Rescue of proinflammatory cytokine-inhibited chondrogenesis by the antiarthritic effect of melatonin in synovium mesenchymal stem cells via suppression of reactive oxygen species and matrix metalloproteinases

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Abstract

Cartilage repair by mesenchymal stem cells (MSCs) often occurs in diseased joints in which the inflamed microenvironment impairs chondrogenic maturation and causes neocartilage degradation. In this environment, melatonin exerts an antioxidant effect by scavenging free radicals. This study aimed to investigate the anti-inflammatory and chondroprotective effects of melatonin on human MSCs in a proinflammatory cytokine-induced arthritic environment. MSCs were induced toward chondrogenesis in the presence of interleukin-1β (IL-1β) or tumor necrosis factor α (TNF-α) with or without melatonin. Levels of intracellular reactive oxygen species (ROS), hydrogen peroxide, antioxidant enzymes, and cell viability were then assessed. Deposition of glycosaminoglycans and collagens was also determined by histological analysis. Gene expression of chondrogenic markers and matrix metalloproteinases (MMPs) was assessed by real-time polymerase chain reaction. In addition, the involvement of the melatonin receptor and superoxide dismutase (SOD) in chondrogenesis was investigated using pharmacologic inhibitors. The results showed that melatonin significantly reduced ROS accumulation and increased SOD expression. Both IL-1β and TNF-α had an inhibitory effect on the chondrogenesis of MSCs, but melatonin successfully restored the low expression of cartilage matrix and chondrogenic genes. Melatonin prevented cartilage degradation by downregulating MMPs. The addition of luzindole and SOD inhibitors abrogated the protective effect of melatonin associated with increased levels of ROS and MMPs. These results demonstrated that proinflammatory cytokines impair the chondrogenesis of MSCs, which was rescued by melatonin treatment. This chondroprotective effect was potentially correlated to decreased ROS, preserved SOD, and suppressed levels of MMPs. Thus, melatonin provides a new strategy for promoting cell-based cartilage regeneration in diseased or injured joints.

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Articular cartilage is a highly specialized tissue that has very poor self-repair capacity after injury or disease because of its avascular structure. Approximately 1% of the world’s population has either of two forms of major arthritides, that is, osteoarthritis (OA) and rheumatoid arthritis (RA), which may result from trauma, genetic mutations, or damage to the meniscus and ligaments [1]. OA is characterized by the degradation and loss of articular cartilage function that might be caused by trauma when left untreated [2]. RA is an autoimmune disease characterized by abnormal synovial proliferation and cartilage destruction by the body’s immune system [3]. Although the origins of these two diseases are different, they involve similar destructive proteinases and inflammatory mediators that mediate the pathological changes in the joints [4].

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** Abbreviations: OA, osteoarthritis; RA, rheumatoid arthritis; SMSC, synovium mesenchymal stem cell; ROS, reactive oxygen species; SOD, superoxide dismutase; GPx, glutathione peroxidase; MMP, matrix metalloproteinase; ECM, extracellular matrix; DDC, diethyldithiocarbamate; 2-MeOH2, 2-methoxyestradiol

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Proinflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α), which are produced by macrophages, synovial fibroblasts, and chondrocytes during the pathogenesis of OA and RA, induce local inflammation in articular cartilage [5,6]. There is extensive literature that demonstrates elevated levels of IL-1β and TNF-α during the cartilage destruction cascade in synovial tissue and fluid [7]. TNF-α also induces cell apoptosis in chondrocytes, as evidenced by increased expression of caspase-8 [8]. Moreover, both IL-1β and TNF-α inhibit cartilage matrix synthesis (e.g., aggrecan and type II collagen), leading to further cartilage destruction [9].

The arthritic inflammatory response induces excessive generation of reactive oxygen species (ROS), which seem to be an important mediator in the pathogenesis of joint diseases and aggravate the breakdown of articular cartilage components. A significant increase in ROS is observed in the early stages of OA progression and is accompanied by loss of cartilage and subchondral bone surface [10]. NADPH oxidase, as a major source of ROS, produces superoxide anions and free radicals to activate several signaling pathways such as the mitogen-activated protein kinase pathway; this results in the overexpression of inflammatory cytokines [11]. Moreover, high levels of ROS that are found in inflamed joints not only induce chondrocyte cell death and oxidative DNA damage [12] but are also responsible for the destruction of cartilage extracellular matrix (ECM) through the activation of matrix metalloproteinases (MMPs) [13].

Mesenchymal stem cells (MSCs) provide a feasible cell source for cartilage repair because they can be harvested from various tissues and can differentiate into multiple lineages, including chondrocytes. Synovium mesenchymal stem cells (SMSCs) have been shown to have robust proliferative capacity and superior chondrogenic potential, thus attracting increased interest in cartilage tissue engineering and clinical utilization [14]. More importantly, the phenotype profile and chondrogenic potential of MSCs are not affected by age or OA etiology [15]. However, cartilage regeneration relying on the in situ chondrogenesis of MSCs, in many instances, occurs in an inflammatory environment and hence is compromised by proinflammatory cytokines. Although MSCs from adipose tissue express anti-inflammatory and immunosuppressive characteristics in collagenase-induced OA [16], high levels of oxidative stress in an inflammatory environment remain challenging for stem-cell-based cartilage repair, as evidenced by suppressed differentiation by IL-1β and TNF-α via activation of the NF-kB pathway [17] and inhibited cartilage formation in the presence of hydrogen peroxide [18]. Therefore, the arthritic microenvironment containing proinflammatory mediators causes failure of cartilage repair: The development of suitable therapies that enhance antioxidant and antiarthritic characteristics of MSCs would promote cartilage regeneration in damaged joints.

Melatonin (N-acetyl-5-methoxytryptamine) is primarily synthesized in the pineal gland and is well known for its antioxidant properties involving direct scavenging of free radicals [19]. Exogenous melatonin also exerts protective effects against oxidative stress in motoneurons by preserving superoxide dismutases (SODs) [20]. The SOD enzyme system processes superoxide radicals to hydrogen peroxide, which is subsequently neutralized by catalase and glutathione peroxidases (GPx’s) [21]. Melatonin has been shown to protect the nervous system by reducing ROS levels [22] and improving the resistance of MSCs to hydrogen peroxide-induced apoptosis [23]. In terms of its action on cell differentiation, melatonin has been proven to enhance ECM formation in primary chondrocytes in vitro through activation of the transforming growth factor β (TGF-β) signaling pathway [24]. In the presence of IL-1β, melatonin improved osteogenic differentiation of MSCs by alleviating the effects of intracellular ROS [25]. Animal studies have also established the promotive effect of melatonin on bone formation and its inhibitive effect on bone resorption [26]. More importantly, it has been shown that melatonin inhibits the expression of MMP-9 and MMP-3 in the animal model of acute gastric ulcers [27] and suppresses the activation of MMPs in RAW264.7 macrophages by inhibiting NF-kB translocation [28]. However, limited information is available regarding the potential protective effect of melatonin on MSC chondrogenesis in diseased joint microenvironments.

The purpose of this study was to investigate whether melatonin protects chondrogenic-specific differentiation of SMSCs from the deleterious influences of catabolic factors in an in vitro arthritic model. To create the in vitro arthritic cell model, two representative proinflammatory cytokines, IL-1β and TNF-α, were added to cell culture to retard chondrogenesis. We hypothesized that melatonin would have an antioxidant effect and reverse the inhibitory effect of cytokines on chondrogenic differentiation. We focused on examining the production of intracellular ROS, activities of antioxidant enzymes, chondrocyte-specific markers, and levels of MMPs in the presence of melatonin, IL-1β, and TNF-α. A melatonin receptor antagonist and SOD inhibitors were used to determine whether the melatonin-induced chondroprotective effect could be blocked. Our long-term goal is to use melatonin to promote stem-cell-based cartilage regeneration, especially for preventing and treating joint diseases such as OA and RA.

Materials and methods

Reagents

Synovium mesenchymal stem cells were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). Dexamethasone, proline, l-ascorbic acid, melatonin, luzindole, dimethyl sulfoxide (DMSO), diethyldithiocarbamate (DDC), 2-methoxyestradiol (2-MeOH2), and 2,7'-dichlorofluorescein diacetate (DCF-DA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). TGF-β1, IL-1β, and TNF-α were obtained from PeproTech Asia (Rehovot, Israel).

Cell culture and chondrogenesis of SMSCs

SMSCs were cultured in 12-well tissue culture plates (Corning, Tewksbury, MA, USA) in α-minimum essential medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and Fungizone (0.25 μg/ml) at 37 °C with 5% CO2. On reaching 95% confluence, the culture medium was replaced with serum-free chondrogenic differentiation medium. The medium contained high-glucose Dulbecco’s modified Eagle’s medium with 40 μg/ml proline, 100 nM dexamethasone, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml Fungizone, 0.1 mM l-ascorbic acid, ITS Premix (BD Biosciences, San Jose, CA, USA), and 20 ng/ml TGF-β1. The medium was changed every 3 days and the cells were induced to differentiate for 14 days.

Proinflammatory cytokine-inhibited cell assay

SMSCs were induced to chondrogenesis and simultaneously exposed to 1 μM melatonin (MT), 10 ng/ml IL-1β, or 10 ng/ml TNF-α. Cells incubated only in chondrogenic differentiation medium served as a controls (CTRL). To assess the anti-inflammatory effect of melatonin, cells were treated with melatonin on coexposure to IL-1β (IL-1β+MT) or TNF-α (TNF-α+MT). To block the melatonin receptor signaling pathway, cells were treated with 1 μM luzindole as a melatonin receptor antagonist (Luz) on coexposure to melatonin (MT+Luz), IL-1β (IL-1β+MT+Luz), or TNF-α (TNF-α+MT+Luz). Cells incubated in 0.1% DMSO were included as a vehicle control (Sigma).
**Measurement of intracellular ROS accumulation**

Intracellular ROS production was quantified by a DCF-DA fluorescence method as previously described [29]. Briefly, DCF-DA was dissolved in methanol and then diluted with phosphate-buffered saline to obtain a final concentration of 10 μM. Cells (n = 4; 2 × 10^5) were incubated in DCF-DA for 10 min at 37 °C, and the fluorescence intensity of DCF, which was activated by intracellular ROS, was measured by a BD dual laser FACScalibur (BD Biosciences). Ten thousand events of each sample were collected. The mean fluorescence intensity of DCF was calculated using the WinMDI 2.9 software (Windows Multiple Document Interface for Flow Cytometry).

**Measurement of hydrogen peroxide accumulation**

The accumulation of hydrogen peroxide in cells was detected with an Amplex Red Hydrogen Peroxide Assay Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Cellular extracts (six-well plates; n = 3) were collected and incubated with Amplex red reagent for 30 min. Fluorescence was then measured with a fluorescence microplate reader (BioTek, Winooski, VT, USA) using excitation at 530 nm and emission detection at 590 nm.

**Superoxide dismutase activity assay**

SOD activity was determined by using a commercially available SOD assay kit (Sigma) according to the manufacturer’s instructions. Cells (six-well plates; n = 3) were trypsinized and suspended in cell lysis solution. Total sample proteins were quantified using a BCA Protein Assay Kit (Invitrogen). The supernatant was mixed with WST solution and incubated at 37 °C for 20 min. The absorbance at 450 nm was measured using a microplate reader (BioTek).

**Catalase activity assay**

Catalase activity was measured with a commercially available catalase assay kit (Sigma). Briefly, total proteins were extracted from cells (six-well plate; n = 3) and quantified with the BCA Protein Assay Kit (Invitrogen). The amount of H2O2 consumed by each sample was measured with a microplate reader by reading the absorbance at 520 nm. The activity of catalase was then calculated according to the manufacturer’s instructions.

**Cell viability assay**

Cells in 12-well plates (n = 3) were collected and digested at 60 °C for 4 h with 125 μg/ml papain in 100 mM phosphate, 10 mM EDTA, pH 6.5, buffer containing 10 mM L-cysteine. The amount of DNA content was measured with a Quant-IT PicoGreen dsDNA Assay Kit (Invitrogen) with a SynergyMx multimode reader (BioTek) according to the manufacturer’s instructions. The DNA content of each group was normalized to that in the control group.

**Antioxidant inhibitor assay**

Cells were pretreated with antioxidant inhibitors before incubation with melanin or proinflammatory cytokines as previously described [30]. Cells were treated with 50 μM DDC (CuZn-SOD inhibitor) for 1.5 h or 1 μM 2-MeOH2 (Mn-SOD inhibitor) for 24 h. Alcian blue staining and Sirius red staining

Cells were fixed in 4% paraformaldehyde overnight. Alcian blue (1%; pH 2.5; Sigma) staining was used to detect sulfated glycosaminoglycans, and Sirius red (1 mg/ml; Sigma) was used to stain collagens. Images were captured with an Olympus IX71 microscope (Olympus, Tokyo, Japan) and processed by Image-ProPlus software (Media Cybernetics, Rockville, MD, USA).

**Total RNA extraction and quantitative real-time reverse transcription–polymerase chain reaction (real-time RT-PCR)**

Total RNA was extracted from cells (n = 3) on days 7 and 14 using TRIzol reagent (Invitrogen). For each sample, 1 μg of total RNA was reverse transcribed with a PrimerScript RT Reagent Kit (TaKaRa, Mountain View, CA, USA). To quantify the mRNA, cDNA equivalent to 20 ng of total RNA was detected by real-time PCR analysis using a GoTaq qPCR Master Mix kit (Promega, Madison, WI, USA). Chondrogenic markers including aggrecan, type II collagen (COL II), cartilage oligomeric matrix protein (COMP), and Sox-9: antioxidant enzyme genes (CuZn-SOD, Mn-SOD, and catalase); and matrix metalloproteinase genes (MMP-1, MMP-2, MMP-9, and MMP-13) were detected. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. The primer sequences are listed in Table 1.

**Statistical analysis**

All data are expressed as the mean ± standard error (SE). Statistical differences between two groups were determined by one-way analysis of variance followed by Student’s unpaired t test with SPSS software (SPSS, Inc., Chicago, IL, USA). P values less than 0.05 were considered statistically significant.

**Table 1**

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Fig. 1. Effects of melatonin and proinflammatory cytokines on the accumulation of ROS and hydrogen peroxide. (A) Intracellular ROS production in SMSCs under the treatments with IL-1β and TNF-α within 24 h. (B) ROS generation when cells were treated with melatonin (MT), IL-1β, IL-1β and melatonin (IL-1β+MT), TNF-α, and TNF-α and melatonin (TNF-α+MT). Serum-free chondrogenic medium served as a control group (CTRL). Cells were labeled with DCF-DA and measured with flow cytometry (top). Gray lines represent the treatments with control, IL-1β, and TNF-α. Red lines represent the treatments with melatonin, IL-1β and melatonin, and TNF-α and melatonin. The mean fluorescence intensity was quantified (bottom) to represent ROS production. (C) Cellular extracts from SMSCs were labeled with Amplex red probe and the fluorescence was measured to represent hydrogen peroxide production. ROS results are representative of four independent experiments. Hydrogen peroxide results are representative of three independent experiments. Values are the mean ± SE. *P < 0.05; #P < 0.05 versus CTRL.
Results

Intracellular ROS and hydrogen peroxide in response to melatonin and proinflammatory cytokines

We first examined the effects of proinflammatory cytokines on ROS production in SMSCs. DCF fluorescence detected by flow cytometry showed that the levels of ROS significantly increased after treatment with catalytic factors (Fig. 1A). Treatment with IL-1β (10 ng/ml) induced the highest level of ROS at 15 min; this effect decreased by 30 min and recovered to the level in the control group by 24 h. However, SMSCs exposed to TNF-α (10 ng/ml) showed a significant increase in ROS accumulation in a short time, peaked within 30 min, continued to decrease, and reached a low level within 24 h.

To determine whether exogenous melatonin regulated the production of ROS in the presence of IL-1β or TNF-α, SMSCs were treated with melatonin at a concentration of 1 μM (Fig. 1B) in the presence of IL-1β or TNF-α. The addition of melatonin alone decreased the level of ROS by 26.6% compared to that in control cells. Similarly, incubation with melatonin attenuated oxidative stress in SMSCs by 14.5 and 30.3% on coexposure to IL-1β and TNF-α, respectively. The accumulation of hydrogen peroxide was measured with an Amplex red probe. As shown in Fig. 1C, on supplementation with melatonin, the intracellular hydrogen peroxide was reduced by 13.5% compared to that in the control group, without significance (P=0.08). In the presence of IL-1β, melatonin treatment decreased the hydrogen peroxide level by 13.3% compared to that in the IL-1β group. However, melatonin treatment did not affect hydrogen peroxide accumulation in SMSCs on exposure to TNF-α (P=0.48).

Effects of melatonin and proinflammatory cytokines on antioxidant enzymes

We investigated the effects of melatonin on SOD mRNA expression and activity. As illustrated in Fig. 2A, treatment with melatonin increased SOD activity by 35.0 and 48.7% in contrast to that in the control and IL-1β groups, respectively. However, it did not affect SOD activity in TNF-α-treated SMSCs. When SMSCs were exposed to IL-1β and TNF-α, the mRNA expression of CuZn-SOD in SMSCs decreased by 44.4 and 25.8%, respectively, compared to that in the control group. However, melatonin treatment significantly increased CuZn-SOD expression (by 46.0% vs the CTRL group, by 15-fold vs the IL-1β group, and by 1.1-fold vs the TNF-α group; Fig. 2B). We found that proinflammatory cytokine treatments increased the level of Mn-SOD, compared to that in the control cells (by 2.2-fold in the IL-1β group and 11.0% in the TNF-α group). Cotreatment with melatonin also increased gene expression of Mn-SOD (by 2.0-fold vs the CTRL group, by 47.9% vs the IL-1β group, and by 86.5% vs the TNF-α group; Fig. 2C).

Catalase is one of the major antioxidant enzymes that scavenges hydrogen peroxide; therefore, we further examined the effect of melatonin on catalase activity and mRNA expression. The activity of catalase slightly increased with melatonin treatment (P=0.11), significantly decreased by 28.1% when exposed to IL-1β, and decreased by 13.0% on exposure to TNF-α. However, melatonin supplementation restored catalase activity after coexposure to proinflammatory cytokines (by 38.5% vs the IL-1β group and by 10.2% vs the TNF-α group; Fig. 2D). The gene expression of catalase was downregulated by treatments with proinflammatory cytokines (by 15.6% in the IL-1β group and by 17.7% in the TNF-α group), but melatonin supplementation significantly increased catalase levels by 18.0 and 19.1% compared to that in the IL-1β group and the TNF-α group, respectively (Fig. 2E).

Melatonin reverses proinflammatory cytokine inhibition of chondrogenesis

IL-1β had no effect on cell viability during SMSC differentiation into a chondrogenic lineage, whereas TNF-α significantly decreased cell viability by 38.7% compared to that in the control group. Melatonin treatment alone decreased DNA content by 22.2%. However, the
Fig. 3. Chondrogenesis of SMSCs on exposure to melatonin and proinflammatory cytokines. SMSCs were induced to chondrogenic differentiation with or without melatonin treatment on coexposure to 10 ng/ml IL-1β or 10 ng/ml TNF-α. (A) Cell viability of SMSCs was determined by PicoGreen dsDNA assay and the DNA content was normalized to the level of control group. (B) Synthesis of glycosaminoglycans in SMSCs was assessed by Alcian blue staining. (C) Synthesis of collagens was assessed by Sirius red staining. DNA results are representative of three independent experiments. Values are the mean ± SE. Scale bar, 100 μm in (B) and (C). *P<0.05; #P<0.05 versus CTRL.
addition of melatonin reversed the TNF-α-induced inhibitory effect on cell viability by 47.7% compared to that in the TNF-α group (Fig. 3A).

To evaluate cartilage-specific ECM production, sulfated glycosaminoglycans (Fig. 3B) and collagens (Fig. 3C) were detected by Alcian blue staining and Sirius red staining, respectively. When differentiated SMSCs were stimulated with IL-1β or TNF-α, they showed a significantly diminished deposition of glycosaminoglycans and collagens, but melatonin treatment induced a glycosaminoglycan-rich and collagen-rich matrix surrounding the differentiated cells, indicating that melatonin rescued the formation of cartilage ECM, which was inhibited by proinflammatory cytokines.

We next examined mRNA expression of chondrocyte-specific marker genes, such as COL II, aggrecan, COMP, and Sox-9, on days 7 and 14 by quantitative RT-PCR. After treatment with IL-1β or TNF-α, gene expression of aggrecan significantly decreased by 95.1 and 74.2%, respectively, compared to that in the control group (Fig. 4A); gene expression was remarkably restored by melatonin treatment (7.6-fold vs the IL-1β group and 2.7-fold vs the TNF-α group). The trends for COL II (Fig. 4B) and COMP (Fig. 4C) mRNA expression were similar to those for aggrecan gene expression. Sox-9 is an essential transcription factor in the early stage of chondrogenic differentiation. Treatment with IL-1β or TNF-α inhibited the level of Sox-9 (by 21.3 and 61.0%, respectively, vs the CTRL group), but melatonin treatment upregulated Sox-9 gene expression by 87.4% in the presence of TNF-α (Fig. 4D). The data on the expression of aggrecan, type II collagen, COMP, and Sox-9 on day 7 also indicated that melatonin promoted chondrogenesis (Supplementary Figs. 1A–1D).

Melatonin suppresses the expression of MMPs

To assess the effect of melatonin on cartilage ECM degradation, gene expression of MMP-1, MMP-2, MMP-9, and MMP-13 was examined. MMP-1 is a critical proteinase that targets native collagens in articular cartilage. We found that IL-1β treatment markedly increased the expression of MMP-1 by 18.7-fold compared to that in the control group. The addition of melatonin significantly decreased MMP-1 expression by 20.2% in the presence of IL-1β (Fig. 5A). MMP-2 expression was significantly upregulated by both IL-1β and TNF-α treatments (by 65.4 and 15.6%, respectively, vs the CTRL group), but was downregulated in the presence of melatonin (by 25.9% vs the IL-1β group and 34.1% vs the TNF-α group; Fig. 5B). However, neither IL-1β nor TNF-α treatment affected MMP-9 expression, whereas melatonin treatment reduced MMP-9 by 56.3% compared to TNF-α treatment (Fig. 5C). Melatonin treatment significantly reduced MMP-13 expression by 37.4% compared to that in the CTRL group. The level of MMP-13 was also significantly increased by treatment with IL-1β or TNF-α (by 45.4 and 32.3%, respectively, vs the CTRL group), but was downregulated by melatonin treatment (by 42.8% vs the IL-1β group and 59.7% vs the TNF-α group; Fig. 5D). The trend in the expression of MMP genes on day 7 was the same as that obtained on day 14 (Supplementary Figs. 2A–2D).

The melatonin receptor antagonist blocks antioxidant and chondroprotective effects

To confirm that the effect of melatonin on chondrogenesis and MMPs was mediated through a melatonin receptor pathway, luzindole, a specific melatonin receptor antagonist, was added to the chondrogenic differentiation medium. Collagen synthesis was suppressed by the addition of luzindole in the presence of IL-1β or TNF-α, as evidenced by Sirius red staining (Fig. 6A). Quantitative RT-PCR showed that treatment with luzindole significantly blocked...
the effect of melatonin on gene expression of aggrecan by 88.1 and 19.2% in the presence of IL-1β and TNF-α, respectively (Fig. 6B). The levels of COMP (Fig. 6C) and Sox-9 (Fig. 6D) also showed the same tendency. In addition, the expression of MMP-1, MMP-2, and MMP-13 was significantly upregulated by luzindole treatment (Fig. 7A–C). Furthermore, intracellular ROS suppression by luzindole treatment was significantly reduced (Fig. 7D).
melatonin treatment was offset by the treatment with luzindole when cells were coexposed to IL-1β or TNF-α (Fig. 7D–F).

SOD inhibitors increase ROS levels and suppress chondrogenesis

To verify the roles of CuZn-SOD and Mn-SOD in melatonin-restored chondrogenesis, two SOD inhibitors, DDC and 2-MeOH2, were used to treat cells that were concurrently treated with proinflammatory cytokines and melatonin. Both inhibitors reversed the antioxidant effect of melatonin by increasing intracellular ROS (Fig. 8A and B). In particular, we found that 2-MeOH2 was more potent at upregulating oxidative stress. Despite the fact that melatonin significantly enhanced chondrogenic differentiation, inhibitors of DDC and 2-MeOH2 downregulated the expression of chondrocyte-specific genes, such as aggrecan (Fig. 8C and D) and Sox-9 (Fig. 8E and F), indicating that high ROS levels suppressed chondrogenic lineage maturation of SMSCs. Treatment with SOD inhibitors also upregulated the mRNA levels of MMP-1 (Fig. 9A and B) and MMP-2 (Fig. 9C and D), suggesting that depleting SOD contributed to MMP-dependent cartilage ECM destruction.

Discussion

In many cases, application of stem-cell-based cartilage regeneration takes place within an inflammatory environment, which prevents chondrogenic maturation and accelerates cartilage breakdown. Release of proinflammatory cytokines induces elevation of oxidative stress, which is considered to cause chondrocyte senescence and contribute to cartilage pathogenesis [31]. Although melatonin has been suggested to protect against free radical species in various cell types and suppress the production of proinflammatory mediators in lipopolysaccharide-stimulated vascular smooth muscle cells [32], the potential anti-inflammatory effect on chondrogenesis of MSCs in an arthritic environment was unknown. To our knowledge, this study is the first to show that melatonin rescues impaired human SMSC chondrogenesis induced by IL-1β and TNF-α. Moreover, the molecular mechanism by which this occurs involves the alleviation of intracellular ROS via preservation of SOD and suppression of MMPs by melatonin treatment. Our findings provide potential applications for melatonin in the treatment of joint diseases and promotion of cartilage repair.

An increasing amount of evidence implicates IL-1β and TNF-α as important proinflammatory mediators in joint diseases. These cytokines play a vital role not only in modulating subchondral bone resorption but also in shifting cartilage homeostasis toward catabolism. As shown by our data, both treatments with IL-1β and TNF-α caused significant reduction in cartilage-specific matrix synthesis and downregulation of chondrogenic marker genes. More interestingly, IL-1β was found to have a more potent effect than TNF-α on the inhibition of chondrogenesis, in agreement with a recent report [33]. During chondrogenesis, these cytokines initiate the activation and nuclear translocation of NF-κB, which then inhibits phosphorylation of Smad3/4, a key signaling mediator in response to TGF-β [34]. Moreover, our data showed that treatment with IL-1β and TNF-α suppressed Sox-9, an essential transcription factor in early chondrogenesis that is also responsible for subsequent inhibition of chondrogenic maturation. Therefore, the inflamed environment present in diseased joints might result in failure of cartilage defect repair. This suggests that blocking the catabolic effect of proinflammatory factors would promote the effectiveness of stem-cell-based cartilage regeneration strategies.

The effects of proinflammatory cytokines and melatonin on cell viability were examined. Our study suggested that IL-1β did not affect cellular viability, whereas TNF-α remarkably inhibited it, as assessed by DNA assays. Consistent with our data, previous studies demonstrated that TNF-α induced cell apoptosis via a caspase 3- and caspase 8-associated cascade [8]. Melatonin protected MSCs...
from cytokines by adjusting the balance between proapoptotic and antiapoptotic factors. Kim et al. [35] showed that melatonin exhibited a protective effect on myoblast cells against NO-induced cell death with increase in Bcl-2 expression and inhibition of Bax expression. In addition, in this study, we incubated MSCs in a serum-free chondrogenic medium to minimize any serum-induced interference because autologous conditioned serum has been demonstrated to be a potential anti-inflammatory agent for treating arthritis and muscle injuries [36].

Evidence of increased generation of ROS and subsequent oxidative stress has been reported in pathological cartilage from OA and RA patients. Proinflammatory cytokines in diseased joints upregulated oxidative stress reactions and led to chondrocyte apoptosis through the caspase signaling pathway [37]. Elimination of superoxide radicals is significantly dependent on the expression of SOD under normal physiological conditions; however, Scott et al. [38] evaluated three SOD genes (CuZn-SOD, Mn-SOD, and EC-SOD) in an OA animal model and reported that OA progression resulted in significant reduction in the levels of SOD. In the present study, we observed that oxidative stress in SMSCs was elevated by proinflammatory cytokine treatments, but melatonin provided a chondroprotective effect by preventing oxidative damage and upregulating the expression of CuZn-SOD and Mn-SOD. The use of luzindole, a melatonin receptor antagonist, significantly counteracted the antioxidant effect of melatonin, indicated by the increased level of ROS in SMSCs. Moreover, hydrogen peroxide, as a product of scavenging of superoxide radicals by the SOD enzyme systems, functioned as an important signaling messenger to affect cellular survival and growth. Kim et al. reported [39] that short-term treatment with hydrogen peroxide at a concentration...
of 100 μM stimulated cell proliferation in fibroblasts through activation of Jun-N-terminal kinase and p38 mitogen-activated protein kinases. In contrast, long-term incubation with hydrogen peroxide at a concentration even as low as 50 μM resulted in cell growth arrest [40]. Melatonin offered protection against cellular oxidative stress injury, particularly that induced by hydrogen peroxide, depending on the inhibition of JAK2/STAT3 phosphorylation [41]. Although intracellular ROS generated by NADPH oxidases was necessary for chondrogenic differentiation [42], the inhibitory effect of external oxidative stress on chondrogenesis of stem cells has been demonstrated in numerous studies. By creating oxidative stress with hydrogen peroxide, Pei et al. [40] showed that chondrogenic maturation of human adult stem cells was significantly suppressed and that the level of type X collagen, a typical hypertrophy marker, increased. Our data also suggest that high levels of intracellular ROS cause suppressed chondrogenic differentiation, but that melatonin treatment successfully restored the inhibitory effect owing to its antioxidant properties. The decreased expression of chondrogenic marker genes in the presence of SOD inhibitors confirms that the expression of superoxide radicals is an important determinant for the poor outcome of MSC chondrogenesis in OA or RA. The high level of cellular hydrogen peroxide was responsible for the senescence and diminished osteogenic potential of adult MSCs because of its effect on c-MAF and PKC-p21 activities [43]. The anti-inflammatory effect of melatonin was possibly related to ERK and p38 signaling pathways because the phosphorylation of these kinases was suppressed in the presence of melatonin [44]. However, the underlying molecular mechanism that regulates this process needs to be elucidated in future studies.

To determine whether SOD participates in the anti-inflammatory effects of melatonin, we analyzed the levels of Cu/Zn-SOD and Mn-SOD and found that they decreased after treatment with IL-1β or TNF-α, suggesting a relationship between suppression of chondrogenic differentiation and reduction in SOD expression. Gavrilidis et al. [45] demonstrated that Mn-SOD depletion was increased. Therefore, the reversal of pro-inflammatory cytokine-induced inhibition of chondrogenic differentiation by melatonin was correlated with low oxidative stress through the SOD antioxidant functions of SOD. The results suggested not only that chondrogenesis was impaired but also that the levels of MMPs increased. Thus, the reversal of pro-inflammatory cytokine-induced inhibition of chondrogenic differentiation by melatonin was correlated with low oxidative stress through the SOD antioxidant functions of SOD. The results suggested not only that chondrogenesis was impaired but also that the levels of MMPs increased. Therefore, the reversal of pro-inflammatory cytokine-induced inhibition of chondrogenic differentiation by melatonin was correlated with low oxidative stress through the SOD antioxidant functions of SOD. The results suggested not only that chondrogenesis was impaired but also that the levels of MMPs increased. Therefore, the reversal of pro-inflammatory cytokine-induced inhibition of chondrogenic differentiation by melatonin was correlated with low oxidative stress through the SOD antioxidant functions of SOD. The results suggested not only that chondrogenesis was impaired but also that the levels of MMPs increased. Therefore, the reversal of pro-inflammatory cytokine-induced inhibition of chondrogenic differentiation by melatonin was correlated with low oxidative stress through the SOD antioxidant functions of SOD. The results suggested not only that chondrogenesis was impaired but also that the levels of MMPs increased. Therefore, the reversal of pro-inflammatory cytokine-induced inhibition of chondrogenic differentiation by melatonin was correlated with low oxidative stress through the SOD antioxidant functions of SOD. The results suggested not only that chondrogenesis was impaired but also that the levels of MMPs increased. Therefore, the reversal of pro-inflammatory cytokine-induced inhibition of chondrogenic differentiation by melatonin was correlated with low oxidative stress through the SOD antioxidant functions of SOD. The results suggested not only that chondrogenesis was impaired but also that the levels of MMPs increased. Therefore, the reversal of pro-inflammatory cytokine-induced inhibition of chondrogenic differentiation by melatonin was correlated with low oxidative stress through the SOD antioxidant functions of SOD. The results suggested not only that chondrogenesis was impaired but also that the levels of MMPs increased. Therefore, the reversal of pro-inflammatory cytokine-induced inhibition of chondrogenic differentiation by melatonin was correlated with low oxidative stress through the SOD antioxidant functions of SOD. The results suggested not only that chondrogenesis was impaired but also that the levels of MMPs increased. Therefore, the reversal of pro-inflammatory cytokine-induced inhibition of chondrogenic differentiation by melatonin was correlated with low oxidative stress through the SOD antioxidant functions of SOD.

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catalase and GPx's. This maintains the balance of ROS in MSCs; however, the role of GPx's in melatonin-mediated cytoprotection and enhanced chondrogenesis will be analyzed in future studies.

Evidence has been provided that degradation of cartilage matrix in joint diseases involves MMPs that include several subtypes (e.g., collagenases, gelatinases, stromelysins, metmyelotins) depending on their specific substrates. MMP-1 (collagenase-1) and MMP-13 (collagenase-3) are rarely expressed in healthy joint tissues and are important mediators of cartilage destruction in OA and RA. They uniquely target native type II collagen, which compromises joint tensile strength and makes collagen chains much more susceptible to further degradation by other MMPs [50]. MMP-13 is also considered to be a chondrocyte hypertrophy marker; its expression is accompanied by that of denatured type II collagen during the development of cartilage pathology [51]. With cytokine treatment, the expression of MMP-1 and MMP-13 markedly increased but was successfully inhibited by melatonin. In addition, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) recently attributed more interest in OA progression, because they target fully or partially denatured type II collagen other than gelatin [52]. Our data confirmed that the mRNA expression of MMP-2 was upregulated by exposure to proinflammatory factors; however, MMP-9 showed no significant difference in the presence of IL-1β, possibly because MMP-2 was present at a much higher level and was more deleterious than MMP-9 in OA-diseased joints [53].

The inhibitory effect of melatonin on the activation of MMPs has been demonstrated in RAW 264.7 and BV2 cells. Activation of NF-κB is required to induce the expression of MMPs, and melatonin effectively inhibits NF-κB translocation and binding activity to suppress MMP levels [28]. Our data suggest that melatonin significantly suppressed the levels of MMPs, including MMP-1, MMP-2, and MMP-13, accompanied by restoration of MSC chondrogenesis. Although the relationship between oxidative stress and MMPs is unclear in OA and RA, Martinez-Lemus et al. [54] suggested that, in cremaster arterioles, activation of MMPs was dependent on the production of ROS, whereas inhibition of ROS prevented the activity of MMPs. In this study, the elevated production of MMPs involved a high level of ROS as an obligatory secondary messenger [55]. Thus, scavenging of free radicals by melatonin possibly contributed to the decrease in MMP levels and the delay of cartilage degradation. Collectively, with the strong inhibition of MMPs, use of melatonin could be a promising therapeutic strategy to protect neo-cartilage undergoing repair from proteolytic enzymes.

In summary, we demonstrated that melatonin reversed the inhibitory effects of IL-1β and TNF-α on SMSC chondrogenesis. Interestingly, the chondroprotective effect of melatonin was potentially related to the decreased level of oxidative stress, the preserved expression of SOD, and the suppressed levels of cytokine-induced MMPs in restraining the degradation of cartilage-specific ECM. Our results suggest that therapeutic strategies that use melatonin may be effective in treating joint disease and promoting MSC-based cartilage regeneration.

**Authors’ contributions**

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. He and Dr. Gong have full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design were done by F.H. and Y.G. Acquisition of data was done by X.L., Y.Y., S.C., Y.Y., K.Z., F.H., and Y.G. Analysis and interpretation of data were done by X.L., Y.Y., S.C., Y.P., F.H., and Y.G.

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**Appendix A. Supplementary Information**

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2013.12.012.

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