Review

Melatonin and the intervertebral disc: a potential intervention for lower back pain?

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ABSTRACT

Lower back pain is a common disability associated with aging that continues to carry a huge economic and health burden globally. Importantly, lower back pain is strongly associated with diseases involving intervertebral discs (IVDs), and many of the treatment options for the repair and maintenance of the IVDs are insufficient. Being a well-tolerated and endogenously produced molecule, melatonin is a suitable candidate for the treatment and prevention of a wide variety of skeletal conditions. In this review, we have evaluated current updates regarding melatonin's activities in IVD degenerative disease and discuss multiple mechanisms related to its effects on inflammation, oxidative stress, autophagy and senescence that contribute towards its support of the IVDs as well as its benefits in the treatment of IVD disease.

Key words: Melatonin; intervertebral disc, reactive oxygen species, senescence, inflammation

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1. INTRODUCTION

Lower back pain continues to be the most prevalent disease in the United States and globally; it is the leading cause of years lived with disability since the 1990s (1). More than 568.4 million cases of lower back pain have been recorded globally, with 223.5 million new cases between 1990 to 2019 (2). It carries an immense economic burden on the health system. Lower back pain and neck pain together cost the United States $134.5 billion in treatment in 2016 and were the highest source of health care expenditure among 154 conditions examined (2-4). Productivity losses further contribute to the total societal cost of lower back pain. Therefore, identifying novel therapies for the treatment and prevention of lower back pain are urgently needed to improve the quality of life.

Intervertebral disc (IVD) disease is considered to be a major cause for lower back pain (5); however, current treatment options for IVD disease are focused on pain management and, if nothing else works, surgical intervention (6). Melatonin supplementation is reportedly beneficial in ameliorating several skeletal problems (7-9) and has been recommended for improving the health of the intervertebral discs in a previous report (8). Recent discoveries have added breadth to the known activities of melatonin in this context, warranting an update.

Herein, we discuss current knowledge as well as future directions of research for evaluating the therapeutic potential of melatonin for lower back pain.

2. INTERVERTEBRAL DISCS: AN OVERVIEW

The spine consists of 33 vertebral bodies arranged as a column. Most of these vertebral bodies are interconnected by IVDs. The IVDs are a three-part system (Figure 1) that consists of a centrally located, well-hydrated, and weakly collagenous nucleus pulposus (NP) layer, a surrounding, rigid, annulus fibrosus (AF) layer, and thin layers of hyaline cartilage that prevent direct interaction between vertebrae and NP layer, termed the cartilaginous endplates (CEP).

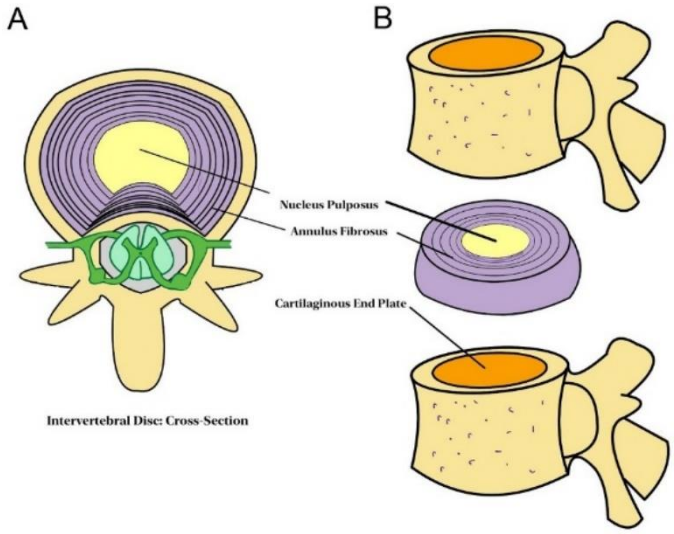

Fig. 1. Anatomy of the intervertebral disc. A. Cross-sectional (top) view. B. Longitudinal view of intervertebral disc located between lumbar vertebrae.

The nucleus pulposus (NP) is a well-hydrated structure due to its glycosaminoglycan (GAG) content. During aging, a change in GAG content of the NP hinders its ability to absorb and retain water. The annulus fibrosus (AF) is a more rigid structure containing type I collagen fibers that surround the inner type II collagen fibers. Both of these structures sit between cartilaginous endplates (CEP), which is a well-nourished structure containing primarily type II collagen fibers. Disruption of any of these structures is associated with IVD disease.

The AF layer is composed of 15-25 ligament fibers that are arranged in a criss-cross fashion surrounding the NP (10, 11). The fibers are primarily composed of type I collagen bundles surrounding inner, type II collagen bundles and are interspersed between progenitor cells and ground substance made up of proteoglycans such as glycosaminoglycans (GAG). The proteoglycan content of the extracellular matrix (ECM) is higher in the outer portion of the AF than in the inner portion, where the proportion is relatively constant during aging (12, 13). The AF maintains the integrity of the disc, thereby creating a finite space for movement of the NP cells and mitigating mechanical stress laterally. Loss or weakening of the AF results in degeneration of IVDs, leading to chronic back pain and disability (14).

The NP is a jelly-like structure located centrally in the disc and is composed primarily of water, a few cells that resemble chondroblasts and chondrocytes, and an ECM made up of randomly arranged type II collagen fibers and GAGs (chondroitin and keratan sulfate) (8). The NP provides mechanical support to the spine, preventing vertical compression of the disc. There is a progressive change in the proportion of GAGs during aging, resulting in dehydration and loss of volume in the disc. The NP functions in mitigating the mechanical stress placed on

the spine. IVD degeneration through compression and aging can result in a local inflammatory environment that further promotes ECM degradation, deposition of type I collagen fibers, accelerated dehydration, and hardening of the NP. Both IVD and ECM disorders are highly prevalent in aging individuals, with most herniations occurring at the mobile sections of the spine (cervical and lumbar regions) can lead to impingement of the nerves in the surrounding tissue (15). It is important to note that a healthy NP environment is avascular and lacks neurons; the presence of either neural or vascular structures is, therefore, considered to be pathological and is implicated in degenerative IVD disease.

The CEP is a thin structure situated between the body of a vertebra and the IVD. It consists of hyaline cartilage, a few end plate chondrocytes (EPCs), and an ECM rich in GAGs and water (10, 16). It is a well-nourished structure due to the diffusion of nutrients through nutrient canals into the avascular NP and inner AF. CEP functions to prevent biomechanical damage to the NP that can arise due to herniation, shear, and tensile forces (16, 17). The CEP has been widely identified as an instigator of diseases involving the IVD, as impaired nutrient dispersion and consequent loss of structure can prove detrimental to overall IVD health. During aging, the ECM of the CEP changes in morphology and functionality. Common histological findings of the CEP with aging include a decrease in width, depolymerization of GAGs, and occlusion of nutrient canals (10, 18). Additionally, progressive calcification of the articular cartilage, commonly seen with IVD disease, reduces the ability of nutrients to diffuse through the CEP, thereby contributing to its degeneration (16).

Physiologically, the structures of the IVD are highly rhythmic and interconnected. IVD metabolism exhibits both an active and a resting phase, with hydration and repair occurring during the latter phase (19). IVD thickness and volume peak in the morning and decrease as the day progresses, largely due to dehydration of the NP; recovery of both metrics occur during the night (20). Inflammation, manifested during IVD disease, disrupts the rhythm and prevents reparative processes that normally occur at night (21).

3. ROLE OF MELATONIN IN IVD DEGENERATION

Melatonin plays multiple roles in supporting the IVD. Here we discuss various molecular mechanisms underlying melatonin function that contribute towards ameliorating those conditions in the different components of the IVD. We begin with melatonin's effects on the nucleus pulposus (Table 1).

Table 1: Major biomolecules modulating the Nucleus Pulposus (NP).

Cytokine/ Pathway	Experimental Model	Role	Ref.
TGF-β1	Human NP cells, Swiss Albino rats	Expression enhanced by melatonin, thereby, promoting the expression of COL2A1, ACAN, and SOX9 for ECM restoration; supports NP structure and mitigates inflammation	(22, 25, 26)
NF-κB	Rat models, Human NP cells, New Zealand white rabbit cells	Inhibited by melatonin, thereby, decreasing inflammation, increasing autophagy, and preserving NP health, possibly via ERK ½ signaling	(27, 34)
IL-1β and TNF-α	Human NP cells	Induce inflammation and contribute to NP degeneration; melatonin disrupts IL-1β/NF-κB-NLRP3 inflammasome loop	(32, 38, 39)

Cytokine/ Pathway	Experimental Model	Role	Ref.
SIRT1	Human NP cells	Regulates oxidative stress and mitochondrial function in NP cells; indirectly enhanced by melatonin	(51)
PI3K/Akt	Female Wistar rats, Sprague Dawley rats, NP-derived mesenchymal stem cells	Activated by melatonin; decreases oxidative stress-induced apoptosis of NP cells. Maybe involved in melatonin-mediated iNOS suppression	(35, 53)
BMAL1	Human NP cells, Mouse models	Expression restored by melatonin; enhances NP cell health and potentially alleviates compression-induced degeneration.	(81)
VEGF	Human NP cells	VEGF binding to its receptor in HUVEC prevented by melatonin	(75)

3.1. TGF-β1 upregulation in NP cells.

Turgut *et al*. documented that pinealectomized chickens, which have low serum melatonin levels, exhibit IVD degeneration and spinal malformation, indicating the importance of melatonin in maintaining spine homeostasis (22). Maintenance of the IVD by melatonin has been associated with the cytokine, transforming growth factor-beta 1 (TGF-β1) (23-25). In rat models with experimentally damaged AF, a slight increase in TGF-β1 expression in AF chondrocytes as well as a reduction in the organization of AF collagen fibers were observed (24, 25). Subcutaneous injection of 30 mg/kg/day of melatonin induced a large increase in TGF-β1 as well as improved the organization of collagen fibers; the appearance of the IVD resembled that of the control, validating its potential for reversing vertebral damage (25).

Melatonin also increases the expression of COL2A1, ACAN, and SOX9 by modulating TGF-β1, as shown in cultured normal human NP cells (26). COL2A1 and ACAN genes encode for type II collagen and aggrecan respectively, both of which are critical for maintaining the structure and tensile strength of the NP. SOX9 is a well-known transcription factor in skeletal development that plays a critical role in the synthesis of type II collagen, aggrecan, and other proteins involved in the proliferation of NP cells (27). Treatment of degenerative human NP cell cultures with TGF-β1 decreased the expression of A Disintegrin And Metalloproteinase with Thrombospondin motifs 4 and 5 (*ADAMTS-4* and *ADAMTS-5*) that degrade the extracellular matrix (ECM), while promoting the expression of tissue inhibitor of metalloproteinase 3 (TIMP-3), a prominent aggrecanase inhibitor (28), thereby maintaining the ECM structure.

While the mechanisms underlying TGF-β1-mediated benefits in NP cells are not completely known, it is thought to function by inhibiting NF-κB activity, likely acting through the ERK1/2 signaling pathway that modulates I-κB phosphorylation (29, 30). Rabbit NP cells co-cultured with bone marrow stem cells (BMSC) showed increased expression of TGF-β1 and decreased phosphorylation of I-κB, thereby decreasing the activity of the NF-kB pathway. These results were reversed via the addition of SB431542, a selective TGF-β1 inhibitor (29). Yang *et al*. showed that the beneficial activity of TGF-β1 in inhibiting MMP-3 was suppressed following downregulation of ERK1/2 using PD98059 (a MAPK inhibitor) and U0126 (a p-ERK inhibitor) in the lumbar NP of Wistar rats (30). Conversely, melatonin at concentrations higher than 1 μM enhanced ERK1/2 activity in a dose-dependent manner, with step-wise increase in type II collagen and aggrecan expression in NP cell cultures (31). These results were replicated in the

New Zealand white rabbit IVD disease model (31). Additionally, melatonin downregulated NP cell senescence markers (including p21 and p27) and prevented apoptosis by promoting the expression of anti-apoptotic Bcl-2 while downregulating BAX in human NP cell cultures; these effects were reversed by ERK1/2 inhibitors (31). Importantly, melatonin also appears to prevent ferroptosis of NP cells; however, the exact mechanism is unclear (32). Studies exploring the activity of melatonin while selectively inhibiting TGF-β1 in NP cells is warranted, as they could provide additional key mechanistic information regarding factors downstream of melatonin (Figure 2).

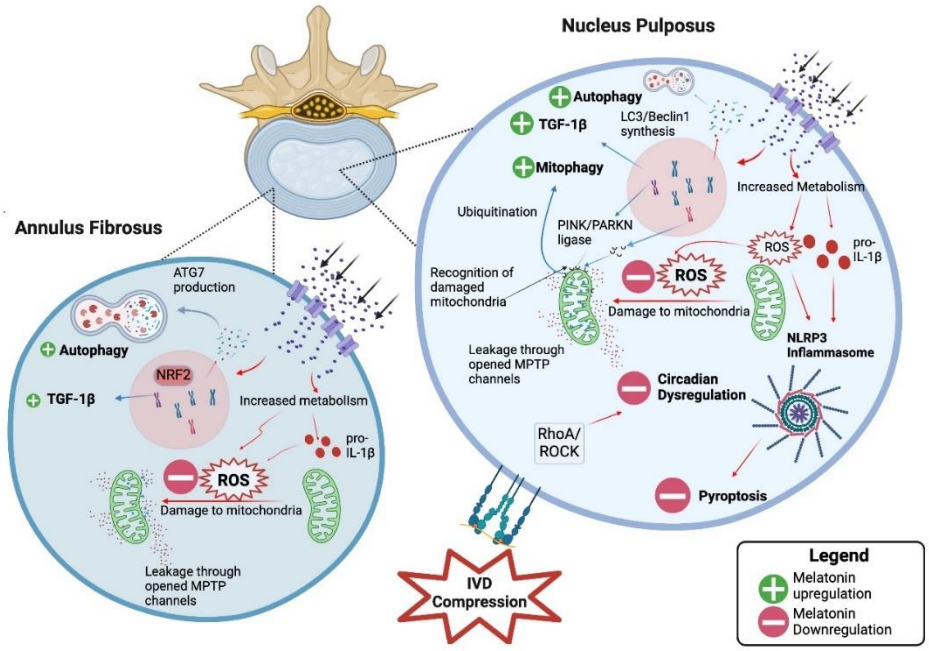

Fig. 2. Multiple actions of melatonin on the nucleus pulposus and annulus fibrosus.

Melatonin restricts apoptosis, ferroptosis (32), and pyroptosis through multiple mechanisms. It inhibits senescence in NP cells by modulating autophagy and mitophagy. In the annulus fibrosus, it upregulates ATG7 and thereby upregulates autophagy. Additionally, de-novo synthesis of melatonin in the mitochondria (not shown) provides additional possibilities for intracellular melatonin action, either through secondary signaling or through direct antioxidant activity. Adapted from a template by BioRender.com (2024). Retrieved from https://app.biorender.com/biorender-templates.

3.2. NF-κB and NP cell receptor activity.

IVD degenerative disease involves high NP cell turnover, senescence, and aggregation of NP cells into large clusters (33, 34). Li *et al*. reported that melatonin, via its membrane receptors, inhibited aberrant NP cell proliferation while enhancing gene expression of collagen and aggrecan, thereby improving ECM integrity in human NP cell cultures (35). Melatonin appeared to restrict cell proliferation by promoting a decrease in the phosphorylation of PI3K p85, PDK1, and AKT subunits; however, the specific melatonin receptor subtype involved was not identified and the benefits of melatonin in an inflammatory environment were not further investigated (35, 36). Qiu *et al*. recently indicated that the MT1B receptor mediates melatonin's activity in reversing TNF-α activity in human degenerative NP tissue and that the addition of the selective antagonist of the MT1B receptor, 4P-PODT, prevented the actions of melatonin (36). Furthermore, it was proposed that melatonin utilizes the guanine nucleotide binding

protein, Gai2, as a secondary messenger to prevent the upregulation of LATS1/2, which functions downstream of TNF-α and phosphorylates YAP for eventual degradation (37). This was tested by silencing Gai2 that resulted in a decrease in melatonin function following administration of TNF-α. Gai2 decreases YAP phosphorylation and its degradation (36). YAP complexes with the co-activator, TEAD translocate to the nucleus, resulting in increased transcription of I-kBα, which prevents the phosphorylation and nuclear translocation of p65, and upregulation of MMP-13 and ADAMTS-5 (36).

These results are consistent with previous findings that showed that injection of melatonin into an IVD disease rat model led to the inhibition of NF-κB via a reduction of p65 phosphorylation (38). This downregulation likely explains the drop in inducible nitric oxide synthase (iNOS) reported previously following administration of melatonin to ovariectomized rats (39, 40), as NF-κB is a known mediator of iNOS expression in most tissues. Further investigation is required to determine if the MT1B receptor is also involved in the inhibition of aberrant NP cell proliferation via the PI3K/p85 axis in non-inflammatory environments and if it decreases iNOS expression.

More recently, melatonin has been shown to function through retinoic acid-related orphan receptors (RORs), a family of nuclear receptors that play crucial roles in human development, circadian rhythm regulation, and in responses to oxidative and inflammation-induced stress (41). These receptors are expressed differentially across tissue types and have been implicated in several human pathologies, making them promising targets for therapeutic interventions (41). Specifically, RORα expression is elevated in the nucleus pulposus of patients presenting with intervertebral disc degenerative disease (42). Interestingly, RORα expression and function appears to be regulated by melatonin; however, whether melatonin or its metabolites directly regulate RORα is unclear (41). Further investigation into whether melatonin exerts its therapeutic effects on the nucleus pulposus, either directly or indirectly, through the activation of RORα receptors is required and may reveal novel mechanisms of melatonin-mediated maintenance of IVD.

3.3. IL-1β, TNF-α, and the inflammatory environment in the NP.

Both IL-1β and TNF-α are known to induce inflammation in IVD, correlating with degeneration (43). IL-1β, an inflammatory cytokine, has attracted interest because it is commonly upregulated during IVD degeneration (44). IL-1β appears to exacerbate the degenerative process, acting to expedite NP cell apoptosis, increase NP cell mitochondrial damage, and promote aggrecan breakdown via the promotion of aggrecanases (19, 28, 45, 46). A significant contributor to IL-1β pathology in severe IVD disease appears to be the NLRP3 inflammasome, given that protein synthesis associated with inflammasome priming is increased in severe IVD disease (45). In this condition, IL-1β would likely serve as a positive feedback loop to increase NLRP3 inflammasome protein synthesis and independently elevate intracellular ROS generation, thus enhancing the inflammatory response (45). Nicotinamide phosphoribosyl transferase (NAMPT), which is known to catalyze the first step in NAD biosynthesis from nicotinamide, was shown to promote NLRP3 inflammasome activity through the MAPK and NF-κB pathways (47). Both IL-1β and TNF-α are known to induce NAMPT expression, which upregulates the NLRP3 inflammasome (47, 48).

Melatonin has been shown to play a multifactorial role in decreasing inflammation in IVD degenerative disease (49, 50). Specifically, plasma melatonin concentration significantly decreased in cases of IVD degeneration and lower levels of melatonin were associated with higher levels of inflammatory cytokines as well as with increased disease severity and duration (49). Melatonin also inhibits polarization of macrophages to the pro-inflammatory M1-type by reversing the inhibition of the SIRT1/Notch pathway; therefore, the expression of pro-

inflammatory cytokines is decreased (50). Melatonin appears to have anti-inflammatory properties through its regulation of autophagy, mitophagy, and mitochondrial reactive oxygen species (ROS) (45, 51). Melatonin regulates autophagy by targeting the NF-κB signaling pathway, specifically by modulating p65 expression and I-κBα activity (38), resulting in an increase in autophagy markers, LC3B and Beclin-1, and a reduction in p62 protein expression in human NP cells harvested from patients with IVD disease. These results have been replicated in IVD rat models, indicating that melatonin promotes autophagy via inhibition of the NF-κB signaling pathway (38).

The upregulation of the cytokine, TNF-α, in IVD degeneration is believed to an important step in the disease process, leading to ECM degradation and NP cell death (52). Huang *et al* . found that melatonin ameliorated TNF-α-induced activation of the NLRP3 inflammasome and ECM degradation by modulating NAMPT signaling of the NF-κB pathways in NP cells; benefits accrued with melatonin treatment were reversed following administration of luzindole, a melatonin receptor blocker (47). The beneficial effects of melatonin in preventing TNF-α signaling were similar to those of APO866, a prominent inhibitor of NAMPT activity (47). Additionally, APO866 has been shown to prevent IL-1β induction, likely through its role in inducing autophagy (48). While a comparative study using melatonin or APO866 relative to NAMPT activity in IL-1β treated NP cells has not been performed, a deeper understanding of the mechanisms may result from such an investigation.

The function of the NLRP3 inflammasome requires a priming step and an activation step. The priming step signals up-regulation of NLRP3 protein expression and association with apoptosis-associated speck-like protein containing a CARD domain (ASC) and caspase-1, while the activation step results in oligomerization of the newly-formed protein aggregates and processing of pro-IL-1β into functional IL-1β (53). Inflammatory cytokines, such as IL-1β and TNF-α, are known mediators of the priming step in NP cells. Mitochondrial damage generally increases ROS production, which then acts as a signal for activation of the NLRP3 (45). Melatonin administration prevents the IL-1β/NF-κB-NLRP3 positive feedback loop in cultured severely diseased IVD human NP cells treated with IL-1β by preventing both priming and activation. Melatonin likely prevents priming of the NLRP3 inflammasome by inhibiting phosphorylation of I-κB and p65 that hinder NF-κB signaling as well as its activation via its antioxidant-promoting properties, as replicated in rat models of IVD disease (45). Melatonin also prevents pyroptosis by upregulating Nuclear factor-erythroid 2 (NF-E2) p45-related factor-2 (NRF2), a transcription factor that promotes the synthesis of antioxidant enzymes (54).

A different mechanism by which melatonin reduces inflammatory cytokine production is by enhancing the expression of the long non-coding RNA, maternally expressed gene 3 (MEG3), which inhibits the microRNA, miR-15a-5p; the latter suppresses the PGC1α-SIRT1 pathway and promotes the production of IL-1β, TNF-α, ADAMS, and MMPs, among others. (55). MEG3 is known to reduce inflammation and is downregulated in IVD disease (55).

Melatonin has been shown to decrease oxidative stress-induced NP cell apoptosis and an increase in ECM content has been found following melatonin treatment (56). A recent study shows the involvement of the PI3K/Akt pathway in melatonin-mediated rescue of NP-derived mesenchymal stem cells from oxidative stress, mitochondrial dysfunction and senescence induced by hydrogen peroxide (57). Induction of the PI3K/Akt pathway by melatonin decreased oxidative stress-induced apoptosis of the stem cells (57). Similarly, in a high glucose environment as is found in diabetes, melatonin promoted the phosphorylation of Akt in NP cells and was associated with decreased apoptosis as well as a decrease in excessive autophagy (58).

Of significant importance is melatonin's ability in promoting mitophagy as a mechanism to curtail ROS-induced damage. The *tert*-butyl-hydroperoxide, a free-radical generating agent, increased apoptosis of NP cells by inhibiting mitophagy; this response was reversed by

melatonin (51). The beneficial effects of melatonin in preventing NP cell apoptosis were inhibited by the addition of cyclosporine A, a mitophagy inhibitor (51). The PINK1/PARKIN axis is a well-known mitophagy regulator and is understood to be induced by excessive ROS production by the mitochondria. Blocking PARKIN using siRNA reduced melatonin's protective effect in inhibiting apoptosis in a rat radiculopathy model (59). Interestingly, an increase in PINK1 in melatonin-treated NP cell cultures was not reported, raising the question of how melatonin upregulates PARKIN (51). A study by Xiao *et al*. showed that sufficient ROS can affect the translocation of PARKIN to mitochondria without elevating the levels of PINK1 (60). However, excessive ROS and accumulation of apoptotic proteins are associated with the induction of apoptosis while inhibiting mitophagy, acting to remove severely damaged cells (61). It has been proposed that melatonin, by removing excessive ROS, disinhibits mitophagy; whether this is due to the direct scavenging of ROS by melatonin, or due to upregulation of antioxidant activities by melatonin, or both, has yet to be determined. However, with the discovery that melatonin is likely produced in the mitochondria, a combination of mechanisms has become more probable (62, 63).

3.4. SIRTs in NP cells.

Sirtuins are evolutionarily conserved, NAD+-dependent histone deacetylases involved in multiple cellular functions and have been associated with several aging-related diseases (64). Among the seven known sirtuins, SIRT1, SIRT2, SIRT3, and SIRT6 are known to be involved in regulating oxidative stress, inflammation and mitochondrial function in IVD diseases (64). We will briefly discuss the role of melatonin-induced SIRT1 and SIRT3 in IVD disease.

Sirtuin 1 (SIRT1) is primarily localized in the cytoplasm and nucleus of most cells. It acts to modify histones in the transcriptional regulation of genes involving redox homeostasis and longevity (65). Melatonin's actions in a number of different tissues suggest that it enhances SIRT1 levels in normal tissues, which in turn increases mitophagy while reducing ROS damage and inflammasome activity, with some evidence of PINK1/PARKIN induction (66-69). SIRT1 is a regulator of mitophagy and an upstream inducer of PINK1/PARK proteins in NP cells (70). In addition, IL-1β-mediated mitochondrial damage increased ROS and induced the NLRP3 inflammasome, which were prevented when SIRT1 was over-expressed (70). The authors proposed that melatonin-mediated improvements in IVD disease may be partially explained by its upregulation of SIRT1 in NP cells, and that investigations of the melatonin receptor-mediated upregulation of SIRT1 in NP cells should be pursued.

Sirtuin 3 (SIRT3) is found in the mitochondria and is involved in maintaining mitochondrial ROS homeostasis. SIRT3 has been reported to be decreased in IVD disease (71), likely contributing to the rise in ROS generation seen in severe IVD disease. Melatonin appears to work both in tandem with and by increasing SIRT3 activity, as shown by its stimulatory effects on SIRT3 in oocytes, follicular cells, endothelial cells, and hepatocytes.(63, 69). In IVD degeneration models induced with IL-1β, SIRT3 increased antioxidant FOXO3a/SOD2 pathway expression in the nucleus pulposus with an associated increase in collagen II mRNA expression (71). Song *et al.* reported that SIRT3 and mitochondrial antioxidants were involved in the protection of NP cells from oxidative stress-induced apoptosis mediated by advanced glycation end products, by inducing the AMPK/PGC-1α pathway (72). Consistent with these findings, Lin *et al.* observed that SIRT3 administration led to SOD2 deacetylation, which enhanced ROS scavenging as well as prevented ROS-mediated cellular senescence, increased levels of type II collagen and aggrecan in the ECM, and decreased matrix metalloproteinase-13 expression (73). It was proposed that the induction of the AMPK/PGC-1α pathway mediated the beneficial actions of SIRT3 on the IVDs (72). Melatonin is produced within the mitochondrial matrix, a common area for antioxidant proteins such as SIRT3 (63, 74).

Additionally, melatonin has been shown to promote AMPK/PGC-1α in a number of tissues (75, 76). While melatonin's ability to stimulate the AMPK/PGC-1α pathway and SIRT3 has not been confirmed specifically in NP cells, it could explain melatonin's multifactorial benefits to IVD disease.

3.5. VEGF and vascularization of NP.

As previously stated, maintaining an avascular and aneural NP environment is critical in maintaining the homeostasis of the IVD. A pathological indicator in NP cells is the production of cytokines and vascular endothelial growth factor (VEGF), thereby, vascularizing the IVD (77). IL-1β induces VEGF, Nerve Growth Factor (NGF), and Brain-Derived Neurotrophic factor (BDNF) in human IVD NP cells (77, 78). Remarkably, melatonin prevents the vascularization of human umbilical vein endothelial cells (HUVEC) (26, 79). Shen *et al*. found that melatonin prevented VEGF binding to its receptor in HUVEC co-cultured with NP cells while promoting the expression of ECM-related genes (26). Similar effects of melatonin in preventing VEGF signaling have been shown in studies using other cell types such as chondrocytes, likely mediated via the MT1 receptor (80-82). A second mechanism by which melatonin could perhaps inhibit neovascularization is by upregulating TGF-β1 which can induce TIMP3. TIMP3 overexpression has been shown to suppress angiogenesis in NP under conditions of inflammation, without the involvement of VEGF regulation (83). TIMP3 also inhibited the expression of substance P in NP, thereby decreasing inflammation (83). Further investigations of the anti-vascular effects of melatonin would be important in delineating the underlying molecular mechanisms. Notably, cells cultured from human patients with IVD disease showed a minor but not significant increase in type II collagen synthesis following melatonin administration (26). This may be due to decreased expression of melatonin receptors in degenerative NP cells (36).

3.6. BMAL1 and cyclic regulation of NP cells.

Given the potential benefits of melatonin in maintaining the general health and treatment of the IVD, it makes sense that many critical maintenance processes are cyclic and synchronized by circadian rhythms. Period Circadian Regulator 2 (PER2) is a protein whose activity is strongly linked to the circadian rhythm and is critical in maintenance of cell homeostasis (84). PER2::Luciferase reporting of circadian rhythm activity in human NP and AF cells indicated that an autonomous circadian clock exists in both cell lines (19). Additionally, mice with BMAL1 gene knockout, a major genetic regulator of circadian cycles, exhibited increased age-related IVD degeneration indicating that the IVD health is reliant on stable circadian rhythms (19). However, the mechanisms associated with circadian rhythms and IVD disease remain largely unknown. Recently, Wang *et al*. indicated that compression-mediated IVD disease leads to induction of the Rho/ROCK and lowers BMAL1 transcription, where melatonin restores the expression of BMAL1 and relieves the compressive IVD disease (85). It is well known that BMAL1 expression is perturbed in advanced age, but correlates negatively with moderate IVD disease (19); this has led to an investigation of excessive mechanical loading on the spine and its role in the IVD. Cultured human NP cells with the PER2::Luc reporter gene identified rhythmic oscillations in healthy NP tissues (85). Cyclic compression of NP cells reduced BMAL1 expression and increased NP cell apoptosis; both effects were mediated the Rho/ROCK pathway (85). Melatonin partially restored BMAL1 expression and ameliorated NP cell apoptosis, while expectedly increasing aggrecan expression and decreasing MMP-13 expression (85). Melatonin also suppressed the expression of p-MLC, a RhoA/ROCK pathway activation marker, in the compression group (85). These results were replicated *in vivo* in a

night compression-induced rat model, which showed a recovery of BMAL1 expression and aggrecan, with a reduction in MMP-13 expression following intradiscal injection of melatonin (85). Together, these results provide strong evidence that the circadian clock is critically important in the maintenance of NP cells during IVD disease, and that melatonin enhances the expression of the BMAL1 gene in such conditions. Additionally, TGF-β1 has also been shown to suppress negative regulators of the circadian clock while increasing the expression of BMAL1 via SMAD3 (86). Thus, melatonin, by inducing TGF-β1 expression, can regulate BMA1 and its protective effects in compression-related IVD disease. Further investigation of TGFβ-1-BMAL1 axis in NP cells is needed to fully understand melatonin function in compression-related IVD disease. Whether the action of melatonin in this situation is receptor-mediated or receptor-independent has yet to be specifically determined; however, this may involve the MT1B receptor as previously indicated in NP cell cultures (36). These observations also suggest a link between age-related decline of melatonin and the increased susceptibility to IVD disease. Aged individuals with low melatonin levels may, therefore, be potentially more susceptible to IVD disease due to loss of nightly reparative activity.

3.7. IVD disease, autoimmunity and melatonin.

Autoimmune reactions have been recently implicated in the pathogenesis of IVD disease (87, 88). It is thought that neovascularization, which occurs during the pathogenesis of IVD disease, results in greater exposure of the avascular NP, which is typically granted immune privilege by the expression of FasL, to the immune system, resulting in FasL-Fas-mediated apoptosis of the disc cells (89). Another postulated mechanism is via IgG autoantibodies targeting extracellular matrix proteins in the NP such as collagen types I, II, and V, as well as aggrecan (90). Melatonin is known for its role in modulating inflammatory cytokine levels in the IVD but it's function in possibly mitigating autommunity-mediated IVD disease has not yet been fully explored. Given its treatment efficacy in several autoimmune-mediated diseases and its effect on regulating Th1 and Th2 balance, melatonin may potentially be a viable treatment option (91). Another possible mechanism by which melatonin may modulate autoimmunity is via its downregulation of RORα receptor activity (41), that results in inhibition of differentiation into Th17 cells (92). Since Th17 cells are known to be induced in, and contribute to, the severity of IVD disease, downregulation of Th17 cells appears to be another viable option (93, 94).

3.8. Melatonin and the CEP.

Most melatonin-mediated effects on NP cells are also applicable to chondrocytes in the CEP. Similar to NP cells, the health of chondrocytes, and thus cartilaginous structures, also are influenced by BMAL1 expression (95). BMAL1 null mice showed accelerated degenerative changes in knee and hip articular cartilage chondrocytes, as well as decreased SOX9, COL2A1, and ACAN expression in the ECM (95). Similar results were reported by Song *et al*. who showed that circadian clock disruption in chondrocytes harvested from the knee exhibited increased MMP-3, MMP-13, ADAMTS-4, and β-catenin expression in the ECM (96). Recently, it was shown that IL-1β and TNF-α, which are found in the inflammatory environment of IVD disease, are disruptors of circadian clock genes, BMAL1 and PER2, in chondrocytes (19, 97). Exogenous application of melatonin led to increased expression of BMAL1 in chondrocytes retrieved from mouse ribcage; this response was receptor-mediated (62). Given that melatonin is a prominent anti-inflammatory agent in such environments, melatonin may have improved the chondrocyte environment in the CEP suppressing IL-1β expression and preventing disruption of the circadian rhythm (98).

Autophagy is critical for maintaining the health of the CEP by preventing early apoptosis of chondrocytes in response to elevated oxidative damage (99, 100). Discoveries related to melatonin's action on autophagy in EPCs are few, but hints of its activity have been shown in studies of osteoarthritis. Chen *et al.* reported that melatonin was anti-apoptotic and promoted autophagy in rat chondrocytes treated with H_2O_2 (100). Melatonin treatment was shown to upregulate SIRT1, an autophagy regulator, in EPCs exposed to H_2O_2, resulting in decreased apoptosis and decreased calcification of the CEP (101). Selective inhibition of either SIRT1 (with EX527) or autophagy (with 3-methyladenine) prevented melatonin from inhibiting CEP calcification and EPC apoptosis (101). Liao *et al.* found that, similar to melatonin, SIRT1 expression in chondrocytes also decreased with age as well as in osteoarthritis and directly correlated with autophagy in chondrocytes (102). Specifically, SIRT1 interacted with Atg7 and induced Beclin-1 expression in chondrocytes (102). Also similar to decreased melatonin expression with aging, Bernick and colleagues (1982) showed that aging was associated with calcification of articular cartilage, obstructing nutrient passage to the disc (18). Further investigations of the influence of the chondrocyte inflammatory environment and its relation to melatonin are justified.

The role of melatonin receptors in the regulation of autophagy, apoptosis, and the ECM content of the CEP has not been studied extensively. Fu *et al.* (2019) indicated that endochondral ossification is dictated by mitochondrial melatonin in chondrocytes harvested from mouse ribs (62). Melatonin application was shown to increase COL2A1, Aggrecan, and SOX9 expression, where benefits to the ECM were shown to be mediated by the MT2 receptor. Zhang *et al.* showed that AF puncture disrupts the CEP in MRIs of the spine of Sprague-Dawley rats, with corresponding dramatic increases in inflammatory cytokines and visible degradation in the CEP in histological staining (101). Treatment of injury to the AF with melatonin indicated no evidence of endplate injury based on X-ray, MRI, or histological staining (101), indicating a strong association between the health of the EPCs and the surrounding IVD, and that inflammatory cytokines, much like in the NP, mediate much of the degradation in the CEP. Melatonin also reduced the inflammatory environment by reducing protein expression of both IL-1β and TNF-α in both animal and human fibroblasts via the PI3K/Akt and ERK signaling pathways (98). Similarly, melatonin was shown to inhibit TNF-α and IL-8 expression in synovial fibroblasts harvested from patients with knee osteoarthritis and the synovium of rats with severe osteoarthritis (80). Melatonin functioned as an anti-inflammatory agent in both fibroblasts and chondrocytes via the MT1 receptor, whereas benefits mediated by modulation of ECM occurred via signaling from the MT2 receptor (103). Additionally, Wu *et al.* confirmed that human EPCs contain higher levels of MT1A and MT1B receptors than do cells derived from rats (104); this difference in the density of the melatonin receptors may be relevant to the differential actions of melatonin in mediating the observed autophagy effects and ECM benefits.

In addition to autophagy, melatonin promotes the replenishment of chondrocytes by promoting the differentiation of bone marrow stem cells (BMSCs), a response that involves both the MT1 and MT2 receptors on the membranes of the chondroblasts (105). Similar to other cells involved in IVD pathology, BMSCs exhibit impaired function and decreased chondrogenesis in inflammatory environments induced by IL-1β and TNF-α (106). Melatonin protected BMSCs from both IL-1β and TNF-α inflammatory environments and rescued their ability to differentiate into chondrocytes, showing decreased MMP-1, MMP-2, MMP-13 and ADAMTS4 expression and increased ACAN and SOX9 expression (106, 107). Melatonin also upregulates microRNAs that inhibit SMAD7 expression. Since SMAD7 is a prominent inhibitor of the BMP/SMAD chondrogenesis axis, melatonin thereby promotes chondrogenesis (108). These findings further elucidate melatonin's multiple actions in promoting healthy cartilage and supports future research exploring its specific actions in EPCs of the CEP.

3.9. Melatonin and the AF.

It has been frequently noted that melatonin improves the organization of collagen fibers in the AF and that TGF-β1 is an important mediator in its positive effects (Table 2), but investigation of AF cells during IVD disease and corresponding beneficial effects of melatonin have not been a focus of such investigations (25). Dudek *et al*. observed that loss of BMAL1 expression in AF cells treated with IL-1β resulted in translocation of p65 in AF and NP cells into the nucleus and consequent activation of the NF-κB pathway (19). Autophagy regulation in the AF is not fully known. Human AF cells exposed to IL-1β under conditions of serum deprivation exhibited increased autophagy in a dose-dependent manner; however, IL-1β alone, without serum starvation, did not induce autophagy (109). However, in AF cells cultured from human patients with IVD degeneration, a reduction in autophagy and an increase in apoptosis is observed (110). The application of melatonin reverses these changes (110). Similarly, rat AF disc cells cultured with TNF-α displayed a degenerative disc pathology, with increased ROS production, cell senescence, and reduced NF-κB activity (111). Melatonin has a significant effect on the expression of all of these markers, indicating its beneficial effects on the maintenance of AF in the context of IVD disease (111). Given that stability of the AF is critical for the maintenance of NP structure and prevention of herniation, the benefits of melatonin offer promise for preventing additional injury. A recent study using a rat IVD degeneration model showed that incorporation of melatonin within a self-healing hydrogel composed of β-cyclodextrin and thiolated gelatin reduced the inflammatory effects of IL-1β on regeneration of AF, boosted mitochondrial energy metabolism, and enhanced antioxidant expression, resulting in preservation of ECM synthesis and improved NP hydration status (112). The authors identified melatonin-induced expression of NRF2, as an important downstream factor involved in rescuing the cells from oxidative stress (112). A similar study using melatonin incorporated in a hydrogel composed of bioactive glass/sodium alginate was also found to be effective in reducing IL-1β- mediated inflammation and oxidative stress in NP cells derived from Sprague-Dawley rats (113)

Table 2: Major biomolecules modulating the Annulus Fibrosus (AF)

Cytokine/ Pathway	Experimental Model	Role	Ref.
TGF-β1	Swiss Albino rats	Improves the organization of collagen fibers in AF; supports AF integrity	(22)
NF-κB	Sprague-Dawley rats	Inhibition by melatonin may preserve AF health by reducing inflammation.	(99)
IL-1β	Human NP cells	Melatonin upregulates NRF2 and reduces IL-1β-mediated inflammation	(100, 101)

4. CONCLUSION

The IVD is a highly dynamic structure that is markedly altered by an inflammatory environment, resulting in degenerative changes. Degeneration of the IVD is a common cause of lower back pain in aging individuals, affecting not only the individual but also placing a burden on public health. IVD health is linked to the circadian rhythms and the availability of melatonin, which is involved in the maintenance of the cellular and extracellular components in the NP, AF, and CEP. Melatonin is a non-toxic molecule that provides promise for

alleviating degenerative changes in the IVD and improving the quality of life (45, 70, 114, 115). While the complete mechanistic picture of melatonin's activities in reducing IVD disease is incomplete, the available data certainly suggest it has potential, especially in the aged population where melatonin levels are greatly diminished, to preserve a more youthful spinal function.

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AUTHORSHIP

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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