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The role of melatonin in bone regeneration: A review of involved signaling pathways

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ABSTRACT

Increasing bone resorption followed by decreasing bone mineralization are hallmarks of bone degeneration, which mostly occurs in the elderly population and post-menopausal women. The use of mesenchymal stem cells (MSCs) has raised many promises in the field of bone regeneration due to their high osteoblastic differentiation capacity and easy availability from abundant sources. A variety of compounds, including growth factors, cytokines, and other internal factors, have been combined with MSCs to increase their osteoblastic differentiation capacity. One of these factors is melatonin, whose possible regulatory role in bone metabolism and formation has recently been suggested by many studies. Melatonin also is a potential signaling molecule and can affect many of the signaling pathways involved in MSCs osteoblastic differentiation, such as activation of PI3K/AKT, BMP/Smad, MAPK, NFkB, Nrf2/HO-1, Wnt, SIRT/SOD, PERK/ATF4. Furthermore, melatonin in combination with other components such as strontium, vitamin D3, and vitamin K2 has a synergistic effect on bone microstructure and improves bone mineral density (BMD). In this review article, we aim to summarize the regulatory mechanisms of melatonin in osteoblastic differentiation of MSCs and underling involved signaling pathways as well as the clinical potential of using melatonin in bone degenerative disorders.

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Abbreviations

AKT	Protein kinase B
ATF4	Activating transcription factor 4
BMD	Bone mineral density
BML	Bone marrow lesions
BMPs	Bone morphogenetic proteins
BMSCs	Bone marrow stromal cells
CDK	Cyclin-dependent kinase
CHOP	CCAAT-enhancer-binding protein homologous protein
Col1a1	Collagen 1a1
CREB	cAMP response element-binding protein
CRP	C - reactive protein
Dex	Dexamethasone
eIF2α	Eukaryotic initiation factor 2α
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ERS	Endoplasmic reticulum stress
ESC	Embryonic stem cells
FGF-2	Fibroblast growth factor 2
FSS	Fluid shear stress
hFOB	Human fetal osteoblastic
HIF-1	Hypoxia-inducible factor 1
HO-1	Heme oxygenase-1
IkB	Inhibitor Kb
IKK	IkB kinase
iPSC	Induced pluripotent stem cells
JNK	c-Jun *N*-terminal kinase
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MHC II	Major histocompatibility class II
MSCs	Mesenchymal stem cells
MSDK	melatonin, strontium (citrate), vitamin D3, vitamin K2
mTOR	Mammalian target of rapamycin
NFATC1	Nuclear Factor Of Activated T Cells 1
NPY1R	Y1 receptor
NPY	Neuropeptide Y
Nrf2	Nuclear factor-erythroid 2-related factor 2
OPG	Osteoprotegerin
OVX	Ovariectomized
PDGF	Platelet-derived growth factor
PERK	Protein kinase-like endoplasmic reticulum kinase
PI3K	Phosphoinositide 3-kinase
POSTN	Periostin
PRKD1	Protein Kinase D1
PTEN	Phosphatase and tensin homolog
RANKL	Receptor activator of nuclear factor _ B ligand
RAP	Rapamycin
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
Runx2	Runt-related transcription factor 2
SIRT	Silent information regulator type
SOD	Superoxide dismutase
SPRY	Sprouty RTK Signaling Antagonist
STIM	Stromal-interacting molecule
TGF	Transforming growth factor-beta
TKA	Total knee arthroplasty
VEGF	Vascular endothelial growth factor

1. Introduction

Bone with its self-renewal ability can regenerate and repair itself. However, we should not ignore that there is a limited ability of the human body to regenerate itself [1]. Bone as a complex tissue contains specific cells, including mesenchymal stem cells (MSCs), osteoblasts, osteocytes, and osteoclasts, and also bone extracellular matrix (organic and inorganic phase). MSCs as multipotent non-hematopoietic stromal cells can divide asymmetrically and differentiate into osteogenic, chondrogenic, and adipogenic tissues [2,3]. Osteoblasts are 4–6% of bone cells that are derived from MSC. Indeed, MSC expresses Runx2 (Runt-related transcription factor 2) and Col1a1 (Collagen 1a1) genes to become osteoblasts progenitors.

Runx2, Osterix, Spp1, and BGLAP, as the osteogenic transcription factors, play a significant role in the commitment of MSCs to differentiate into osteoblast lineage. Moreover, Osteocytes are a result of the last stage of osteoblasts differentiation and they localize on the bone matrix. Osteoclasts originate from hematopoietic stem cells. Macrophage colony-stimulating factor (M-CSF) and RANKL (receptor activator of nuclear factor_B ligand) stimulate these stem cells to differentiate into osteoclasts to directly modulate bone resorption. Mechanistically, osteoblasts, stromal cells, immune cells express RANKL, osteoclast precursors, and osteoclasts express the RANK receptor. The interaction between this ligand and receptor has a pivotal role in bone remodeling processes. Meanwhile, osteoprotegerin (OPG) can inhibit this interaction and consequently inhibit bone loss [4,5].

Bone regeneration has attained remarkable clinical consideration in order to treat various bone degenerative diseases such as osteoporosis, osteoarthritis (OA), and rheumatoid arthritis. There are various strategies have been used in regenerative medicine to overcome these disorders and recover lost tissues. According to their capacity to differentiate towards multiple mesenchymal

lineages including bone, MSCs have great potential for use in bone regeneration in pursuit of successful treatment.

Melatonin (*N*-acetyl-5-methoxytryptamine) is a hormone secreted by the pineal gland that affects circadian rhythm, regulates the sleep-wake cycle, inhibits tumor growth, and regulates immunity. In most mammals, including humans, melatonin receptors have two subtypes, MT1 and MT2, which belong to the G protein-coupled receptor family, which made melatonin capable to regulate multiple intracellular signaling cascades and lead to the activation of different ion channels [6]. Today, melatonin as a drug or supplement has an important place in pharmaceutical studies, which could links health practices to chemical sciences [7,8]. Furthermore, melatonin's possible regulatory role in bone metabolism has been taken into consideration by researchers in recent years. Melatonin induces osteoblastic differentiation of MSCs, which is one of the most important strategies in the field of bone regenerative medicine. Melatonin is also involved in protecting osteoblasts against stress-induced apoptosis and could stimulate the mineralization of pre-osteoblastic cells [9]. Some recent studies demonstrated that oral administration of melatonin can increase bone formation and bone strength in ovariectomy-induced degenerated bone and aged mice with osteoporosis [10,11]. Moreover, the mechanism and signaling pathways involved in melatonin's effects on osteoblastic differentiation also has been investigated in numerous recent articles. Summarizing these signaling pathways can help to better understand the bone biology under the melatonin treatment. In this regard, in this review article, we will explain the signaling pathways involved in increasing the differentiation and survival of osteoblasts that are activated by melatonin.

2. Bone degenerative disease

Osteoporosis, osteoarthritis, rheumatoid arthritis, and idiopathic scoliosis are common disabling conditions that cause many difficulties for individuals and increase the health burden in societies. Osteoporosis is a chronic and long-term disorder conducting to increases the incidence of fragility fractures [12]. Genetics, age, hormonal change, smoking, calcium, and vitamin D deficiency are introduced as the main risk factors for this systemic skeletal disease [13]. There are some underlying mechanisms behind osteoporosis including loss of optimal strength during bone growth, increasing bone resorption, and replacement failure. In another word, bone remodeling has two phases: bone resorption and osteoblastic replacement. Bone resorption is a shorter phase compared with osteoblastic replacement. Therefore, an increase in bone remodeling causes bone loss and consequently osteoporosis [14]. Studies have proven that pro-inflammatory cytokines, including IL-1, IL-6, and TNF-α, play a pivotal role in the bone resorption phase and bone mass in humans. In addition, osteoblasts and osteoclasts are two responsible cells for bone remodeling. Osteoblasts have an essential role in producing and secreting organic and inorganic parts of the extracellular bone matrix. Also, osteoblasts modulate bone resorption by the secretion of macrophage colony-stimulating factor (*M-CSF*), receptor activator of nuclear factor kappa-B ligand (RANKL), and osteoprotegerin (OPG). Mechanistically, RANKL interacts with its receptor RANK on osteoclasts and causes osteoclast differentiation. However, OPG as a decoy protein blocks RANK/RANKL interaction in order to inhibit osteoclast differentiation. Disregulation of this process leads to bone disease especially osteoporosis [14,15]. Estrogen deficiency in postmenopausal women is an underlying cause of the inadequate bone formation and consequently osteoporosis [16]. There is an argument that menopause, osteoporosis, and melatonin reduction are interrelated. Therefore, investigations suggest that nightly melatonin treatment can alleviate menopause symptoms and increase BMD in postmenopause osteoporosis cases [17,18]. The protective effects of melatonin therapy against bone loss in diverse models of osteoporosis are shown in Table 1. However, more clinical trials should be conducted in the future to examine the effects of melatonin therapy in human patients suffering from osteoporosis and reach a general agreement on the doses and estimated side effects, because these, are essential information, that help clinicians to find the best method in osteoporosis therapy.

Osteoarthritis, the most common joint disease, is prevailed among people over 60. The whole joint, involving cartilage, subchondral bone, and synovium play important role in OA pathogenesis in association with systemic inflammation. Indeed, there is an imbalance between repair and destruction of the joint. To sum up, in OA cartilage loss occurs and bone turnover rises in subchondral bone [19]. Moreover, IL-1β and TNF-α are mentioned as the most secreted cytokines in OA [20].

Rheumatoid arthritis, a chronic inflammatory joint disease, is pathologically occurred in the presence of autoantibodies. In rheumatoid arthritis, Joint swelling is the cause of synovial membrane inflammation and consequently immune system activation that leads to leucocyte infiltration. Therefore, during joint inflammation, synovitis consists of innate and adaptive immune cells. RANKL expresses on T cells, B cells, and fibroblasts, also, RANK expresses on macrophages, dendritic cells, and pre-osteoclasts. Taken together, this synovial inflammation promotes chondrocyte catabolism and synovial osteoclastogenesis leading to more joint damage and symptoms [21]. As a result, Healthcare providers and policymakers try to confront the increasing rate of bone diseases.

Idiopathic scoliosis (IS) the most common scoliosis is a complex disease that is associated with deformity of the spine and it is common among adolescents aged between 10 and 16. Although the etiology of IS is not fully understood, it has been suggested that it is related to biomechanical, neuromuscular, genetic, and environmental conditions [22–24]. Observations show that not only BMD in IS patients is low, but also the osteogenic differentiation of idiopathic scoliosis MSC cells decreases compared to normal cells [25,26]. During the past two decades, some treatments, including surgical treatment have been suggested but these strategies are challenging. In this regard, more investigations need to find the most efficient therapy for this disease [27].

3. Stimulating MSC differentiation to osteoblasts as a strategy for bone regeneration

“Regenerative medicine” was declared for the first time by Kaiser et al., in 1991 [28]. As inferred from its name, regenerative medicine is the regrowth of injured or lost tissues or organs. On this basis, tissue regeneration especially bone regeneration has been the subject of lots of research for the last two decades. A large number of these researches have been conducted to determine the main factors, which require for bone regeneration. The researchers have introduced three factors including stem cells, growth factors, and scaffold materials [29] (Fig. 1). Besides creating mechanical strength, scaffolds can provide an osteoconductive platform to increase cell adhesion, and provide a matrix for cell proliferation [30]. There are two categories of scaffold materials: 1- natural bone grafts, and 2- synthetic bone grafts. Natural bone grafts are divided into three subtypes based on their origin, including autografts (the biopsy of the host), allografts (same species, different individual), or xenografts (different species such as bovine). Particularly, the use of autografts has been declared the gold standard method because of decreasing the risks of tissue rejection and transmission of diseases [31,32].

However, one of the most important factors in bone

Table 1
Evidence of melatonin's effects on osteoporosis improvement.

Model	Case of Study	Kind of Administration	Dosage	Duration	Melatonin Effect	Additional information	Reference
Healthy Mice	Mice	Oral gavage	10 or 100 mg/kg body weight/d	6 weeks	Regulates bone mass (↑) Cures ovariectomy-induced bone loss	*MT2*−/− cells did not respond to melatonin addition	[141]
Postmenopausal osteoporosis model (ovariectomized)	Mice	Intraperitoneal injection	60 mg/kg per day	8weeks	BMD (↑) BV/TV (↑) upregulates ZIP-1 promotes citrate secretion		[142]
Induced osteoporosis (ovariectomized)	Mice	Intraperitoneal injection	10 or 50 mg/kg	8weeks	Promoted osteoblast differentiation inhibit NLRP3	Via mediating the Wnt/β-catenin pathway	[143]
Type 2 Diabetic Osteoporosis	Mouse MC3T3-E1 cells	Incubated with melatonin	100 nM	–	Rescued cell proliferation Decreased high glucose-induced late apoptotic Alleviates High Glucose-Induced ER Stress	The incidence of osteoporosis is significantly higher In type 2 diabetes patients	[144]
Age-related osteoporosis	Male BALB/c Mice	Orally administered	100 μg/ml	4–20 months of age	Increase plasma melatonin improve both bone strength and trabecular bone density	Detection of MT2 in both osteoblasts and osteoclasts of the mice femur melatonin deficient mice	[145]
Postmenopausal osteopenic women	Clinical trial	Oral administered	1,3 mg	1year	femoral neck BMD (↑) trabecular thickness in tibia (3 mg) ↑ volumetric bone mineral density (vBMD) in the spine(3 mg) ↑	in a dose-dependent manner	[146]
Induced osteoporosis (ovariectomized)	SD Rats and BMMSCells	Tail vein injection	10 mg/kg	3month (twice a week)	ameliorated the bone micro-architecture intracellular oxidative stress ↓ intracellular antioxidant enzymes (SOD2, GPX1)↑	SIRT1 was involved in the melatonin-mediated recovery of osteogenesis and antioxidant functions	[147]
		Incubated with treatment	1 μM 14 and 100 μM	14 days	osteogenic potential ↑		
Experimental autoimmune encephalomyelitis (EAE) model of MS	C57BL/6 mice	Intraperitoneal injection	10 mg/kg/day	13 days	Serum levels of vitamin D, calcium, and osteocalcin (OCN) reverted back to nearly normal levels	MS promotes osteoporosis and Melatonin increases bone formation by increasing OCN levels in vivo	[148]
Ovariectomized (OVX) rats	SD Rats	Intraperitoneal injection	50 mg/kg body weight	4 weeks	Periprosthetic bone mass, implant fixation intensity↑ The expression of oxidative stress markers (NAPDH oxidase 2 and cytochrome *c*) ↓	Expression levels of the alkaline phosphatase, osteocalcin, and osterix ↑ Melatonin ameliorated oxidative stress in mitochondrial via the SIRT3/SOD2 signaling pathway	[149]
Ovariectomy	Mouse bone marrow cells(in vitro)	Intraperitoneal injection	5–25 mg/kg	8 weeks	Blocked RANKL-induced osteoclastogenesis by inhibiting PRMT1 and asymmetric	The anti osteoclastogenic effect of melatonin	[150]

(*continued on next page*)

Table 1 (*continued*)

Model	Case of Study	Kind of Administration	Dosage	Duration	Melatonin Effect	Additional information	Reference
					dimethylarginine (ADMA) expression	was mediated by a cascade of inhibition of RANKL-induced TRAF6, JNK, PRMT1, and NF-κB signaling in melatonin receptors-independent pathway	
	Female C57BL/6 mice				Alleviated the ovariectomized (OVX)-induced bone loss by inhibiting bone resorption		
Glucocorticoid-induced osteoporosis(GIO)	Wistar female rats	Intraperitoneal injection	5 mg/kg	28 days.	Positive effects melatonin on bone metabolism in rats with GIO	Glucocorticoids are the most common cause of drug-induced osteoporosis.	[151]
Retinoic acid induced OP	C57 mice	Intraperitoneal injection	5–50 mg/kg/d	8week	Promoted bone formation and inhibited bone resorption. Promoted the antioxidant level and suppressed the level of oxidant molecules	Play a role in the ERK/SMAD and NF-κB pathways	[152]
Aged rats with OP	SD	intragastric administration	40 mg/kg/d	12week	Bone density of lumbar vertebra and bilateral femur and bone mineral level ↑	Elevate serum calcium level and reduce bone mineral loss	[153]
Type 2 Diabetic	Rats MC3T3-E1 Cells	Incubated	1, 10, or 100 μM	48 h incubated	Significantly reduced the level of ferroptosis and improved the osteogenic capacity of MC3T3-E1 through activating the Nrf2/HO-1 pathway in vivo and in vitro	High glucose induces ferroptosis via increased ROS/lipid peroxidation/glutathione depletion	[154]
Osteoporosis	SD rats	Intraperitoneal injection	10–50 mg/kg				

BMD: bone mineral density; BV/TV: Bone volume fraction; OP: Postmenopausal osteoporosis.

regeneration is cells. Several cell types like osteoblasts, embryonic stem cells (ESC), induced pluripotent stem cells (iPSC) and MSC can use for bone regeneration. In particular, there is a general agreement that MSC is the best candidate cell among them. It has three specific reasons. First, MSCs do not have problems like the low potential of proliferation, immunologic incompatibility, teratoma possibility, and clinical application difficulties. Secondly, MSCs are responsible for the normal process of bone healing. MSCs can produce and release factors that have a role in the managing of chemotaxis, angiogenesis, and osteogenesis. Also, they can differentiate to osteoblasts and chondrocytes. Finally, these cells have lower levels of the major histocompatibility class II (MHC II) markers, which enable them to escape from the immune system and consequently make them a great factor for therapeutic strategies like bone regeneration [29,33]. In a systematic review, researchers assessed 23 studies and they concluded that MSC exosomes have therapeutic efficacy for bone regeneration without considering the type of exosomes. Indeed, MSC exosomes promote osteogenic differentiation to osteoblasts and osteocytes as well as give rise to osteogenic markers like Osteocalcin (OCN) and RUNX2 [34]. Practically, the result of 5–15 years follows up of 140 OA patients (between 65 and 90 years old) showed that MSCs injection in subchondral bone of OA cases decreased bone marrow lesions (BML) volume and consequently reduced or postponed the need for total knee arthroplasty (TKA) significantly to just 18% of total patients in a mean of ten years. Also, subchondral bone MSCs treatment is more applicable for patients with BML lower than 3 cm^3 without severe malalignment [35].

Encouraging results from osteoporotic animal model studies have proved that MSC transplantation enhances BMD, osteogenic markers, like ALP and OCN, and finally, bone formation, which these findings suggest new cell-based therapy for osteoporosis [36,37].

The other important factor in bone regeneration is growth factors. The growth factors including, BMPs (bone morphogenetic proteins), TGF_β (transforming growth factor-beta), VEGF (vascular endothelial growth factor), and PDGF (platelet-derived growth factor) as morphogenic signals play a significant role in bone regeneration and vascularization. BMPs are secreted from MSCs, osteoprogenitor cells, osteoblasts, and chondrocytes. Then, BMPs reside in ECM to modulate MSC cell proliferation and differentiation into osteoblasts and chondrocytes. Platelets produce and discharge TGF-β and PDGF at the beginning of the fracture healing process. TGF-β has three main functions in the bone healing process, including osteogenic cell proliferation, BMP synthesis induction, and osteoclast in-activation and apoptosis (20).

In conclusion, the application of MSC has shown a promising role in bone regeneration and bone healing processes, and growth factors can increase MSCs survival rate and osteoblastic differentiation whereas scaffolds can provide a platform for their growth.

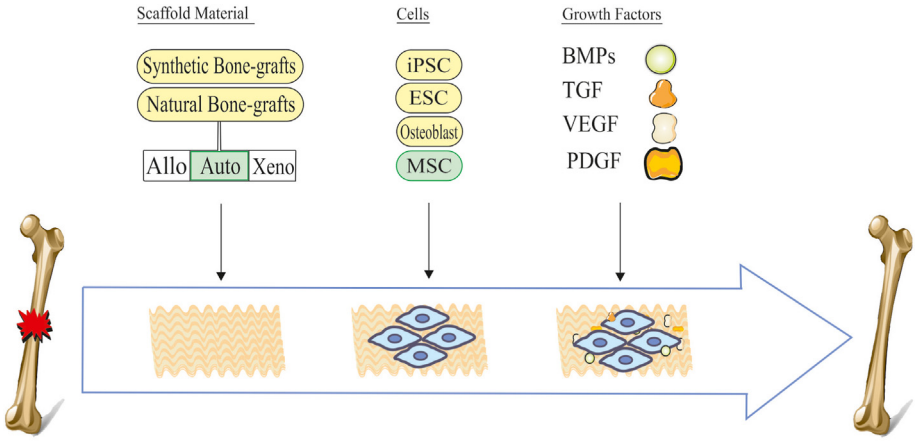

Fig. 1. Schematic diagram of bone regeneration strategies. This figure shows the chosen components that are more efficient in bone regeneration process. The final goal is either restoring the lost function or introducing a new function to the damaged tissue.

4. Melatonin and osteoblastic differentiation

Different aspects of the cellular functions of melatonin have been widely investigated in recent years. It has been proven that melatonin contributes to anti-inflammation, anti-oxidant, circadian, and endocrine rhythm regulation. As such, it has been demonstrated that melatonin has a pivotal role in the regulation of cell survival, proliferation, differentiation, and apoptosis. For instance, melatonin has an imperative role in ESC, iPSC, and especially MSCs differentiation to osteoblasts lineage. In this regard, melatonin has become the center of attention in recent decades and it opens a new window in bone regenerative medicine and fracture healing [38].

There are a variety of mechanisms with which melatonin can induce osteogenesis in these stem cells, for example, melatonin can increase chromatin accessibility of the osteogenic genes, promote osteogenesis of BMSCs in vitro, and alleviate osteoporosis progression in-vivo [39]. In addition, a recent study showed that the potential application of melatonin in osteoporotic bone defect repair comes from its promoting effect in osteogenesis–angiogenesis coupling. In such a way that, besides increasing osteogenesis differentiation, melatonin also has angiogenic effects, accelerating bone regeneration. BMSCs cells are treated with melatonin and have high expression levels of both osteogenesis-related markers such as ALP, OCN, Runx2, and angiogenesis-related markers such as VEGF, angiopoietin-2, and angiopoietin-4 [40]. However, melatonin is a molecule with a dual role in the control of angiogenesis, and depending on the conditions, for example, the hypoxic state of tumor cells that causes the overexpression of angiogenic factors, it can exert anti-angiogenic or pro-angiogenic effects. There are many factors such as signaling pathways, dosages used, or cell type used in the study (whether it is a cancer cell or a stem cell) involved in determining which effect melatonin has on angiogenesis (reviewed in Ref. [41]).

Melatonin can be used as a supplement candidate for improving osteoporosis and OA by increasing MSCs' osteoblastic differentiation. The findings of a study indicated that melatonin can inhibit the production of ROS during bone formation and differentiation of BMSC, and antagonizes TNF-α-induced ROS production. While the concentration of melatonin is 100 μM, its pro-osteogenic, anti-inflammatory, and antioxidative effects can be maximized [42]. Another study shows that melatonin promotes bone formation differentiation in human MSC and restores oxidative stress-inhibiting bone formation through AMPK signaling pathway activation in human MSC, which may characterize osteoporosis metabolism. These findings propose a promising new therapeutic strategy for treating osteoporosis. When human MSCs treated with melatonin (100 μM) for two weeks, the level of calcium deposition was significantly increased in-vitro. These results indicate that melatonin has an effect on osteoblast differentiation and can be used as a bone formation promoter in stem cell-based therapies [43]. In addition, due to its powerful biological function, melatonin is associated with the development of several degenerative diseases. Concerning OA, melatonin promotes cartilage matrix synthesis, inhibits chondrocyte apoptosis, weakens the inflammatory response, and suppresses matrix degradation by regulating the TGF-β, MAPK, or NF-κB signaling pathway has been reported [44].

Furthermore, melatonin mediates osteoblastic differentiation of MSCs dose-dependently and time-dependently through the regulation of downstream signaling pathways, including PI3K/AKT, BMP/Smad, MAPK, NFkB, Nrf2/HO-1, PERK-eIF2α-ATF4, Septin4 and 7, SIRT1/SOD2, and Wnt pathways. Thus, we will discuss the cross-talk between melatonin and these pathways' role in bone regeneration.

4.1. PI3K/AKT and BMP/Smad signaling

PI3K/AKT signaling pathway involves various cellular processes like apoptosis, proliferation, and differentiation. Also, multiple studies have reported the function of PI3K/AKT signaling in bone regeneration and osteoblastic differentiation. First, PI3K/AKT appears to increase the expression level of VEGF protein, an essential component in bone regeneration and osteoblastic differentiation, through HIF-1α expression [45] (Fig. 2-a). Secondly, PTEN as a tumor suppressor can inhibit PI3K/AKT signaling pathway and its function. As such, PTEN inhibition enhances fracture healing processes in mature osteoblasts. In this regard, researchers have suggested that PTEN inhibition participates in bone repair due to PI3K/AKT pathway activation [46]. Finally, there is some evidence that the activation of PI3K/Akt and BMP/Smad pathways increases the expression level of osteogenic transcription factors, like Runx2, leading to the induction of osteoblastic differentiation [47,48] (Fig. 2-a). A study showed that 100 μM dexamethasone (Dex) reduces Runx2 expression and consequently decreases osteoblast differentiation and mineralization in MC3T3-E1 cells. However, 1 μM melatonin treatment is able to reverse these effects and gives a rise to osteogenic differentiation and calcium deposition. Also, in this study, researchers have tested 8 signaling pathway inhibitors

to find which ones participated in the melatonin-induced osteogenic differentiation. Results have shown that PI3K/AKT and BMP/Smad signaling cascades have a positive correlation with melatonin treatment and osteogenesis. Taken together, melatonin as an osteogenic agent mediates its function in Dex-induced MC3T3-E1 cells by PI3K/AKT and BMP/Smad signaling pathways [48]. This specific study just focused on cell lines and, regarding the differences in in-vivo situation, it is recommended to apply this method in vivo on mice to compare with these results.

Platelet-derived growth factor (PDGF) is one of the important growth factors required for cell proliferation and bone formation. Previously, it has been revealed that PDGF can activate downstream signaling pathways like the AKT signaling pathway in order to mediate cell proliferation activation [49,50]. A recent study reported that the administration of melatonin causes osteoblastic differentiation of MC3T3-E1 cells through PDGF/AKT signaling pathway. Moreover, the fracture healing process in melatonin-treated mice has been observed in this study, and the experiment results confirmed the involvement of the PDGF/AKT signaling pathway in the fracture healing process of melatonin-treated mice [51] (Fig. 2-a).

4.2. MAPK signaling pathway

Mitogen-activated protein kinase (MAPK) family consists of three members including extracellular signal-regulated kinase (ERK), p38, and c-Jun *N*-terminal kinase (JNK). These elements have been proven to participate in cellular processes like proliferation and differentiation. Relatively, MAPKs are introduced as key signal transducers responsible for regulating bone mass [52,53].

Melatonin serves as a modulator for different signaling pathways including MAPK signaling in order to regulate osteoblastic differentiation. On the other side, as we mentioned above, the dual effect of melatonin on osteoblast proliferation depends on its concentration and time of exposure. For example, 1 mM melatonin is able to cause cell cycle arrest and inhibit osteoblast cell proliferation. Therefore, a study revealed that melatonin concentration, ERK pathway, and osteoblast proliferation are interrelated. In this study, researchers revealed that 1 mM melatonin suppresses ERK phosphorylation, and activation, conducting down-regulation of gene expression of cyclin D1 and CDK4 (G1 phase arrest) as well as cyclin B1 and CDK1 (G2/M phase arrest), which overall leads to osteoblasts proliferation inhibition [54] (Fig. 2-b). Not only osteoblasts differentiation but also osteoclasts differentiation requires activation of ERK signaling at the early stage of fracture healing [55]. This study on adult zebra fish also confirmed that melatonin exposure (100 nM) can inhibit ERK signaling, consequently reducing osteoblasts and osteoclasts differentiation and finally causing fracture healing impairment. As a result, we can conclude that although melatonin reduction leads to bone loss and osteoporosis, excessive melatonin appears to repress the fracture healing process [55]. This specific study revealed that high doses of melatonin can be toxic and exacerbate bone loss. Thus, it will be necessary to confirm the therapeutic dosage based on in-vivo studies. On the other hand, by inactivation of MAPK, melatonin inhibits osteoclast differentiation, which suggests that melatonin could be a suitable therapy for bone loss and imply a potential role of melatonin in bone health. In a recent study melatonin (300 μM) inhibits V-ATPase Vo Domain (Atp6v0d2) through the MAPK signaling inhibition. ATP6v0d2 is known to be an essential component of the osteoclast-specific proton pump, that mediates extracellular acidification in bone resorption. Therefore, these results indicate that melatonin exerts suppressive effects on osteoclastogenesis [56]. However, in lower concentrations melatonin has an activator role for MAPK/ERK signaling. In this regard, a recent in-vitro study revealed that melatonin (100 μM) significantly promoted proliferation, osteogenic gene expression, and ALP, OCN, and RUNX-2 proteins expression of dental pulp-derived MSCs by phosphorylation and activation of MAPK/ERK signaling [57].

Another study was conducted to investigate the role of MEK/ERK signaling on melatonin-induced osteoblast differentiation in MSCs. Analysis of this investigation showed that not only does knocking down of MEK/ERK (1/2) signal transduction have an inhibitory effect on melatonin-induced osteoblast differentiation in MSCs, but also acute melatonin exposure (50 nM) raises MEK/ERK and subsequently increases osteoblastic differentiation. However chronic exposure has converse results. Because it has been found that melatonin receptors, especially MT2 receptors, are involved in the mediation of melatonin function in osteogenesis. So, when MSCs expose to chronic melatonin, melatonin receptors desensitize and cause MEK/ERK signaling inhibition. In conclusion, researchers declared that melatonin induces MEK/ERK signaling activation and translocation, leading to enhancement of ALP expression and activation, which takes part in osteoblast differentiation of MSCs [58] (Fig. 2-b). A recent study showed the role of melatonin in MEK1/2 and MEK5 in mouse and human MSCs and on bone using small-molecule inhibitors and CRISPR/Cas9 knockout approaches. This team demonstrated that MEK1/2 and MEK5 were the primary drivers underlying melatonin's actions on bone density and bone mechanical properties by increasing osteogenic factors (RUNX2, BMP-2, FRA-1, OPG) expression and decreases in PPARγ [59].

We believe that duration, time, and dosage are the determining factors in the effectiveness of melatonin therapy. However, there is a lack of evidence on the protocols of melatonin therapy. This gap should be filled by future examinations.

There is an argument that melatonin has the capacity to reverse the effects of iron overload.

The contribution of iron imbalance is investigated in various diseases including osteoporosis [60,61]. An in-vivo study [60] declared that iron overload can cause an increase in bone resorption, oxidative stress, and inhibition of BMSCs differentiation to osteoblasts, which overall conduct to bone loss and osteoporosis. Therefore, researchers suggested that melatonin as a well-known endogenous anti-oxidant is able to reverse iron overload effects. Mechanistically, ROS accumulation as a result of iron overload increases p53, ERK, and p38 signals, which are responsible to activate downstream genes involving cellular senescence, apoptosis, and differentiation suppression. In this regard, melatonin reverses iron imbalance effects via blocking phosphorylation and activation of the p53/ERK/p38 signaling pathway and consequently enhances BMSCs differentiation as well as inhibition of bone loss [62,63].

Previous studies confirmed that ERK1/2 plays a significant role in both osteoblast differentiation and mineralization [64]. In this regard, encouraging evidence shows that melatonin can provoke osteoblastogenesis from MSCs and their precursors while decreasing osteoclastogenesis through activation and modulation of the ERK1/2 pathway [18,65].

Moreover, transduction of frictional force from blood flow (FSS) by endothelial cells leads to orchestrating signals and regulation of gene expression and consequently controlling cell behavior. It was shown that both fluid shear stress (FSS) and melatonin can stimulate ERK phosphorylation, which conducts to the higher expression level of anabolic proteins in MC3T3-E1 osteoblast cells. On this basis, this study proved that the combination of FSS and melatonin has a stronger effect on osteoblasts compared with melatonin single therapy [66].

Furthermore, it is fully understood that three kinases including p-mTOR, p-ERK, and p-Akt involve in the regulation of autophagy during ER stress and accumulation of unfolded proteins.

Growing evidence shows that FSS and melatonin together

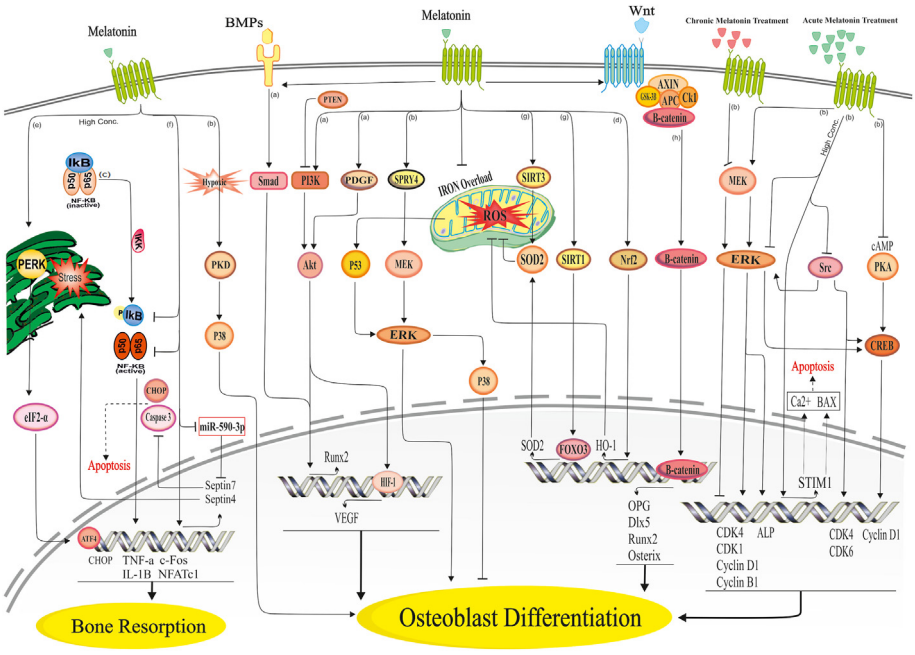

Fig. 2. The beneficial effects of melatonin therapy in osteoblastic differentiation through various signaling pathways including: a) PI3K, SMAD, PDGF b) MEK/ERK, SPRY4, PKA, PKD c) NF-KB d) Nrf2 e) PERK f) SEPTIN g) SIRT h) B-catenin.

appear to activate p-ERK, p-Akt, and p-mTOR proteins in MC3T3-E1 osteoblast cells in order to preserve both cell structure and function [67]. There is some evidence that microgravity (a condition of weightlessness and zero-g) negatively induces bone metabolism and causes bone loss and osteoporosis through the reduction of p-mTOR, p-ERK, and p-Akt proteins. Yeong-Min Yoo et al., demonstrated that melatonin has the capacity to reverse microgravity effects on pre-osteoblast MC3T3-E1 cells by increasing activation and phosphorylation of ERK/Akt/mTOR proteins [68]. Taken collectively, melatonin can regulate osteoblastogenesis, osteoclastogenesis, anti-oxidant balance, and microgravity, which are involved in the modulation of osteoporosis through ERK-specific pathways.

In contrast, hypoxia occurs during aging, inflammation, and bone fracture and causes expression inhibition of bone-forming genes. There is a study that admitted that melatonin is capable to increase bone-forming gene expression, phosphorylate and activate p38, MAPK, and Prkd1 signaling under the hypoxic condition, which these changes show beneficial effects of melatonin on osteoblastic differentiation, mineralization, and bone-forming capacity [69] (Fig. 2-b).

It has been reported that a higher concentration of melatonin has inhibitory effects on osteoblasts proliferation, which helps normal proliferation in scoliosis [70]. A group of researchers put forward the theory that a high level of melatonin can stimulate osteoblast apoptosis through up-regulation of Stromal-interacting molecule 1 (STIM1) and down-regulation of the ERK pathway [71]. STIM1 as an ER Ca2+ sensor activates when ER Ca2+ concentration reduces. Collectively, these events lead to the activation of enzymes, like phospholipases, which create damage to mitochondria and enhance cell apoptosis [72]. In this regard, the experimental analysis demonstrated that melatonin concentration and STIM1 expression have positively correlated. In fact, a higher melatonin level provokes upregulation and activation of STIM1, which has three results. First, STIM1 increases the cytosolic concentration of Ca2+, which reduces mitochondrial membrane potential and causes cell death. Secondly, STIM1 activates pro-apoptotic factors like BAX protein. Finally, there is a suggestion that melatonin inhibits the ERK pathway through up-regulation of STIM1 because the knockdown of STIM1 conducts ERK phosphorylation and activation, which results in increased proliferation and differentiation. In conclusion, a higher concentration of melatonin induces apoptosis in osteoblast cells through the STIM1/cytosolic calcium elevation/ERK pathway [71] (Fig. 2-b). Therefore, although a higher concentration of melatonin puts negative effects on osteoporosis, it might be helpful for scoliosis treatment.

Furthermore, Src as an upstream protein of the MAPK pathway is responsible to regulate some downstream proteins like Ras, c-Raf, or ERK. Moreover, Src can increase the expression and activation of CDK 4/6 complex in order to increase cell proliferation. Additionally, cAMP response element-binding protein (CREB) as a transcription factor regulates cell proliferation through the regulation of cyclin D1 activity [73,74]. Relatively, Src is the upstream factor of p-ERK/p-CREB. However, a higher expression level of melatonin (2 mM) suppresses Src function and reduces cell proliferation. Therefore, evidence confirmed that melatonin inhibits CREB activity and cell proliferation via downregulation of the Src protein. Additionally, cAMP accumulation leads to PKA activation and subsequently CREB function and cell proliferation. This study concluded that a high level of melatonin is associated with both cAMP-PKA and Src pathways regulation, conducting to inhibition of osteoblasts cell proliferation [75] (Fig. 2-b).

There is an argument that there is a correlation between SPRY4, MAPK/ERK signaling, melatonin, and osteogenesis in idiopathic scoliosis. SPRY4 as one of the Sprouty family proteins is a receptor tyrosine kinase (RTK)- associated signaling protein that is involved in osteogenic differentiation through MAPK/ERK signaling pathway [76]. SPRY4 expression in idiopathic scoliosis is significantly low. It has been demonstrated that not only does SPRY4 downregulation in idiopathic scoliosis MSCs conduct osteogenic differentiation impairment, but also SPRY4 upregulation stimulates osteogenesis from MSCs. In vivo evidence has also confirmed the contribution of SPRY4 in MSCs differentiation to osteoblasts. Mechanistically, SPRY4 plays its osteogenic role through regulation of the MEK-

ERK1/2 pathway; because SPRY4 knockdown appears to decrease MEK-ERK1/2 pathway phosphorylation and function while SPRY4 induction enhances MEK-ERK1/2 activation and eventually osteogenesis potential. Moreover, in this study researchers investigated and concluded that SPRY4 and melatonin are positively correlated and conducted to MEK-ERK1/2 pathway induction and osteogenesis from idiopathic scoliosis MSCs [26](Fig. 2-b). In this regard, it can be concluded that one of the melatonin mechanisms of action in osteogenesis induction might be through the activation of the SPRY4-MEK-ERK1/2 pathway.

4.3. NFkB signaling

NFkB signaling same as other signaling pathways and involves in different cellular processes, especially inflammatory responses. In addition, it has been revealed that this signaling contributes to osteoporosis development [77]. In a normal situation, NF-κB dimers are blocked by IκB. However, after a stimulus, IKKβ (IκB kinase) phosphorylates IκB at Ser32/36, leading to proteasomal degradation of IκB and removing the inhibitory effect of IκB on NF-κB heterodimer (p65/50). Now, NF-κB heterodimer (p65/50) can translocate to the nucleus and play its effects on gene expression patterns mostly involved in inflammation [78]. Moreover, RANKL as an osteoclastogenic cytokine appears to activate the NF-κB signaling cascade, which leads to enhanced osteoclastogenesis. In another word, the NF-κB pathway has an essential role in the RANKL-RANK axis. As we mentioned melatonin plays its osteogenic function through its anti-inflammatory capacity and the NF-κB pathway is the most important inflammatory pathway involved in osteogenesis regulation. Thus, this information generates the hypothesis that melatonin may play its anti-inflammatory role through the regulation of the NF-κB pathway. In addition, melatonin at lower concentration (1–50 μM) has a positive effect on bone balance. For example, 10 nM of melatonin can increase the expression and protein level of osteogenic markers and also decrease inflammatory cytokine, TNF-α. Taken collectively, the cross-talk between melatonin and NF-κB signaling has been investigated in osteoporosis recently. Melatonin suppresses the MT2-dependent NF-κB signaling pathway and RANKL production to inhibit osteoclastogenesis and accelerate osteogenic differentiation from MSCs [79]. It has been demonstrated that melatonin inhibits inflammatory cytokines secretion and NF-κB signaling orchestrating through blocking IκB and p65 phosphorylation to reduce bone resorption and prevent bone formation impairment [80] (Fig. 2-c). Another similar study revealed that melatonin downregulates NF-κB signaling by blocking IKKα/β, IκBα, and p65 phosphorylation to inhibit the production of inflammatory cytokines TNF-α and IL-1β and alleviate OA disease [81]. It has also been illustrated that melatonin can suppress the inflammatory osteolysis of titanium particles [82], but the underlying mechanisms were obscure [83]. Moreover, the NF-kB signaling pathway is responsible to activate during inflammation and enhance osteolysis. Therefore, a study was carried out and found the relationship between melatonin, NF-kB signaling, and osteolysis prevention. Evidence showed that melatonin suppresses IkB-α and p65 by blocking their phosphorylation, which leads to NF-kB signaling pathway inhibition, decreasing the expression level of transcription factors c-Fos and NFATc1 and consequently results in reducing inflammation and bone resorption as well as increasing bone formation [82]. Overall, the NF-kB signaling pathway is one of the critical signalings in osteogenesis regulation, which can be inhibited by melatonin and gives us a clue to consider strategies for bone disease treatment and regeneration.

4.4. Nrf2/HO-1 signaling pathway

Nuclear factor-erythroid 2-related factor 2 (Nrf2) has been revealed to have antioxidant properties, a protective effect on osteoblasts, and eventually reduce bone loss. Indeed, Nrf2 has a direct role in maintaining cellular redox homeostasis and the balance of oxidative mediators by regulation of antioxidant responsive element gene expression. In addition, the analysis showed that inhibition of Nrf2 increases osteoclasts differentiation via up-regulation of RANKL, leading to bone loss. Moreover, Nrf2is involved in the regulation of antioxidant enzymes production as a defense mechanism against oxidative stress-mediated osteoporosis [84,85].

As we mentioned before iron overload and ROS increase bone loss. Therefore, ferroptosis has harmful effects on bone microstructure. Previous data showed that Nrf2 protects cells against ferroptosis. Taken together, an in-vivo and in-vitro investigation revealed that melatonin appears to decline ferroptosis and promote the osteogenic capacity of the MC3T3-E1 cell line due to activation of the Nrf2/HO-1 pathway (Fig. 2-d). Moreover, although a higher concentration of glucose in diabetes mellitus halted osteogenic differentiation through intracellular ROS accumulation, melatonin can reverse this effect and increase osteogenic markers via Nrf2/HO-1 signaling pathway [86,87]. Another study's results show that melatonin supports the anabolic metabolism of cartilage matrix in OA chondrocytes by increasing protein levels of NRF2 through miR-146a inhibition. Melatonin-mediated activation of the NRF2/HO-1 axis prevents cartilage degeneration and is a promising therapeutic target for the treatment of early OA [88]. Therefore, we need more investigation in this field to use the potential ability of Nrf2/HO-1 signaling and melatonin in bone regeneration and bone healing processes.

4.5. PERK-eIF2α-ATF4 pathway

Protein kinase-like endoplasmic reticulum kinase (PERK) as a transmembrane protein is located in ER. It transduces a signal from ER to cytosol or nucleus to prevent the accumulation of unfolding proteins in ER. Activating transcription factor 4 (ATF4) as a member of cAMP response element-binding (CREB) has an essential role in bone formation, development, and preservation. It is fully understood that misfold protein accumulation is an important characteristic of ER stress. After that, PERK can phosphorylate and activate eukaryotic initiation factor 2α (eIF2α), which follows two separate ways: G1 phase cycle arrest or increasing translation of ATF4. Indeed, PERK is responsible for phosphorylation of eIF2α, conducting ATF4 translation, and consequently the expression of genes including genes involved in osteogenesis. Thus, the PERK-eIF2α-ATF4 signaling pathway plays a significant role in osteoblast differentiation and bone formation [89]. However, during long-term ER stress, ATF4 upregulates gene expression of CCAAT enhancer-binding protein homologous protein (CHOP) to promote apoptosis. High glucose concentration in type 2 diabetes patients causes endoplasmic reticulum (ER) stress.

(ERS) and consequently gives rise to PERK-eIF2α-ATF4-CHOP signaling pathway and finally cell death in osteoblastic cell line MC3T3-E1. This evidence confirmed that the incidence of osteoblasts apoptosis leading to bone loss and osteoporosis is high in type 2 diabetes patients. As a result, melatonin has protective effects against osteoblast apoptosis and osteoporosis by suppression of PERK-eIF2α-ATF4-CHOP signaling [90].

However, a high concentration of melatonin (4 mM) is associated with ERS, which leads to eIF2α phosphorylation, ATF4 expression, CHOP activation, cleaved caspase-3, and p-JNK upregulation (Fig. 2-e). PERK-eIF2α-ATF4-CHOP signaling pathway,

cleaved caspase-3, and p-JNK upregulation conduct hFOB 1.19 cells to apoptosis and cell death. Meanwhile, this study declared that melatonin concentration and periostin (POSTN) are interrelated [91]. POSTN as an extracellular matrix protein expresses and finds in osteoblasts and the mesenchymal lineage cells and also plays role in raising epithelial-mesenchymal transition (EMT) and cell differentiation [92]. It has been reported that inhibition of POSTN enhances the effects of melatonin in hFOB 1.19 cell apoptosis. Moreover, the report shows that although POSTN suppresses the eIF2α-ATF4 pathway, its concentration is positively correlated with melatonin concentration. Researchers have suggested that this is because POSTN has protective properties against melatonin in order to maintain hFOB 1.19 cells [91]. In conclusion, this pathway plays a significant role in bone mass and regulation accompanied by melatonin.

4.6. Septin4 and 7

Septins family is introduced as GTP binding protein family. One of the subtypes is Septin4, which plays a significant role in apoptosis and proliferation regulation through its tumor suppressive function [93]. A recent study revealed that Septin4 has a direct effect on ERS induction and osteoblasts apoptosis. This study is one of the few studies, which investigated that melatonin appears to increase Septin4 expression dose-dependently to accumulate excessive or prolonged ERS and eventually decrease osteoblasts' cell viability. Idiopathic scoliosis (IS) is prevalent among juveniles whose bones are in fastest-growing state. So, the best prevention and strategy to cure this disease is suppression of osteoblasts proliferation. On this basis, a higher concentration of melatonin stimulates Septin4 expression, leading to ERS accumulation and osteoblasts cell death (Fig. 2-f). This approach has also been suggested for Idiopathic scoliosis (IS) treatment [94].

Septin7 is another member of the Septins family and it is considered an essential factor to regulate cell cycle progression and dead. There is contradictory evidence about SEPT7 function in apoptosis or cell survival after melatonin treatment. In this regard, it has been reported that high melatonin concentration provokes apoptosis in the human fetal osteoblastic (hFOB) 1.19 cell line through induction of ERS. Relatively, Septin7 expression level and melatonin concentration are positively correlated. On this basis, the experimental analysis showed that Septin7 protects osteoblasts cell line from melatonin-induced apoptosis through suppression of apoptotic proteins, including CHOP and caspase-3 as well as inhibition of ERS accumulation [95] (Fig. 2-f). On the other side, a study revealed that SEPT7 is a direct target of miR-590-3p. Also, melatonin increases SEPT7 by decreasing miR-590-3p expression. Evidence shows that melatonin treatment causes MiR-590-3p downregulation and SEPT7 upregulation, which results in increased apoptosis in (human osteoblast cell line) hFOB 1.19 cell line rather than decreased apoptosis based on the previously mentioned study [96]. There is another study declared that melatonin has the capacity to increase intracellular calcium overload, leading to ERS and consequently osteoblast cell dead. Also, this study suggested that melatonin mediates this effect through Septin7 induction. However, upregulation or downregulation of this protein did not have a significant effect on the results. So, there is a theory that maybe other un-investigated factors are involved in this process [97]. Further studies are needed to find out the target factor of melatonin to induce ERS.

4.7. SIRT1 and SIRT3/SOD2 signaling pathway

Human silent information regulator type 1 (SIRT1) has positive effects on cell growth, cell survival, gene silencing, stress resistance, and differentiation in some specific ways [98]. First, SIRT1 enables the regulation of the transcription factors like p53 and FOXO3 through its deacetylase activity to regulate the cellular process. Secondly, SIRT1 reverses the cell senescence process and increases cell proliferation by inhibition of p16INK4α because p53/p21 and MAPK/p16INK4α pathways are confirmed that they are underlying causes of cell senescence. In fact, p16INK4α accumulation and SIRT1 suppression happen during H2O2 exposure and causes significant inhibition of CDK4 and CDK6 cell cycle kinases and induction of the p38 MAPK signaling pathway, leading to cell senescence. In this regard experimental data shows that during H2O2 exposure melatonin appears to increase SIRT1 concentration and consequently reduce p16INK4α protein, resulting in protecting MSCs from cell senescence [99]. H2O2 can spread freely through the cell membrane and is a typical representative of ROS in organisms [100]. Another study showed that melatonin activates SIRT1, and SIRT3 and inhibits p66Shc (a protein that promotes aging by inducing apoptosis and necrosis), which cause to reduces the intracellular ROS levels, stabilizes mitochondria, reduces malondialdehyde levels, increases superoxide dismutase activity, and reduce apoptosis in immature osteoblast cell line treated with H2O2 [101]. It has been widely proved that melatonin plays an antioxidant role in MSC cells and enhances the osteogenic potential of postmenopausal osteoporosis cases. A recent study declared that inhibition of SIRT1 by sirtinol and nicotinamide conducts to the accumulation of ROS and reduces MSCs proliferation. More importantly, SIRT1 suppression reduces melatonin effects on matrix mineralization and osteogenesis of ovariectomized (OVX) rats significantly. Taken together, melatonin in association with SIRT1 upregulation enhances osteogenic potential and reduces bone loss in postmenopausal osteoporosis patients [102] (Fig. 2-g). Therefore, it is estimated that SIRT1 activation is directly related to melatonin. Moreover, SIRT1 is in charge of FOXO3 deacetylation, leading to expression induction of antioxidant enzyme Superoxide dismutase 2 (SOD2), which overall has protective effects on osteoblast cells from apoptosis and inflammation. On this basis, a recent investigation reported that melatonin upregulates and activates the SIRT1/SOD2 signaling pathway in MSCs to preserve these cells and increase MSCs' osteogenic potential [103].

Another study revealed that melatonin plays its anti-inflammatory function in chondrocyte cells of OA animal models through suppression of H2O2-induced SIRT1 mRNA and protein expression [104]. In this regard, melatonin plays its anti-oxidant role and osteogenic capability through the upregulation of SIRT1.

The normal function and homeostasis of cell organelles such as mitochondria are critical for cell proliferation. In this regard, ROS accumulation appears to impair the function of mitochondria and inhibit osteogenesis [105]. Relatively, it has been proven that melatonin with its anti-oxidant propriety can protect cells and enhance osteogenesis. However, one of the underlying mechanisms revealed in 2019 when Wei Zhou et al. [106] demonstrated that melatonin has a protective role against oxidative stresses and improves bone synthesis and bone mass through SIRT3/SOD2 signaling pathway; Because mitochondrial sirtuin 3 (SIRT3), which is located in mitochondria, deacetylases Ac-SOD2 and creates the active form of SOD2 to reduce ROS accumulation [105]. However, during ROS accumulation SIRT3 expression decreases, and Ac-SOD2/SOD2 ratio increases. Evidence shows that melatonin can reverse this situation and enhances bone formation and osteogenesis of MC3T3-E1 cells [106] (Fig. 2-g).

4.8. wnt signaling pathway

Major signaling pathways required for the regulation of bone formation are Wnt/β-catenin and NF-κB. Wnt signaling pathway is

reported to involve in the regulation of bone mass, like induction of osteoblast differentiation from MSCs, bone formation, and inhibition of osteoblasts apoptosis. Wnt activates its downstream transcription factor, β-catenin, which is associated with increased expression of osteoprotegerin (OPG), leading to indirect inhibition of osteoclasts activity and consequently inhibition of bone loss. Also, the Wnt/β-catenin pathway is positively correlated with BMP-2 and its bone formation quality. Finally, the Wnt/β-catenin pathway is associated with the induction of MSCs to become mature osteoblasts through increasing osteogenic markers, including Runx2, Dlx5, and osterix, and reducing adipogenic markers like PPARγ. In this regard, this pathway is one of the well-investigated pathways in bone regeneration and formation [107]. Although the Wnt/β-catenin pathway is among the most essential signaling pathways involved in osteogenesis, a few studies are focused on the impact of melatonin on this specific signaling pathway. Wnt/β-catenin signaling pathway appears to produce ROS in the aging MSCs. Melatonin is confirmed to regulate the Wnt/β-catenin pathway to play its osteogenic role (Fig. 2-h). Melatonin with its anti-oxidant property has a protective role against ROS generation. Moreover, melatonin activates MT2 receptors, which are located on MSC cells, and stimulates the activation of Wnt signaling in order to enhance osteoblastogenesis [108–110]. In conclusion, BMP/ERK/Wnt pathways are considered a key mechanism in the osteogenic capacity of melatonin treatment in MC3T3-E1 cells [111].

Ti-particle causes bone resorption and osteolysis [82]. A group of researchers investigated the effect of melatonin on particle-stimulated osteolysis both in-vivo and in-vitro. They found that melatonin can reverse the titanium particle effects through activation of the Wnt/β-catenin pathway. Indeed, Ti-particle is associated with Wnt/β-catenin degradation while melatonin prevents this degradation to regulate RANKL/OPG ratio, and inhibit osteoclastogenesis and bone resorption [112].

4.9. Neuropeptide Y/Neuropeptide Y receptor Y1 signaling

Neuropeptide Y (NPY) system has an influential role in controlling behavior, immunity, cardiovascular system, and also bone formation, and fracture healing [113]. NPY as a 36-amino acid peptide is a neurotransmitter that it expresses not only in the brain but also in osteoblasts, osteocytes, and chondrocytes. Moreover, the Y1 receptor (NPY1R) appears to express on both osteoblasts and bone marrow stromal cells. Also, it has been revealed that NPY enhances the fracture healing process by increasing the expression level of ALP, OC, PICP, and ICTP [114]. The correlation between melatonin and NPY has not identified until 2018 by Penghong Dong et al. In this study, they have been reported that melatonin can stimulate osteoblastic differentiation and migration of MSCs via up-regulation of NPY and NPY1R in the fracture site. Moreover, inhibition of NPY1R with BIBP3226 leads to suppression of melatonin effects on bone formation and fracture healing process. Taken together, same as other signaling pathways, NPY/

NPY1R signaling involves the function of melatonin in bone formation and osteogenesis [115].

5. Melatonin and other components synergistic stimulating effect on osteoblastic differentiation

There is general agreement that melatonin has considerable effects on bone metabolism, MSCs differentiation, and commitment [38]. However, some studies investigated and evaluated the synergism effect of melatonin and other components on bone microstructure. Furthermore, Bone Morphogenetic Proteins (BMPs) are introduced as a transforming growth factor (TGF) superfamily and divide into 15 subtypes including BMP4 and BMP9 [116]. As it is inferred from their names, these proteins are involved in bone formation and osteogenic differentiation from MSCs. BMP9 can activate transcription factors, like smad1and smad5, in order to enhance the expression level of essential genes for MSCs differentiation and osteogenesis. In this regard, a recent study revealed that the combination of BMP9 and melatonin stimulates ALP activity and matrix mineralization greater than BMP9 or melatonin alone in C3H10T1/2 cells [117].

As we mentioned above both melatonin and BMP-4 have anabolic effects on bone and prevent bone degenerative disease. Relatively, a recent study reported that the combination of melatonin and BMP-4 increases bone formation capacity and osteogenic markers like ALP through up-regulation of the p38/ERK signaling pathway [118].

Also, previous studies declared that Rapamycin (RAP) has positive effects on BMD, bone microstructure, and osteoporosis treatment [119]. Another study also revealed that RAP improves the prevention effects of melatonin on osteoporosis in rat models. As a result, melatonin and RAP synergistically activate OPG expression and reduce RANKL expression to reduce bone loss and enhance bone mass more effectively than melatonin only [120]. Fibroblast growth factor 2 (FGF-2) has an important role in angiogenesis, bone formation, and development [121]. Moreover, it has been reported that melatonin plays an independent function in improving bone formation and osteogenesis from progenitor cells [122]. Collectively, a study suggested that the combination of melatonin and FGF-2 appears to enhance OCN and OPN expression, leading to osteoblastic differentiation, proliferation, and mineralization in MC3T3-E1 cells within IP-CHA constructs [123]. Melatonin might be a potential factor in the induction of osteogenesis. In-vivo and clinical pieces of evidence are required to put this theory into practice.

Previously, it has been demonstrated that calcium or vitamin D supplementation has no significant effect on osteoporosis prevention [124,125]. In addition, the effect of strontium has been evaluated and revealed that strontium can enhance vertebral and femoral bone density in postmenopausal osteoporosis cases [126]. Also, evidence shows that the reduction of bone loss and fracture incidence occurs due to vitamin K2 application in postmenopausal osteoporosis cases [127]. The combination of these factors has been investigated and resulted in increasing BMD [128]. In this regard, studies investigated the combination of four components named MSDK, including melatonin, strontium (citrate), vitamin D3, and vitamin K2 on bone formation and bone loss processes in postmenopausal women cases. These studies show some precious results; first, MSDK improves BMD because a 6.48% reduction in the percentage of osteoporotic fracture risk was observed in treated cases, compared to a 10.82% rise in the control group. Secondly, MSDK not only appears to increase bone-forming markers but also reduces turnover markers. Furthermore, MSDK enhances osteoblastogenesis from MSCs while it decreases osteoclastogenesis. Mechanistically, MSDK gives rise to osteoblastogenesis by raising the OPG: RANKL ratio. In addition, MSDK therapy has a much greater effect on osteogenesis compared with melatonin alone, which is related to the induction of pERK1/2, pERK5, and RUNX2 in osteoblasts after MSDK treatment [129]. Moreover, MSCs can shift to adipogenesis or osteogenesis. PPARγ (Peroxisome proliferator-activated receptor γ), as one of the important factors, which determine MSC cells' destination, improves adipogenesis instead of osteogenesis [130]. In this regard, MSDK downregulates PPARγ expression and its effect, which leads to enhanced osteoblastogenesis from MSCs [129]. Also, it has been revealed that MSDK plays its anti-inflammatory role through the reduction of CRP (*C*-reactive protein) level, which is associated with bone health [131].

Fortunately, MSDK treatment has not shown any harmful effects on the body, such as high blood pressure [129]. Finally, weight fluctuation has negative impacts on bone mass because it is conducted to increase the rate of bone turnover [132]. MSDK is likely to enhance weight stabilization and consequently bone mass [129]. Besides, we believe that the side effects of MSDK should be also evaluated and reported to have a better understanding of this combined therapy on overall health.

Another new strategy for using melatonin in regeneration medicine is to combine it with MSC-derived exosomes. MSC-derived exosomes are content with miRNAs involved in the regeneration and self-renewal properties of MSCs and these advantages can transfer to cells treated with these exosomes [133]. This combination also showed more beneficial effects such as suppression of oxidative stress and apoptosis in vitro, and in vivo inflammation, oxidative stress, DNA/mitochondrial damage, and apoptosis [134]. In this regard, MSC-derived exosomes have attracted a lot of attention in regeneration medicine and its combination with melatonin has also been studied in wound healing [135,136], renal cells regeneration in renal ischemia-reperfusion injury [137], the regenerative potential of chronic kidney disease-derived mesenchymal stem/stromal cells [138], myocardial repair in myocardial infarction [139], which showed promising results. In this regard, melatonin combination with MSC-derived exosomes showed a promising role in improving OA by inducing chondrogenesis. Melatonin-induced cell sleep can significantly increase circular non-coding RNA (ncRNA) circRNA3503. This ncRNA in combination with MSC-derived exosomes can promote chondrocyte renewal and alleviate the progressive loss of chondrocytes [140]. Nevertheless, more study is needed to demonstrate how this approach can be helpful in the osteogenic differentiation of MSCs.

6. Conclusion

Melatonin is a hormone with vast biological functions such as the potential for regeneration of damaged tissues. In the case of bone regeneration, melatonin can shift MSCs from an adipocytic to osteoblastic differentiation, which is an important step in bone regeneration. Melatonin is also a potential signaling molecule that controls and affects many of the signaling pathways involved in MSCs osteoblastic differentiation. It plays a certain role in bone formation and osteogenesis through the regulation of downstream signaling pathways such as PI3K/AKT, BMP/Smad, MAPK, NFkB, Nrf2/HO-1, NPY, Wnt, SIRT/SOD, septins, PERK/ATF4. Melatonin as an osteogenic agent induces osteoblastic differentiation through activation of PI3K/Akt, BMP/Smad, and Wnt/β-catenin pathways, which consequently increases the expression level of osteogenic transcription factors, like Runx2, Dlx5, and osterix and reducing adipogenic markers like PPARγ. However, in different doses and times of exposure, it may have dual effects. For instance, a high concentration of melatonin suppresses ERK phosphorylation and activation, conducting downregulation of gene expression of cyclin D1, CDK4, cyclin B1, and CDK1, which overall leads to osteoblasts proliferation inhibition. Based on clinical evidence melatonin treatment can alleviate bone degenerative diseases like osteoporosis and increase BMD. Several published reports are indicating that combination therapy of melatonin with other components such as BMPs, RAP, FGF-2, strontium (citrate), vitamin D3, and vitamin K2 have beneficial effects on bone and prevent bone degenerative diseases. Herein, it can be postulated that melatonin is a potential candidate for bone regeneration. However, additional studies and clinical trials are needed to determine unknown mechanisms and investigate the optimum dose of melatonin that is safer and more efficient to use as a drug routinely.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any authors.

Declaration of competing interest

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