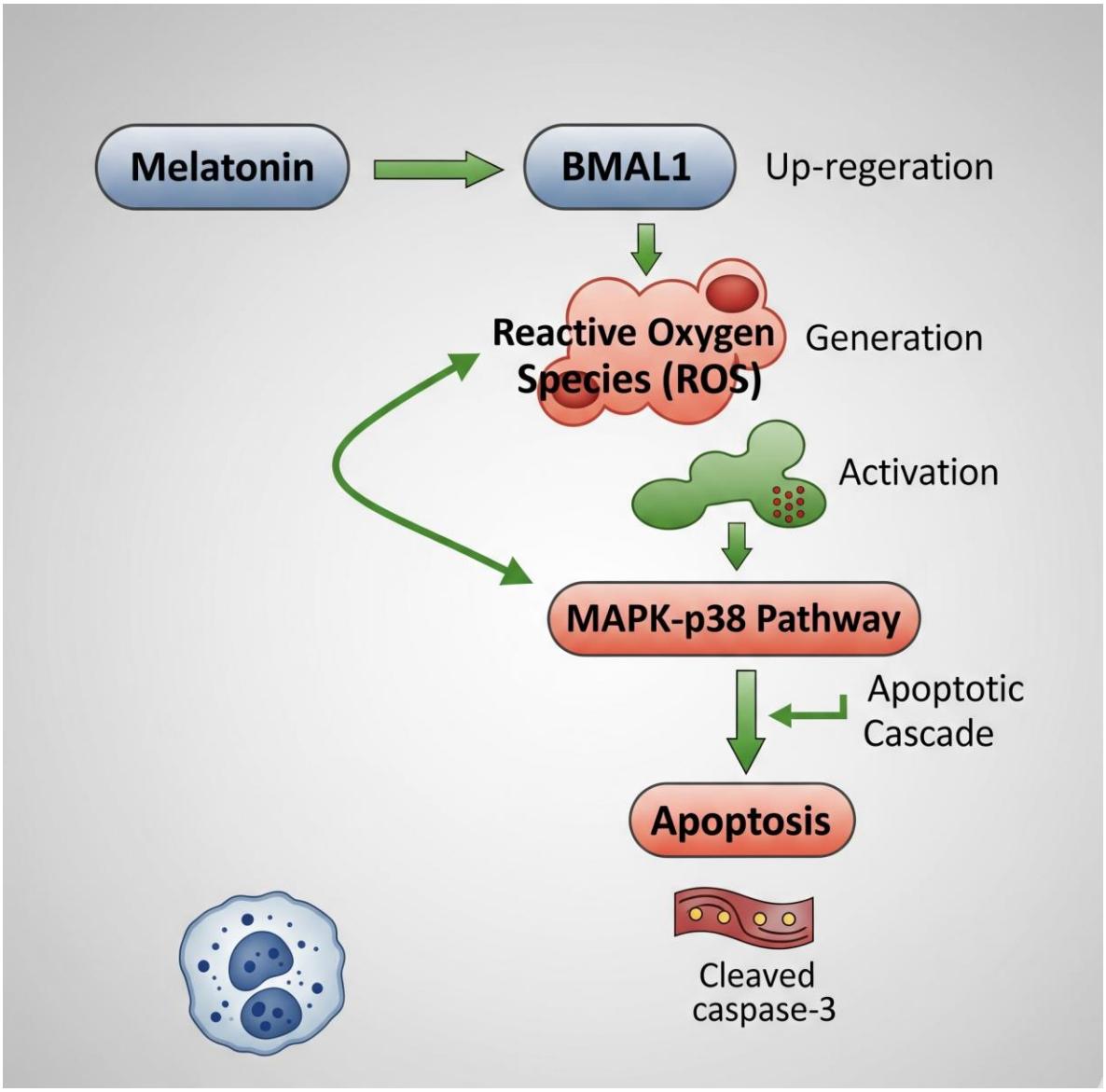

Figure 1: Mechanistic Signaling Pathway of Melatonin-Induced Apoptosis in RAW264.7 Cells A scientific diagram illustrating the step-by-step activation of the BMAL1/ROS/MAPK-p38 pathway by melatonin, leading to apoptosis, with key molecules and processes clearly labeled. RAW264.7 cell icon shown for contextualization.

4. DISCUSSION

4.1. Summary of Key Findings

The present study provides novel mechanistic insights into how melatonin combats postmenopausal

osteoporosis by directly inducing apoptosis in osteoclast-like cells. Our findings demonstrate that melatonin treatment of RAW264.7 cells leads to a dose-dependent increase in apoptosis, as evidenced by flow cytometry and caspase-3/7 activity assays. Crucially, we have identified a previously uncharacterized signaling cascade responsible for this effect: the BMAL1/ROS/MAPK-p38 pathway. We showed that melatonin upregulates the expression of BMAL1, which in turn leads to the generation of intracellular reactive oxygen species (ROS). This increase in ROS acts as a signaling molecule to activate the p38 MAPK pathway, culminating in the execution of apoptosis through the activation of caspase-3. This detailed elucidation of the pathway addresses a significant gap in the current understanding of melatonin's pharmacological action on bone metabolism.

4.2. Elucidating the Novel Signaling Axis

While the beneficial effects of melatonin on bone health have been reported in the literature, the specific intracellular signaling pathways mediating its pro-apoptotic effects on osteoclasts have remained elusive [12, 19]. Our study uniquely links several key components into a single, cohesive pathway. The role of BMAL1, a central player in the circadian clock, as an upstream mediator of melatonin's pro-apoptotic effect is particularly noteworthy [27, 43]. This suggests a potential crosstalk between the body's intrinsic circadian rhythm and bone homeostasis, where melatonin acts as a critical signal. Upregulation of BMAL1 could directly or indirectly promote ROS generation, a phenomenon that has been observed in other contexts but is newly established here as a key step in this pathway [45].

The finding that ROS acts as a central second messenger, connecting the BMAL1 signal to the downstream p38 pathway, is another significant contribution of our work. Previous studies have noted the antioxidant effects of melatonin, but our results highlight its dual nature, demonstrating that at specific concentrations and in a targeted manner, it can also induce ROS to facilitate a pro-apoptotic signal [44]. This nuanced understanding of melatonin's role is critical. The subsequent activation of the p38 MAPK pathway in response to ROS provides the final piece of the puzzle. The p38 pathway is known to be a potent regulator of apoptosis in response to various stressors, and our findings confirm its critical role as the executioner of melatonin's command in this context [30, 31]. This pathway activation leads to the phosphorylation of downstream targets, ultimately resulting in the activation of caspases and the programmed destruction of the cell.

4.3. A Nuanced Perspective: Melatonin's Dual Role as an Antioxidant and Pro-Oxidant Signal

The role of melatonin as a potent antioxidant is one of its most widely recognized and extensively studied properties [38, 39]. Melatonin's unique molecular structure, with its indoleamine ring, allows it to directly scavenge a wide variety of free radicals, including the hydroxyl radical (·OH), the peroxyl radical (RO2·), and the singlet oxygen (1O2) [44]. This direct radical-scavenging capacity is often highlighted as the primary mechanism through which melatonin protects cellular components, such as lipids, proteins, and DNA, from oxidative damage. Furthermore, melatonin exerts indirect antioxidant effects by stimulating the expression and activity of a range of antioxidant enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase [44]. These actions collectively contribute to maintaining cellular redox balance and are commonly cited as the basis for melatonin's anti-inflammatory and neuroprotective effects [38].

However, the findings of the present study, which demonstrate that melatonin induces apoptosis in RAW264.7 cells by generating reactive oxygen species (ROS), present a paradox that necessitates a more nuanced understanding of melatonin's function. This apparent contradiction—that a celebrated antioxidant can also act as a pro-oxidant to drive a cellular process—is not unique to melatonin. It highlights a fundamental principle of cellular biology: a molecule's effect is often context- and concentration-dependent, with the same molecule

serving as both a protective agent and a signaling molecule depending on the biological setting [45]. In this instance, our research posits that melatonin shifts its role from a general scavenger of oxidative stress to a precise generator of a localized ROS signal, which serves as a crucial second messenger to initiate the pro-apoptotic cascade.

The generation of ROS in a controlled manner is a well-established mechanism of "redox signaling" [45]. Unlike the uncontrolled oxidative burst that leads to cell damage, a targeted and transient increase in ROS can trigger specific signaling pathways by modifying the activity of key proteins, such as protein kinases and transcription factors, through the oxidation of specific cysteine residues [52]. Our findings suggest that melatonin exploits this mechanism to selectively activate the p38 MAPK pathway. The precise biochemical steps that link melatonin treatment to this controlled ROS generation are complex and warrant further investigation, but we can hypothesize a potential mechanism. Given that mitochondria are both the primary source of intracellular ROS and a key target for melatonin, it is plausible that melatonin interacts with the mitochondrial electron transport chain (ETC) in a way that promotes a subtle, controlled leakage of electrons to oxygen, thereby generating a transient burst of superoxide radicals (O2·) [52]. Melatonin has been shown to alter mitochondrial membrane potential and influence the activity of complexes within the ETC, suggesting a direct link to this process [38].

A critical component of this signaling is the direct link to the core clock gene, BMAL1, which our study identified as an essential upstream regulator. This connection provides a potential explanation for the switch in melatonin's role. It is plausible that the upregulation of BMAL1 by melatonin modifies mitochondrial dynamics or other cellular metabolic processes, creating a cellular environment where a localized pro-oxidant signal can be effectively generated [27]. For instance, BMAL1 has been shown to regulate the transcription of genes involved in mitochondrial function and energy metabolism [43]. An increase in BMAL1 protein levels could lead to changes in the expression of key mitochondrial proteins, such as components of the ETC or uncoupling proteins, which could, in turn, promote a transient, non-damaging ROS efflux [53]. This sophisticated interplay between the circadian clock and cellular redox state suggests a deep, interconnected regulatory network that controls bone homeostasis.

The activation of the p38 MAPK pathway in our study provides a direct molecular link from this ROS signal to the apoptotic response. The p38 MAPK is highly sensitive to oxidative stress and can be activated by a variety of upstream signals, including ROS [30]. The oxidation of a specific cysteine residue on the p38 kinase, for example, can lead to its conformational change and activation [45]. This redox-sensitive activation is a well-documented mechanism of p38 signaling, and our results—that a ROS scavenger (NAC) blocks melatonin’s effect on p38 phosphorylation—provide direct evidence for this pathway in our system. The subsequent phosphorylation of downstream targets by p38 leads to the activation of the pro-apoptotic machinery, culminating in the cleavage of caspase-3 and the demise of the osteoclast-like cell.

This dual-role concept significantly enhances our understanding of melatonin’s therapeutic potential. Instead of being viewed merely as a general protective agent, melatonin can be considered a highly specific modulator that can drive a desired cellular outcome (apoptosis in osteoclasts) through a precise signaling cascade. This level of specificity is highly desirable in a therapeutic agent. For PMOP, the goal is not to broadly inhibit all cellular processes but to selectively eliminate the hyperactive osteoclasts that are contributing to bone loss. Our findings suggest that melatonin achieves this with remarkable precision. This sophisticated mechanism could be a key reason why melatonin has a more favorable side-effect profile compared to conventional treatments.

This nuanced understanding also opens up new avenues for research and therapeutic development. We can now

ask more specific questions about melatonin's action. For example, what is the precise molecular mechanism by which BMAL1 upregulation promotes mitochondrial ROS generation? Does this mechanism involve a direct physical interaction or a transcriptional regulatory loop? What are the specific targets of p38 that lead to the activation of caspases in osteoclast-like cells? Further studies could use advanced techniques such as proteomics to identify the specific proteins that are oxidized in response to melatonin treatment, providing a more comprehensive map of the redox-signaling network. Understanding this dual-role capacity is a critical step towards harnessing melatonin's full therapeutic potential.

4.4. Comparison with Other Anti-Osteoporotic Agents

Current anti-osteoporotic drugs, such as bisphosphonates and denosumab, primarily work by inhibiting osteoclast activity and survival, thereby reducing bone resorption [18]. While effective, these agents can have potential side effects, including osteonecrosis of the jaw and atypical femoral fractures [19]. Melatonin, being a naturally occurring hormone, offers a potentially safer alternative with a favorable side-effect profile [36]. Our study reveals that melatonin's mechanism of action—inducing apoptosis through a specific, targeted pathway—is a powerful strategy for mitigating bone loss. Unlike bisphosphonates, which can accumulate in bone for years, melatonin's action is transient and precisely regulated by the cell's own signaling machinery [15, 17]. This unique mechanism of action could lead to the development of new, more biocompatible therapeutic approaches for PMOP. The dual effect of melatonin—promoting osteoblastogenesis and inhibiting osteoclast survival—makes it a highly attractive candidate for a holistic bone health therapy [40].

4.5. Implications and Clinical Relevance

Our findings have profound clinical implications. By elucidating the BMAL1/ROS/MAPK-p38 signaling axis, this study provides a molecular foundation for the use of melatonin as a potential therapeutic agent for PMOP. The ability to precisely target and induce apoptosis in osteoclasts without affecting other cells could minimize off-target effects and improve therapeutic efficacy. Furthermore, the identification of key molecular targets within this pathway could pave the way for the development of new small-molecule drugs that mimic or enhance melatonin's action. The link to BMAL1 also suggests that interventions aimed at regulating circadian rhythms could have an ancillary effect on bone health, a concept that warrants further exploration.

4.6. Limitations and Future Directions

While our study provides a comprehensive mechanistic framework, it is not without limitations. Our research was conducted using the RAW264.7 cell line, which is a well-established model for osteoclast differentiation but is not a primary human cell line [46, 47]. The findings from this in vitro study need to be validated in primary osteoclasts derived from human or animal bone marrow to confirm their relevance to human physiology. The use of specific pharmacological inhibitors, while powerful, can sometimes have off-target effects. Therefore, future studies should utilize genetic approaches, such as gene knockout or CRISPR-Cas9 gene editing, to more definitively confirm the role of BMAL1 and p38 in this pathway.

Looking ahead, the next crucial step is to transition from in vitro to in vivo studies. Using a well-established animal model of postmenopausal osteoporosis, such as ovariectomized rats or mice, would allow us to assess the in vivo efficacy of melatonin in preventing bone loss and reducing fracture risk. Such studies could also investigate whether systemic melatonin administration leads to the activation of the proposed BMAL1/ROS/MAPK-p38 pathway in the bone tissue of live animals. Additionally, future research should explore whether BMAL1 acts as a direct transcriptional regulator of genes involved in ROS production or p38 activation,

which would provide a deeper understanding of the molecular mechanics of the pathway.

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