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**Destabilising microtubule polymeriza-
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factor kappa b and β -catenin pathway**

Destabilising microtubule polymerization regulates chondrocyte dedifferentiation and inflammation via nuclear factor kappa b and β -catenin pathway

Yohan Han, Young Seok Eom, Fahad Hassan Shah and Song Ja Kim

Department of Biological Sciences, College of Natural Sciences, Kongju National University, Gongju 32588, Republic of Korea.

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Abstract

The role of cell morphological changes in colchicine-inhibited tubulin polymerization of rabbit articular chondrocytes and their involvement in dedifferentiation were investigated. Colchicine treatment resulted in the dedifferentiation of chondrocytes, which was supported by the loss of type II collagen expression and proteoglycan production. Inhibition of tubulin depolymerization with paclitaxel rescued colchicine-caused dedifferentiation and tubulin polymerization. Additionally, colchicine stimulated β -catenin overexpression, which is characterized by the accumulation of β -catenin into the cytosol determined by immunofluorescence staining. Inhibition of the β -catenin-mediated pathway by siR β -catenin recovered colchicine-caused the suppression of type II collagen expression in the chondrocytes. Treatment with colchicine also induced inflammation, as determined by the increased expression level of cyclooxygenase-2 and decreased I κ B- α expression level by western blot analysis. Modulating the expression levels of pI κ B α and I κ B α via BMS 345541, was able to modulate colchicine-induced inflammatory effect.

Introduction

One of the most common painful and incapacitating chronic degenerative joint illnesses worldwide is osteoarthritis. Age, weight, gender, dietary habits, and joint injuries are a few examples of risk factors for osteoarthritis (Whittaker et al., 2021). However, there is still no perfect treatment for osteoarthritis, and the molecular mechanism is not fully understood. Understanding the molecular pathways governing the differentiation, dedifferentiation, and inflammation of chondrocytes is essential for treating osteoarthritis.

Extracellular matrix components synthesized by the cartilage include collagen, sulphated-proteoglycan, hyaluronic acid, and glycoproteins which are expressed by various cellular regulatory signals stimulating cartilage-specific genes (Shi et al., 2019; Li and Wu, 2021; Xiao et

al., 2021). Homeostatic disruption of extracellular matrix expression in articular chondrocytes involves loss of the differentiated phenotype of chondrocytes during the development of cartilage diseases such as osteoarthritis (Guo et al., 2021). To maintain homeostasis, extracellular matrix synthesis and breakdown must be strictly regulated numerous elements in the chondrocytes' chemical and mechanical state regulate their metabolic and anabolic activities (Zheng et al., 2021; Hodgkinson et al., 2022). The breakdown of chondrocytes is attributed to dedifferentiation, an imbalance between matrix metalloproteases and their inhibitors, apoptosis, the release of pro-inflammatory cytokines, retinoic acid, and monolayer culture (Yu and Kim, 2011; Charlier et al., 2019; Ghosh et al., 2022).

It has been reported that the structure of the cytoskeleton acts as an important factor in determining the



phenotype of chondrocytes (Kim et al., 2012; Han and Kim, 2018). Actin cytoskeleton alterations can be seen in chondrocytes dedifferentiated by retinoic acid or monolayer cultures (Yu and Kim, 2011; Charlier et al., 2019). Disruption of the actin cytoskeleton by cytochalasin D or simvastatin causes redifferentiation from dedifferentiated chondrocytes (Kim et al., 2012; Beier, 2016; Han and Kim, 2018). Microtubules are essential structural components for maintaining cell skeleton and morphology in tumor cells as well as normal cells (Goodson and Jonasson, 2018). They also perform various functions such as organelle-cell migration and micromolecular transport.

β -Catenin is a versatile transcription factor and a crucial structural element of cadherin-based adhesion junctions. It is a key regulator of the conventional Wnt signaling pathway. The Wnt/ β -catenin signaling pathway is crucial for cell differentiation, growth, proliferation, and the development of cancer (Zhang et al., 2020). In the absence of Wnt ligand protein, β -catenin is phosphorylated by the GSK3 complex (Huang et al., 2019). Phosphorylated β -catenin, which is marked with ubiquitin is recognized and degraded by the proteasome. Conversely, the presence of Wnt ligand inhibits the degradation of β -catenin, which accumulates in the cytoplasm and migrates to the nucleus (Zhang and Wang, 2020). The study shows that the regulation of Wnt/ β -catenin signaling affects the differentiation of chondrocytes (Yu et al., 2019). Additionally, it was discovered that osteoarthritis patients have activated β -catenin signaling (Fernández-Torres et al., 2018; Li et al., 2020). In light of this, chondrocyte differentiation and the pathogenic processes of osteoarthritis depend on Wnt/ β -catenin signaling.

Nuclear factor kappa B (NF- κ B) exists in the cytoplasm with heterodimer inhibitor protein of p50 and p65, known as a inhibitor of NF- κ B (I κ B) (Zhang et al., 2021). Nearly all cell types have NF- κ B, which is essential for controlling inflammatory responses. I κ B and homo- or heterodimers of NF- κ B combine in the cytoplasm when there is no stimulation. However, IB kinases (IKK α , IKK β , and IKK γ) phosphorylate I κ B, as a result of numerous stress-inducing substances including cytokines, LPS, or ROS, which causes I κ B to be ubiquitinated and degraded by the proteasome (Zhang et al., 2021) and has been linked to various diseases including arthritis (Malkowski, 2011; Wang et al., 2021; Pannunzio et al., 2018; Ferrer et al., 2019).

This research looked into how the microtubule changes in primary articular chondrocytes regulate chondrocyte development and the inflammatory response.

Materials and Methods

Articular chondrocyte culture and maintenance

Primary articular chondrocytes were isolated from

rabbit articular cartilages which were cultured and maintained by using the reported protocol (Han et al., 2022). Colchicine (CAS:64-86-8) and paclitaxel (CAS: 333069-62-4) were brought from Sigma Aldrich and were added to the cell medium when the density of the cells reached 80% population.

RNA expression quantification

The protocol followed for RNA extraction, and RNA to DNA amplification are reported elsewhere (Han et al., 2022). The primers used to quantify the expression were: Cyclooxygenase-2 (COX-2) (298-bps), 5'-TCAGCCACGCAGCAAATCCT-3' (sense) and 5'-GTGATCTGGATGTCAGCAGC-3' (antisense), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 299-bps), 5'-TCACCATCTTCCAGGAGC GA-3' (sense) and 5'-CACAAATGCCGAAGTGGTCGT-3' (antisense), tubulin (296-bps), 5'-GCACTCTGATTGTGCCTTCA-3' (sense) and 5'-AGGCATTGGTGTGATCTCTGCT-3' (antisense) and type II collagen (370-bps), 5'-GACCCCATGCAGTACATG CG-3' (sense) and 5'-AGCCGCCATTGATGTCTCC-3' (antisense). 1% Agarose gel was used to analyse samples obtained from amplification.

Transfection

siRNA of β -catenin siRNA was transduced in chondrocytes by mixing pure DMEM with TurboFect transfection agent obtained from ThermoFisher Scientific. The siRNA sequences were: negative control (NC) siRNA 5'-ACGUGACACGUAUCGGAGAATT-3' (antisense) and 5'-UUCUCCGAACGUGUCACGUTT-3' (sense), β -catenin siRNA, 5'-AUUUUAUAUCAUCUGAACCCCTT-3' (antisense) and 5'-GGGUUCAGAU GAUUAUAAUUTT-3 (sense).

Alcian blue staining

Cells fixation was done with 3.5 % of formaldehyde solution which was then cleaned thrice with 1x phosphate saline buffer followed by the introduction of alcian blue stain. The stained cells are incubated for overnight and then washed with 1x phosphate saline buffer and then quantified with ELISA plate analyzer at 595 nm (Han et al., 2022).

Immunofluorescence analysis

The cells are fixated and permeabilized with 3.5% paraformaldehyde and 0.1% of triton X-100 and then washed with 1x phosphate saline buffer. The primary antibodies were added to the cells which are type II collagen and β -catenin and incubated for 24 hours. After that, cells were washed with 1x phosphate saline buffer and secondary fluorescent antibodies were added. The results were then visualized with a fluorescent microscopy (Han et al., 2018).

Statistical analysis

Analysis of variance (ANOVA) was used to assess the statistical significance of the difference between the

Box 1: Western Blotting

Principle

Western blotting is based on the principle of immunochromatography where proteins are separated into polyacrylamide gel according to their molecular weight.

Requirements

BCA protein assay kit, Cells pellet, Chemiluminescence reagent, Chemiluminescence detector, RIPA buffer, Protease cocktail, Blocking buffer, Primary antibodies, Secondary antibodies, Phosphate saline buffer, 1x Tris-buffered saline, 0.1% Tween, Sodium polyacrylamide gel, Nitrocellulose membrane, Transfer buffer

Procedure

Step 1: Rabbit articular chondrocytes were treated with colchicine and incubated for 24 hours at 37°C with 5 %carbon dioxide incubator

Step 2: After incubation, cells media was removed and phosphate buffer was added and then cells were scrapped from the cell culturing dish and centrifuged at 13,000 rpm for 10 min at 4°C

Step 3: Phosphate buffer was then removed and RIPA buffer containing protease cocktail was added on to the pellets to extract the proteins by inducing cell lysis

Step 4: Protein concentration obtained from cell lysis was then quantified with BCA protein assay kit

Step 5: Quantified proteins were then added to the 9% polyacrylamide gel to separate the proteins according to their molecular weight

Step 6: Separated protein gel was then transferred to the nitrocellulose membrane with the aid of transfer buffer

Step 7: Nitrocellulose containing protein bands were cleaved according to the specific protein markers

Step 8: Cleaved protein bands were then washed thrice with TBST and then blocked with blocking buffer for 1 hour at room temperature

Step 9: After blocking, bands were washed again with TBST and then added to the primary antibody on a shaker incubator at 4°C overnight

Step 10: The protein bands were then taken out of primary antibody and washed thrice with TBST and then added on to the secondary antibody

Step 11: After exposure to secondary antibody, the protein bands were washed again with TBST and then visualized with chemiluminescence solution using autoradiography detector

Reference

Han et al., 2022

References (Video)

Eslami and Lujan, 2010; Shen et al., 2021; Lim et al., 2021; Liu et al., 2021

control and the comparison groups followed by Tukey's multiple comparisons. P-value <0.05 was considered to indicate statistically significant differences.

Results

Tubulin synthesis and chondrocytes differentiation

The effects of colchicine were explored within the concentration of (0.1-1 μ M) in the articular chondrocytes. The expression of chondrocyte differentiation markers (type II collagen, aggrecan, SOX-9, COX-2, tubulin) and loading control (GAPDH) was quantified in chondrocytes treated with colchicine in concentration- and time-dependent manner.

Western blot analysis indicated that colchicine-treated cells induced dedifferentiation by decreasing the expression of chondrocyte differentiation markers (type II collagen, SOX9 and aggrecans) and disturbed the microtubule polymerizing monomers (tubulin) in a dose- and time-dependently (Figure 1A,C). Whereas, the expression of cyclooxygenase-2 (COX-2) was also increased. These results were further verified with RT-PCR analysis to confirm the relative gene-specific transcript expression. The RT-PCR result corroborated the findings of western blot analysis (Figure 1B,D). The quantitative findings procured from alcian blue staining indicated that colchicine halted the synthesis of sulfated proteoglycans as shown in (Figure 1E).

In the following experiment, the impact on microtubule polymerization and chondrocyte differentiation was investigated in colchicine-treated cells. Treatment with colchicine reduced tubulin synthesis and differentiation of chondrocytes, as compared to control, as demonstrated by immunofluorescence staining (Figure 2). Thus, these results suggest that colchicine induces chondrocyte dedifferentiation and inflammation in primary chondrocytes.

Chondrocytes dedifferentiation via regulating microtubule polymerization

To prove this speculation that colchicine influence on the tubulin depolymerization caused the chondrocyte dedifferentiation paclitaxel was explored which is a microtubule polymerizing agent and counteract their disassembly. The influence of both paclitaxel and colchicine was analyzed with western blotting and Immunofluorescence and verified with RT-PCR and Alcian blue staining. Paclitaxel at a concentration of 5 μ M induced chondrocyte differentiation and ameliorated expression of type II collagen via MAPK pathway (Im and Kim, 2009). The same 5 μ M concentration was used for paclitaxel whereas 1 μ M of colchicine and cotreated together to observe the effects on chondrocyte differentiation. Exposure to colchicine decreased the chondrogenic markers (type II collagen, SOX9, aggrecans) and tubulin expression whereas paclitaxel increased the expression of tubulin and chondrogenic

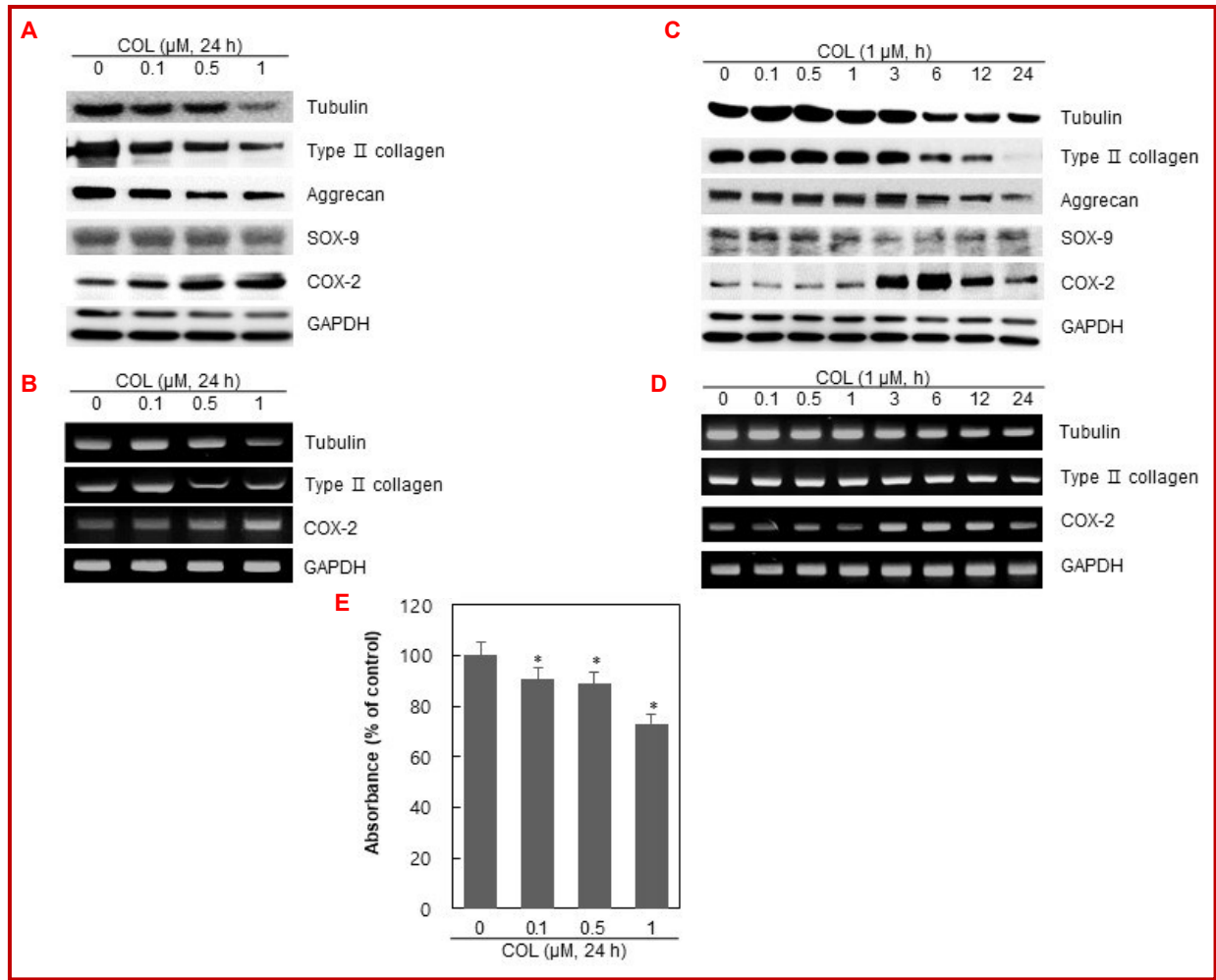


Figure 1: Colchicine reduces tubulin synthesis and chondrocytes differentiation and induces COX-2 expression in articular chondrocytes

Chondrocytes were either left untreated or given different doses of colchicine for 24 hours in (A and B). By using western blot (A) or RT-PCR, the expression levels of tubulin, type II collagen, aggrecan, SOX-9, COX-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were examined (B). GAPDH was used as a loading control. (C and D) For 24 hours, chondrocytes were either left untreated or given different doses of colchicine. By using western blot (C) or RT-PCR, the expression levels of tubulin, type II collagen, aggrecan, SOX-9, COX-2, and GAPDH were examined (D). GAPDH was used as a loading control. (E) For 24 hours, chondrocytes were either left untreated or given different doses of colchicine. Alcian blue staining was used to assess how sulfated proteoglycans were synthesized. The data in (A, B, C, D, and E) indicate the outcomes of a typical experiment, whereas the data in (E) represent mean values with standard deviation. * $p < 0.05$

markers (Figure 3A). But when both these drugs co-treated together in chondrocyte, paclitaxel counteracted the effect of colchicine by rescuing the chondrogenic and tubulin expression as confirmed with RT-PCR analysis (Figure 3B).

The changes in polymerization and depolymerization of microtubules in treated chondrocytes were observed via immunofluorescence staining (Figure 3C). Treatment with paclitaxel increased the differentiation of chondrocytes and tubulin expression, and also increased colchicine-reduced expression of type II collagen (Figure 2C). Staining results also support the mechanism of regulation of microtubule synthesis in chondrocyte differentiation (Figure 3D). These results suggest that colchicine treatment halts tubulin synthesis in

chondrocytes and thereby disrupts the chondrocyte differentiation process (Figure 3).

Expression of β -catenin and *I κ B α*

The experiments showed that colchicine inflicted dedifferentiation in the chondrocytes and increased the expression of COX-2 which made us explore the expression of β -catenin and *I κ B α* in the treated cells. The results indicated that in concentration dependent manner, colchicine ameliorated the expression of β -catenin via phosphorylation of GSK-3 α/β (Figure 4A). The mechanism of colchicine-induced inflammatory reaction was confirmed by treatment with BMS 345541, a highly selective IKK α inhibitor (Figure 6A). It was confirmed with western blot that BMS 345541 not only

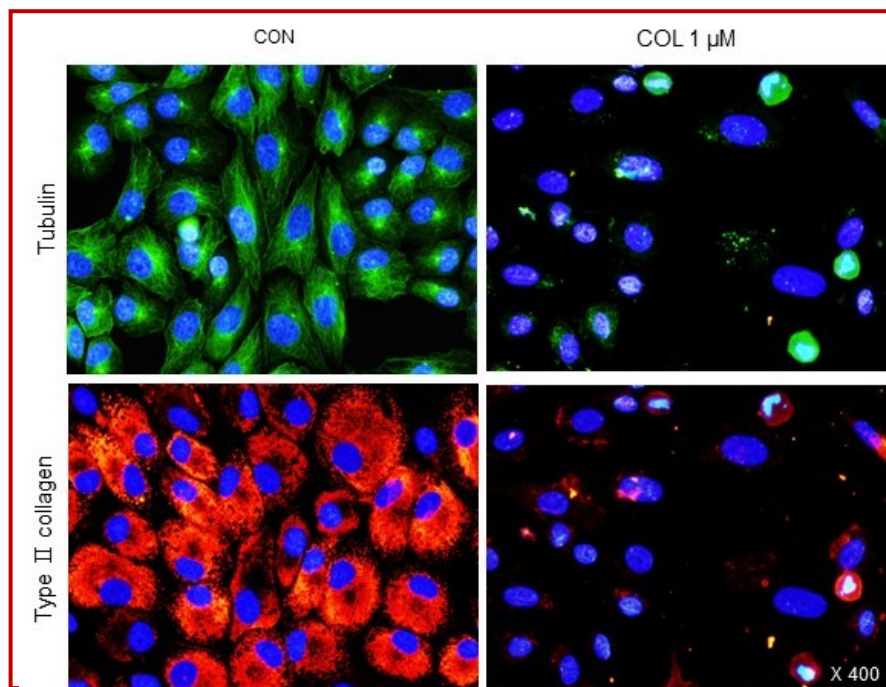


Figure 2: In rabbit articular chondrocytes, colchicine decreases tubulin production and chondrocyte differentiation. For 24 hours, chondrocytes were either left untreated or given 1 mM colchicine. Using immunofluorescence staining, it was possible to measure the expression of type II collagen and tubulin. Cells were labelled with a particular antibody (red or green) and DAPI for nuclear staining (blue). Magnification $\times 400$

significantly reduced the expression of COX-2 and pI κ B α , but also that COX-2 and pI κ B α increased by colchicine were significantly reduced by BMS 345541. The changes of pI κ B α , I κ B α and β -catenin identified by western blot suggest that the NF- κ B and β -catenin pathway involved to inflammatory and dedifferentiating effects via colchicine (Figure 4B).

β -Catenin pathway to induce chondrocyte dedifferentiation

To elaborately study the β -catenin pathway activated by colchicine, siR β -catenin and MG132 (β -catenin activator) were treated with chondrocytes and the results were confirmed with western blot. The reduction in the expression level of β -catenin was observed in the case of siR β -catenin treatment which led to differentiation cotreated with colchicine (Figure 5A). These results were also cross-confirmed with immunofluorescence staining (Figure 5B). Conversely, when the accumulation of β -catenin in chondrocytes was induced by treatment with MG132, it was confirmed that the chondrocytes were de-differentiated due to the decrease of type II collagen. Furthermore, it was confirmed that the simultaneous treatment of MG132 and colchicine further increased the accumulation of β -catenin in chondrocytes, thereby accelerating the de-differentiation of chondrocytes. Identical to western blot results, co-treatment of colchicine with siR β -catenin increased the differentiation of chondrocytes as compared to

colchicine treatment alone (Figure 5B). In addition, β -catenin, which is intensively distributed in the cell membrane, was greatly increased inside the cytoplasm by treatment with colchicine. However, treatment with siR β -catenin reduced the expression of β -catenin increased by colchicine and also restored the expression of type II collagen (Figure 5B). This suggests that colchicine treatment modulated β -catenin pathway to disrupt microtubule synthesis in chondrocytes and thereby inducing chondrocyte dedifferentiation (Figure 5).

Inflammation and differentiation

As described in Figure 3, to understand the mechanism of colchicine was used paclitaxel, which had the opposite mechanism of action than that of colchicine (Figure 6A). As expected, colchicine ameliorated the β -catenin expression by decreasing the phosphorylation of GSK-3 α β tyrosine and serine residues while paclitaxel increased the phosphorylation of GSK-3 α β tyrosine and serine residues. When these two were treated together, paclitaxel not only suppressed the expression of β -catenin but also augmented the phosphorylation of GSK-3 α β serine residues (Figure 5B). In addition, paclitaxel, which induces the polymerization of microtubules as opposed to colchicine, showed similar results as BMS 345541 treatment. The expression level of COX-2 and phosphorylation of I κ B α were decreased by paclitaxel (Figure 6B).

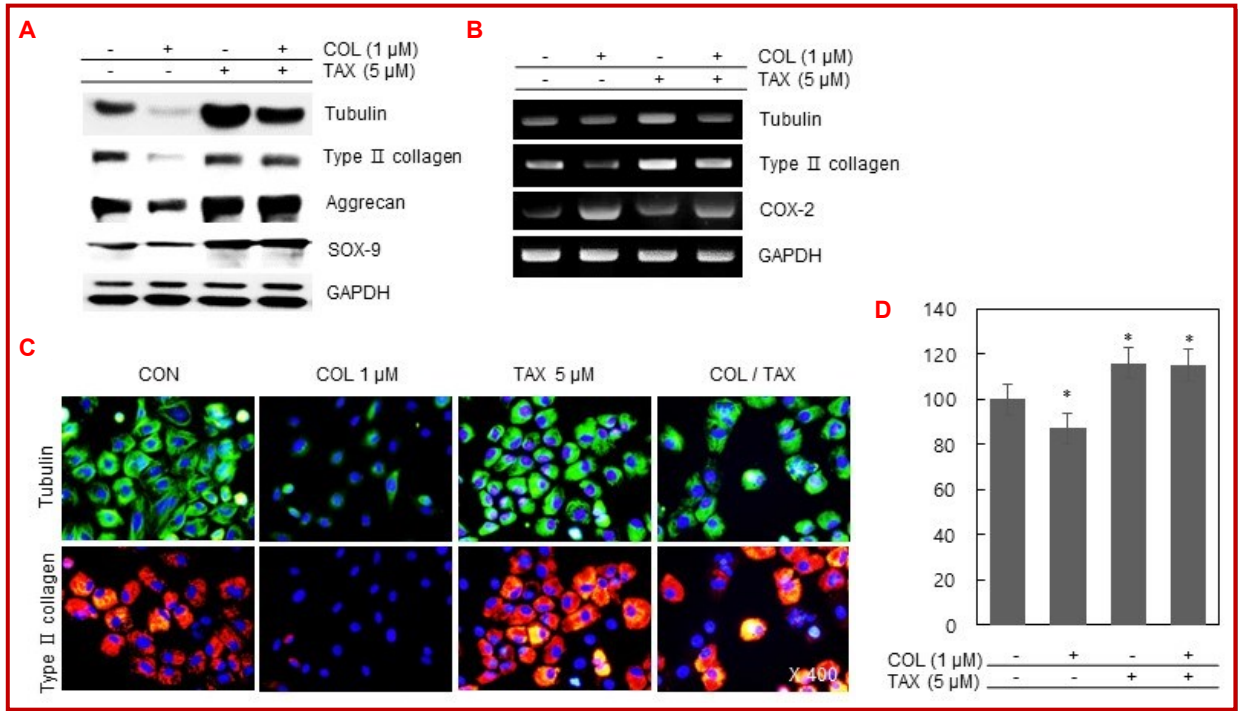


Figure 3: Regulation of microtubule synthesis controls the differentiation of chondrocytes and expression levels of COX-2

Chondrocytes were either left untreated or pre-treated with 5 M taxol for 2 hours before being exposed to 1 M colchicine for 24 hours (A, B, and C). (A) Immunofluorescence labeling was used to identify the expression of type II collagen and tubulin. Cells were labeled with a particular antibody (red or green) and DAPI for nuclear staining (blue). 400 times magnification; (B and C) Western blot analysis (B) was used to assess the expression of tubulin, type II collagen, aggrecan, SOX-9, COX-2, and GAPDH. Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to identify the expression of tubulin, type II collagen, COX-2, and GAPDH (C). We used GAPDH as a loading control. (D) Alcian blue staining was used to assess how sulphated proteoglycans were synthesized. The data are the average values, plus standard deviation, from at least four independent studies; *p < 0.05 in comparison to the control group. The data in (A, B, C, and D) indicate the outcomes of a typical experiment, whereas the data in (D) represent mean values with standard deviation. *p<0.05

Discussion

This study evaluated the specific role that microtubules may play in determining chondrocyte differentiation

using two well-studied microtubule reagents, colchicine, and paclitaxel.

The treatment of colchicine showed a dramatic dedifferentiation effect in chondrocytes in the present

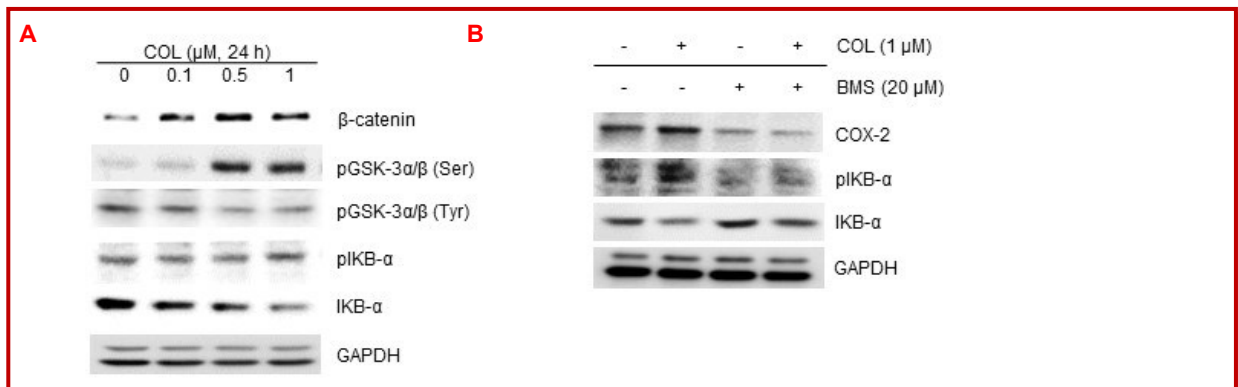


Figure 4: Colchicine cause regulating β-catenin expression via phosphorylation of GSK-3αβ and modulating NF-κB pathway. (A and B) Chondrocytes were either left untreated or subjected to a variety of colchicine treatments for 24 hours (A) or for the given amounts of time (1 μM colchicine) (B). Western blot analysis was used to determine the expression levels of β-catenin, pGSK-3αβ (Ser9/21), pGSK-3αβ (Tyr216/279), pIκBα, IκBα, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH was used as a loading control. The findings of an average experiment or the mean values plus standard deviation from at least four different experiments are represented by the data

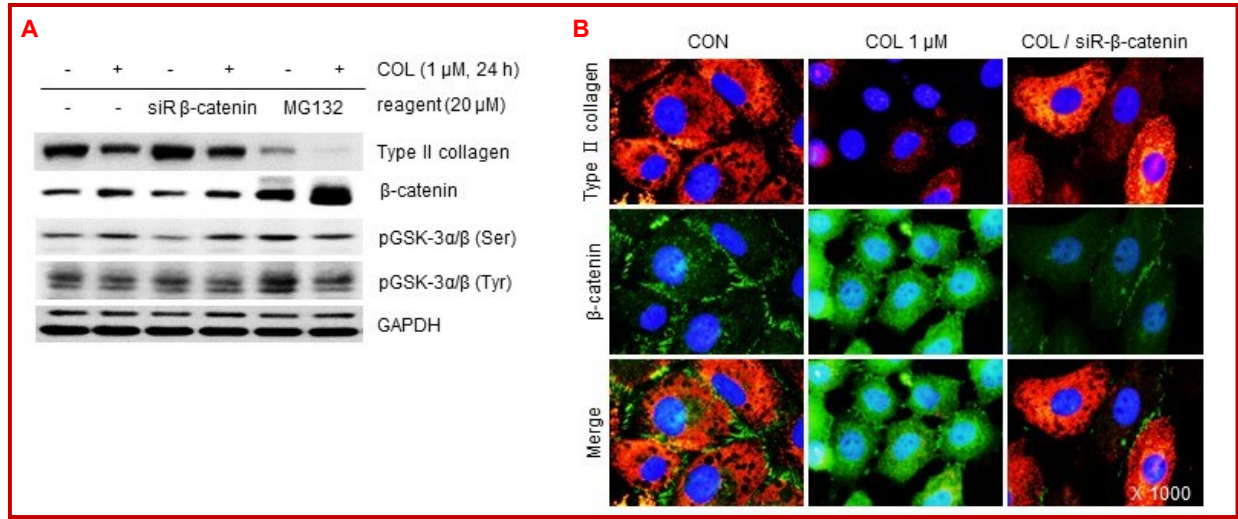


Figure 5: Colchicine causes chondrocyte de-differentiation via regulating the β -catenin pathway

(A) Chondrocytes were treated with 20 μ M MG132 for 12 hours before being exposed to 1 μ M colchicine for 24 hours, or they were transfected for 12 hours with either an empty vector or 0.1 μ M siR β -catenin. Western blot analysis was used to find the expression of type II collagen, β -catenin, pGSK-3 (Ser9/21), pGSK-3 (Tyr216/279), and GAPDH. GAPDH was used as a loading control. (B) Chondrocytes were subjected to 1 mM colchicine for 24 hours after being left untreated or treated for 3 hours with 5 mM TAX. Western blot analysis was used to find expression of pGSK-3 (Ser9/21), pGSK-3 (Tyr216/279), and GAPDH. We used GAPDH as a loading control. (C) Chondrocytes were transfected for 12 hours with either an empty vector or 0.1 μ M siR β -catenin before being subjected for 24 hours to 1 μ M colchicine. Using immunofluorescence labeling, it was possible to measure the expression of type II collagen and β catenin. Cells were labeled with a particular antibody (red or green) and DAPI for nuclear staining (blue). Magnification: x400

study. Microtubule depolymerization caused by KIF-18A activation can induce skeletal destruction of cells and inhibit chondrocyte proliferation (Sun et al., 2020). Those were the results of the inhibition of tubulin polymerization and microtubule depolymerization suggests that it inhibits the differentiation of chondrocytes.

Here, it was demonstrated how colchicine reduced chondrocyte differentiation by inducing β -catenin expression at both the protein and mRNA levels. Furthermore, colchicine's suppression of chondrocyte microtubule polymerization increased in β -catenin expression. The results of the current investigation suggested that colchicine controls the Wnt/ β -catenin pathway, which has been proven to regulate chondrocyte dedifferentiation. According to earlier research that supports other findings, simvastatin causes differen-

tiation via activating the Wnt/ β -catenin pathway (Yu et al., 2019).

It may be helpful for cartilage treatment to understand the mechanism of action of the inflammatory response, which is a significant contributor to cartilage breakdown in osteoarthritis. Additionally, COX-2, a crucial regulator of cartilage inflammation, was verified by recent investigations (Han and Kim, 2018; Yu et al., 2019). These findings suggest that controlling microtubule production and COX-2 expression in chondrocytes may be able to prevent cartilage degeneration.

Many cytokines and inflammatory response mediators produced by the activation of immune cells are one of the common pathogeneses of rheumatoid and degenerative arthritis (Shah and Kim, 2022). The activity of NF

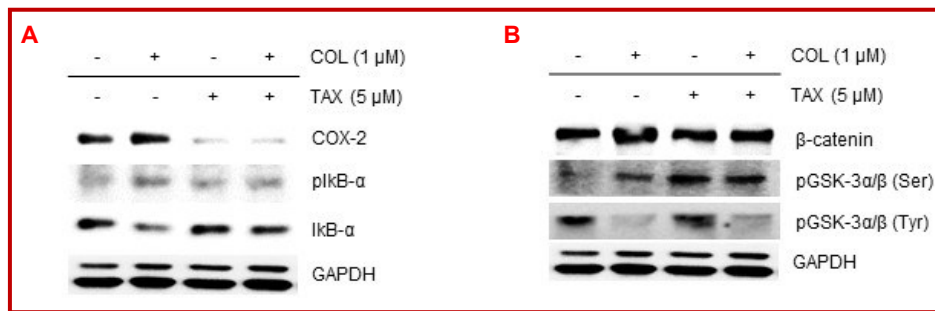


Figure 6: Colchicine causes inflammation via NF- κ B pathway in articular chondrocytes

Chondrocytes were either left untreated or given 20 μ M BMS for 3 hours before being subjected to 1 μ M colchicine for 24 hours (Figure A). Western blot analysis was used to find the expression of COX-2, pI κ B α , I κ B α and GAPDH. GAPDH was used as a loading control. (B) Chondrocytes were subjected to 1 mM colchicine for 24 hours after being left untreated or treated for 3 hours with 5 mM TAX. Western blot analysis was used to find the expression of COX-2, pI κ B α , I κ B α , and GAPDH

- κ B, which is phosphorylated by I κ B, has been linked to the etiology of numerous diseases (Zhang et al., 2021). Additionally, NF- κ B regulates inflammatory regulators like NO and ROS, which promotes the formation of matrix metalloproteases and inhibits the synthesis of extracellular matrix. According to several studies, NF- κ B can control how matrix metalloproteases are expressed in chondrocytes (Li et al., 2012; Ostojic et al., 2021). The NF- κ B pathway must therefore be modulated to cure arthritic conditions like RA and osteoarthritis. These findings further supported this proposed mechanism by showing that the NF- κ B pathway was significantly stimulated in colchicine-added cells compared to control cells.

These findings showed that colchicine increased levels of pro-inflammatory mediators, which may be extremely important for the management of osteoarthritis. It is proposed that the chondrocyte phenotype is modified during monolayer culture is modified by the tubulin polymerization status. The therapeutic ramifications of these data suggest that the key to curing osteoarthritis lies in comprehending how microtubule production alters the chondrocytes' phenotype. To validate this innovative idea, additional research on colchicine's effects on chondrocyte-related cell types and animal models is required.

Conclusion

The regulating of the Wnt/ β -catenin pathway is significant in the modulation of colchicine-reduced microtubule synthesis caused chondrocyte dedifferentiation.

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Ethical Issue

The ethical committee of Kongju National University approved this animal-based study (KNU_2022-04).

Conflict of Interest

Authors declare no conflict of interest

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Author Info

Song Ja Kim (Principal contact)

e-mail: ks85@kongju.ac.kr

First three authors contributed equally