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Original Article

Influence of regulatory intervention of Wnt/β-catenin signaling pathways on the activity of cultured and preserved articular cartilage tissues

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Abstract: Background: The activity of cultured and preserved arthrodial cartilage tissues is gradually weakened with prolonged preservation, thereby restricting the application and development of bone cartilage allotransplantation. Some studies have shown that Wnt/β-catenin signaling pathways are closely linked with proliferation, differentiation, migration, apoptosis, and maintaining the growth homeostasis of chondrocyte. Purposes: To explore the relationships of Wnt/β-catenin signaling pathways with the activity of cultured and preserved arthrodial cartilage tissues and locate the targets for blocking the apoptosis of the cultured and preserved arthrodial cartilage tissues. Moreover, this study provides experimental basis for improving the activity of cultured and preserved arthrodial cartilage tissues. Methods: Arthrodial cartilage tissues were taken from adult pigs and prepared into blocks of cartilages with a volume of approximately 0.5 mm^3 under sterile conditions. The investigated subjects were separated into three groups, namely, the control group (Group A) preserved in the basal culture medium, and Groups B and Group C, to which 10 μmol/L Kenpaullone (a pathway inhibitor) or 10 μmol/L SB-216763 (a pathway activator) were added, respectively. The apoptosis of cartilage tissues of these subjects was observed by using histology, immunohistochemistry, and molecular biology, among others. Results: Except for the subjects that did not exhibit significant changes on Day 1, the cell survival rate and the GSK3β protein content at the remaining points of time (Day 14, 21, 28, 35) were as follows: Group B achieved higher results than the other two groups, and Group C achieved the lowest results ($P < 0.05$). In terms of the apoptosis rate and the contents of apoptotic proteins Caspase-3 and β-catenin, Group C achieved higher results, and Group B obtained lower results; both groups exhibited significant differences from the control group ($P < 0.05$). Conclusions: The addition of 10 μmol/L Kenpaullone and 10 μmol/L SB-216763 in the process of culturing and preserving arthrodial cartilages inhibited and activated Wnt/β-catenin signaling pathways, respectively, with the apoptosis of chondrocytes alleviated and increased.

Keywords: β-catenin, apoptosis, arthrodial cartilages, caspase-3, GSK3β

Introduction

As a result of a lack of vessels and nerves in cartilage tissue, the nutrients of arthrodial cartilages come mainly from synovial fluid and synovial permeability. Restoration of arthrodial cartilages, which have limited abilities to repair themselves, has been always one of the hotspots and difficulties in the research field of sports medicine. The current treatment method is autotransplantation or allotransplantation of cartilage tissues for serious cartilage injury. However, autotransplantation of cartilage tissues may easily lead to secondary damages to patients. Therefore, as a reliable treatment, allotransplantation of arthrodial cartilage tissues is commonly used for articular cartilage injuries nowadays [1, 2]. However, given the graft source shortage and a short in vitro storage time, the development of this technique is limited to a large extent. Tissue culture provides a favorable environment for allogenic arthrodial cartilages [3, 4], but their long-term preservation is not ideal yet. Many studies have shown that Wnt/β-catenin signaling pathways are highly important to maintain the growth homeostasis of chondrocytes in the proliferation, differentiation, migration, and apoptosis [5, 6], because they can regulate the activity of chondrocytes and promote (or inhibit) differentiation of chondrocytes [7]. Moreover, such regulation is two-way, having both promoting and inhibiting actions [8, 9]. The present study is based on previous studies and explores the regulatory effect

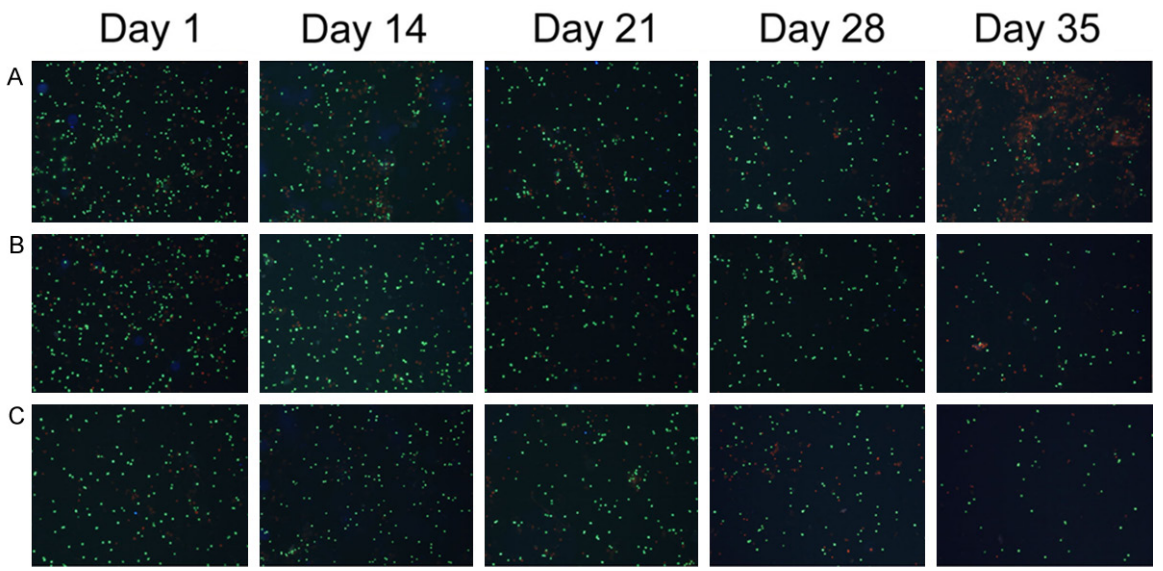

Figure 1. Cell survivals rate of the three groups at different points of time.

Table 1. Survival rate (%) of cells preserved on Days 1, 14, 21, 28, and 35 ($\bar{X}\pm S$, n=10)

Groups	Day 1	Day 14	Day 21	Day 28	Day 35
Group A	94.40±2.07	87.60±1.14	80.40±2.07	70.40±1.14	61.00±2.23
Group B	95.20±1.92	90.00±2.23	83.60±2.30	73.60±2.07	66.80±1.78
Group C	93.40±2.70	84.00±1.58	77.00±2.23	65.40±2.70	55.60±2.07
F	0.797	15.545	11.192	19.860	37.648
P	> 0.05	< 0.05	< 0.05	< 0.05	< 0.05

Note: The within-group F value of Group A is 213.724, $P < 0.05$; that of Group B is 130.542, $P < 0.05$; and that of Group C is 274.936, $P < 0.05$.

Table 2. Apoptosis rate (%) of cells preserved on Days 1, 14, 21, 28, and 35 ($\bar{X}\pm S$, n=10)

Groups	Day 1	Day 14	Day 21	Day 28	Day 35
Group A	6.04±1.09	12.75±0.40	21.90±0.57	30.44±0.79	40.70±0.43
Group B	5.68±0.22	11.18±0.48	16.55±2.33	27.54±0.59	35.40±0.65
Group C	7.93±0.42	16.70±0.37	23.52±0.75	36.36±0.68	45.38±0.48
F	48.76	134.66	21.12	124.92	265.18
P	> 0.05	< 0.05	< 0.05	< 0.05	< 0.05

Note: The within-group F value of Group A is 2095.92, $P < 0.05$; that of Group B is 338.05, $P < 0.05$; and that of Group C is 741.14, $P < 0.05$.

of Wnt/β-catenin signaling pathways on the activity of cultured and preserved arthrodial cartilage tissues.

Materials and methods

Materials

Ten knee joints were taken from 5 healthy adult male or female long-white pigs without joint lesions under sterile conditions. The experimental animals were sent to a sterile lab within two hours after bloodletting slaughter, and the experimental materials were collected from their knee articular cavities under sterile conditions. All the animals used in the experiment have been approved by the Ethics Committee for Animal Experimentation, Taishan Medical University.

Reagents and their origins: Trypsin-EDTA, Annexin V-FITC, rabbit anti-actin PcAb, and HRP-A-labeled goat anti-rabbit IgG were purchased from Beijing Solarbio Science & Technology Co. Ltd., KeyGEN BioTECH, ZSGB-BIO, and Beyotime BioTECH, respectively. Kenpaullone, SB-216763, GSK-3β, β-catenin, and Caspase-3 antibodies were imported from Santa Cruz, USA.

Experimental methods

The blocks of arthrodial cartilage tissues were rinsed and cut into small 0.5 mm^3 pieces under sterile conditions, with at least 100 pieces per knee joint. The cartilage tissue blocks of every knee joint were divided into three groups by the

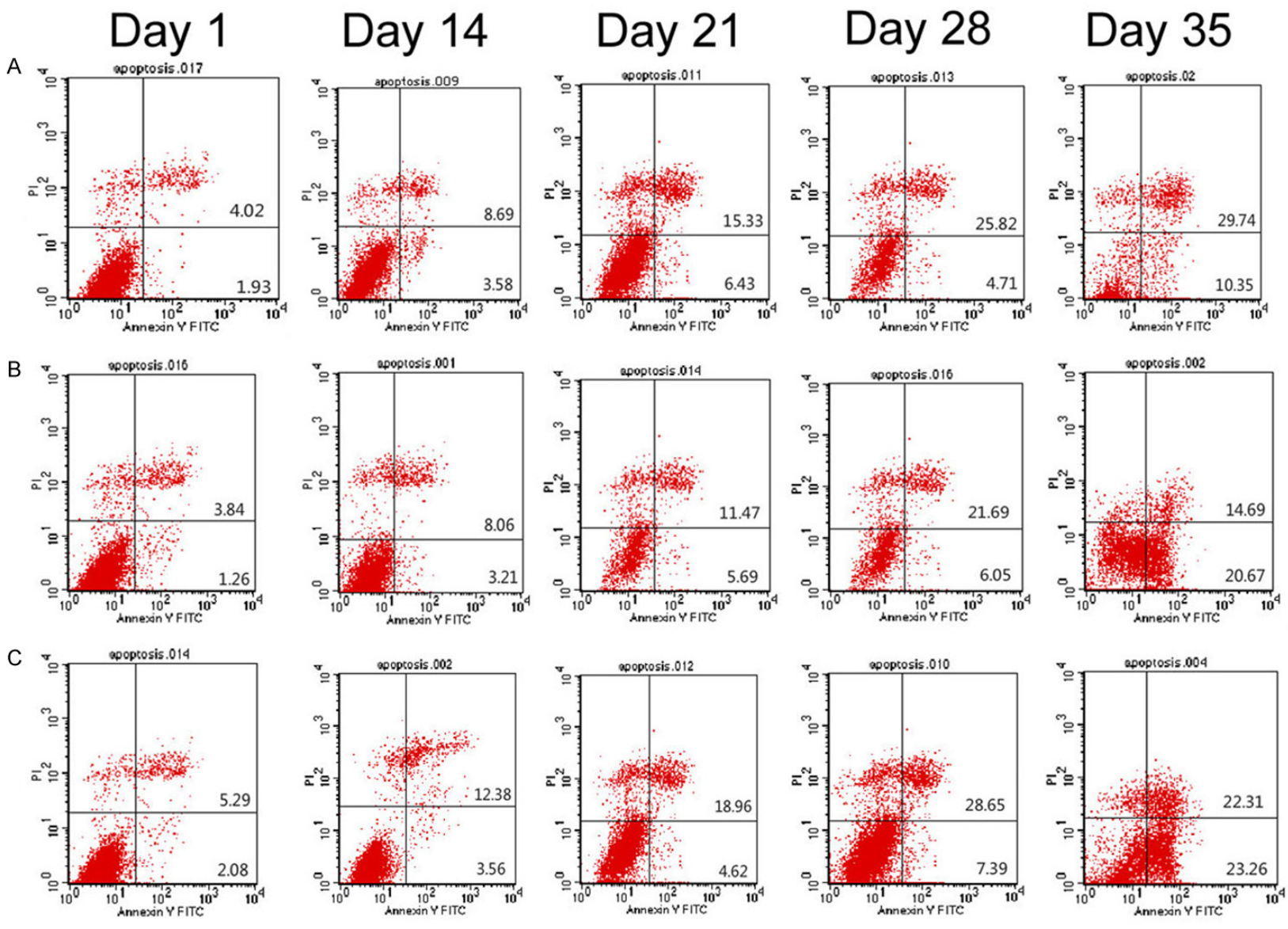

Figure 2. Chondrocyte apoptosis rate of the three groups measured by flow cytometer at different points of time. (Note: Dead and normal cells are located in the upper and lower left quadrants, respectively; late and early apoptotic cells are located in the top right and bottom right quadrants, respectively).

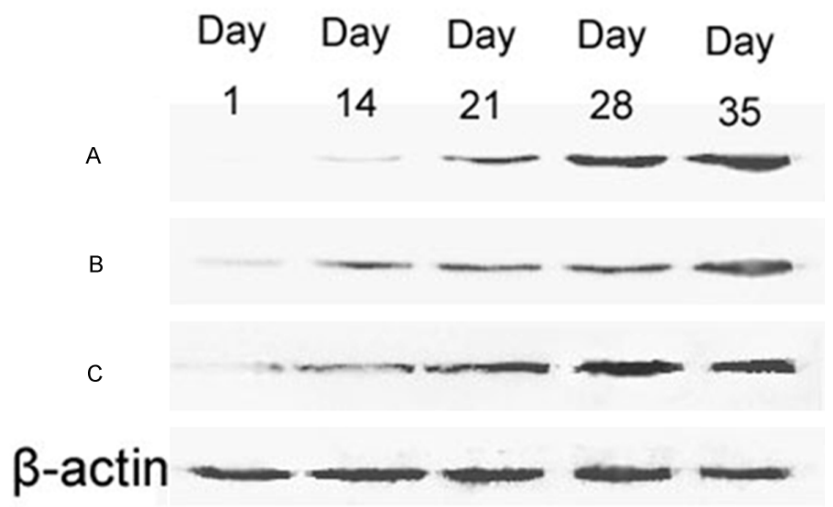

Figure 3. Expressions of Caspase-3 of the three groups measured by Western blot at different points of time (β-actin as the internal control).

Table 3. Caspase-3 contents of the three groups on Days 1, 14, 21, 28, and 35 ($\bar{X} \pm S$, n=10)

Groups	Day 1	Day 14	Day 21	Day 28	Day 35
Group A	0.078±0.032	0.193±0.026	0.327±0.041	0.510±0.023	0.719±0.063
Group B	0.051±0.036	0.138±0.032	0.238±0.039	0.320±0.036	0.507±0.019
Group C	0.106±0.009	0.232±0.029	0.379±0.047	0.600±0.023	0.845±0.029
F	3.391	20.565	23.400	75.704	53.651
P	0.103	< 0.05	< 0.05	< 0.05	< 0.05

Note: The within-group F value of Group A is 169.515, $P < 0.05$; that of Group B is 264.887, $P < 0.05$; and that of Group C is 568.783, $P < 0.05$.

culture liquid composition, with at least 30 blocks per group: (1) for Group A (the control group), arthrodial cartilage specimens were preserved in common sterile MEME culture liquid (pH value 7.2-7.4) that contains 100 u/ml penicillin-streptomycin; (2) for Group B (the treatment group with the inhibitor), 10 μmol/L Kenpaullone (a Wnt/β-catenin signaling pathway inhibitor) was added to the culture liquid; and (3) for Group C (the treatment group with the activator), 10 μmol/L SB-216763 (a Wnt/β-catenin signaling pathway activator) was added to the culture liquid. The three groups were all stored in 5% CO_2 incubators at 37°C, with the culture liquid changed once every other day. Six to ten blocks of arthrodial cartilage tissues were taken from every knee joint for testing on Days 1, 14, 21, 28, and 35.

Main outcome measures

Cell survival rate (EB/FDA staining): Two to three blocks of arthrodial cartilage tissues were taken from every knee joint at each point of time and immersed in 0.25% trypsase-EDTA bath at 37°C for 45 min after being cut into pieces, and in 0.2% II collagenase bath at 37°C for 4 h. After centrifuging, the blocks were immersed separately in EB (10 mg/l) and FDA (50 mg/l) baths at 37°C, and observed under a fluorescence microscope after a reaction in the dark for 20 min.

Apoptosis rate (Annexin V-PI double staining): After being cut into pieces, the blocks of arthrodial cartilage tissues were immersed in 0.25% trypsin-EDTA and 0.2% II collagenase bath to collect cells. The cells were resuspended in 100 μL of 1 × binding buffer, and 5 μL Annexin V-FITC and 5 μL PI staining solutions were added to the cell suspensions and mixed evenly by gentle shaking. Then, 400 μL of 1 × binding buffer was added after a reaction in the dark for 10 min. The specimens were tested by using a flow cytometer within 1 h.

Expressions of caspase-3 and GSK3β (western blot): The extracted tissue proteins were incubated separately in the secondary antibody after loading, electrophoresis, transmembrane, primary antibody incubation, and membrane cleaning. ECL coloration and X-ray photosensitive film development, fixing, flushing, drying, and photographing were performed.

Expressions of β-catenin and GSK3β (immunohistochemistry): The prepared paraffin sections were de-waxed and hydrated as routine, rinsed with PBS for 5 min, and sealed in 3% hydrogen peroxide for 5-10 min. Then, with the addition of a proper amount of goat serum, the sections were sealed at room temperature for 30 min. The sections were then rinsed with PBS after

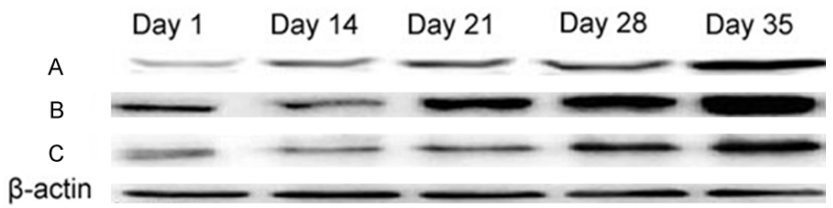

Figure 4. Expressions of GSK3β of the three groups measured by Western blot at different points of time (β-actin as the internal control).

Table 4. GSK3β contents of the three groups on Days 1, 14, 21, 28, and 35 ($\bar{X}$±S, n=10)

Groups	Day 1	Day 14	Day 21	Day 28	Day 35
Group A	0.162±0.025	0.242±0.019	0.493±0.012	0.717±0.020	0.905±0.011
Group B	0.171±0.024	0.311±0.013	0.558±0.013	0.874±0.078	1.041±0.072
Group C	0.145±0.023	0.198±0.022	0.425±0.027	0.616±0.019	0.778±0.043
F	0.332	27.338	55.075	21.778	21.208
P	0.730	< 0.05	< 0.05	< 0.05	< 0.05

Note: The within-group F value of Group A is 849.395, $P < 0.05$; that of Group B is 161.864, $P < 0.05$; and that of Group C is 263.841, $P < 0.05$.

they were stored overnight at 4°C with the addition of the primary antibody solution (β-catenin or GSK3β) and incubated for 30 min at room temperature with the secondary antibodies added separately. Finally, they were mounted with neutral gum after DBA coloration. In the negative control, the primary antibody was replaced with PBS. The specimens were observed under light microscope.

Statistical analysis

SPSS 19.0 was applied in statistical analysis of data obtained in the form of mean ± standard deviation ($\bar{x}$±s). Two-way or one-way ANOVA was adopted for between-group comparison, and one-way ANOVA was chosen for data analysis with a statistical difference ($P < 0.05$).

Results

Survival rate of chondrocytes

Five regions were chosen randomly from each cell smear for analysis by using the Olympus 6.0 image processing system. The survival rate of chondrocytes = (living cells/(living cells + dead cells)) × 100%. EB/FDA staining results are shown in **Figure 1** and **Table 1**.

The above data indicate that the survival rate of chondrocytes decreases with the extension of the culture and preservation, and has a significant statistical difference. The survival rate of Group B is maintained well, whereas that of Group C is maintained poorly.

Apoptosis rate of chondrocytes

A two-parameter dot plot created by using Annexin V-FITC and PI fluorescence can be divided into four regions: (1) mechanical damage cells (Annexin V-/PI+) on the upper left; (2) necrotic or late apoptotic cells (Annexin V+/PI+) on the upper right; (3) normal cells (Annexin V-/PI-) on the bottom left; and (4) early apoptotic cells (Annexin V+/PI-) on the bottom right. Calculating the cell populations of four regions obtains a sum of the cell populations on the top and bottom right equal to the population of apoptotic cells, and statistical analysis is performed (**Table 2**). The test results of the flow cytometer are shown in **Figure 2**.

The above data indicate that apoptosis rate of chondrocytes increases with the extension of the culture and preservation. The between-group comparison indicates that no significant changes were observed on Day 1, but significant statistical differences were found at the remaining points of time. Group B maintains its low apoptosis, and the apoptosis rate of Group C is higher than that of Groups A and B.

Expressions of apoptotic protein caspase-3 and GSK3β: western blot

The gray ratio of the target band to the β-Actin band as the internal control is taken as the final results of the protein expression.

Western blot results of Caspase-3 are shown in **Figure 3** and **Table 3**. The histogram shows that the apoptotic protein Caspase-3 content of chondrocytes increases with the extension of the culture and preservation, and the between-group comparison has significant statistical differences.

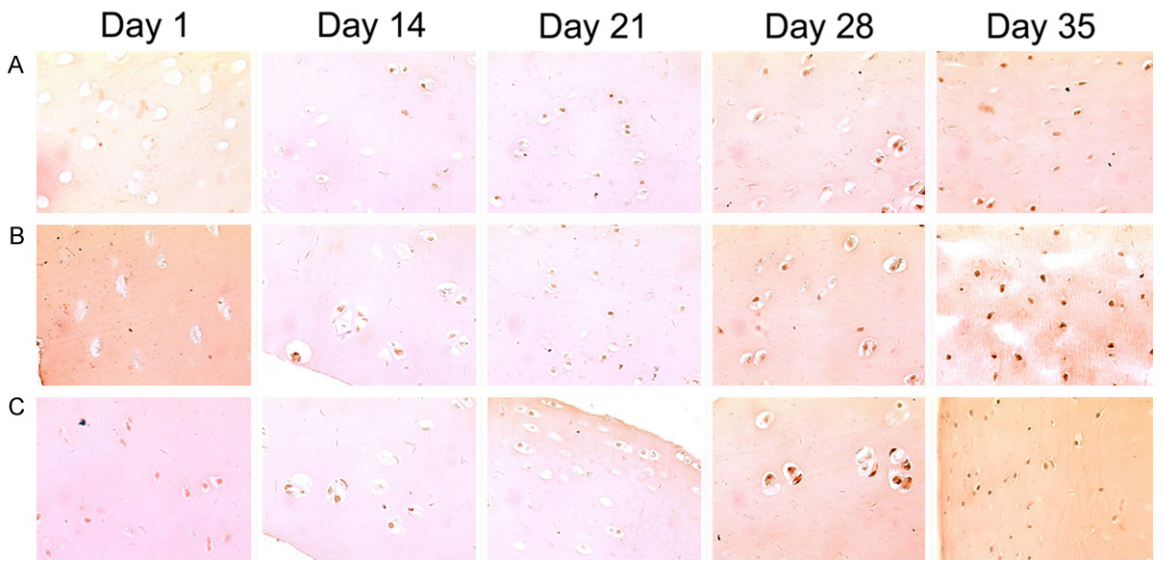

Figure 5. GSK3β expression of the three groups at different points of time.

Table 5. GSK3β contents of the three groups on Days 1, 14, 21, 28, and 35 ($\bar{X}$±S, n=10)

Groups	Day 1	Day 14	Day 21	Day 28	Day 35
Group A	171.60±4.21	149.20±6.61	124.80±4.86	114.20±6.09	91.80±9.62
Group B	174.00±7.58	131.00±6.96	115.80±7.85	103.20±8.52	78.80±6.76
Group C	173.60±5.68	158.60±2.40	133.20±3.27	123.40±4.61	110.40±7.36
F	0.230	30.137	11.819	11.694	25.261
P	0.798	< 0.05E	< 0.05	< 0.05	< 0.05

Note: The within-group F value of Group A is 111.419, $P < 0.05$; that of Group B is 109.943, $P < 0.05$; and that of Group C is 123.092, $P < 0.05$.

Western blot results of GSK3β are shown in **Figure 4** and **Table 4**. The above data and the histogram show that the GSK3β content of chondrocytes increases gradually on the whole and has significant statistical differences at different points of time. The GSK3β content of Group B is the highest, whereas that of Group C content is the lowest, and the between-group comparison indicates significant statistical significances.

Expressions of GSK3β and β-catenin: immunohistochemistry

Image-Pro Plus (IPP) 6.0 software is applied in static image grayscale analysis, with 5 regions chosen randomly from each tissue slice to measure the grayscale value. The final results are obtained by averaging the measured grayscale values at the 5 regions.

The immunohistochemical staining of GSK3β is shown in **Figure 5** and **Table 5**. The immunohistochemical expression of GSK3β is consistent with the performance of molecular biology.

The immunohistochemical staining of β-catenin is shown in **Figure 6** and **Table 6**. The above data indicate that the β-catenin content increases gradually with the extension of the culture and preservation, and has significant statistical significances among Groups A, B, and C. The β-catenin content in Group C is the highest, followed by Group A, and that of Group B is the lowest.

Data correlation analysis

The expressions of GSK3β and Caspase-3 are measured by using Western blot, while that of β-catenin is analyzed by the immunohistochemical grayscale value. **Table 7** shows that the survival rate of chondrocytes is negatively related to the apoptosis rate and to the expressions of Caspase-3, β-catenin, and GSK3β. The apoptosis rate is positively correlated with the protein contents of Caspase-3, β-catenin, and GSK3β. The content of apoptotic protein Caspase-3 is positively correlated with the expressions of β-catenin and GSK3β. The expression of β-catenin is positively correlated with the expression of GSK3β.

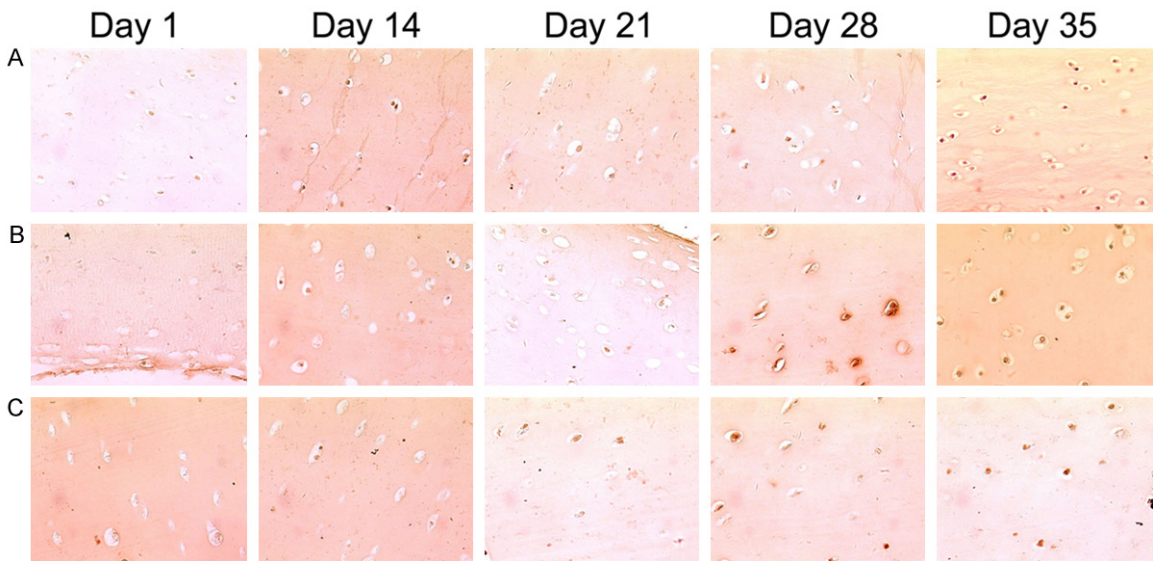

Figure 6. β-catenin expression of the three groups at different points of time.

Table 6. β-catenin contents of the three groups on Days 1, 14, 21, 28, and 35 ($\bar{X}$±S, n=10)

Groups	Day 1	Day 14	Day 21	Day 28	Day 35
Group A	172.20±6.18	147.40±8.79	133.60±6.58	125.00±4.18	100.00±5.52
Group B	169.60±4.61	157.20±5.07	144.40±6.26	135.40±7.23	110.80±9.44
Group C	167.80±11.5	137.60±4.93	124.80±5.11	113.00±8.48	87.80±5.26
F	0.382	11.317	13.287	13.292	13.475
P	0.691	< 0.05	< 0.05	< 0.05	< 0.05

Note: The within-group F value of Group A is 86.512, $P < 0.05$; that of Group B is 54.999, $P < 0.05$; and, that of Group C is 77.573, $P < 0.05$.

Table 7. Correlation analysis of different indicators (r value)

Variable	Cell survival rate	Apoptosis	Caspase-3	GSK3β	β-catenin
Cell survival rate	-	-0.999	-0.983	-0.870	0.979
Apoptosis rate	-0.999	-	0.981	0.875	-0.977
Caspase-3	-0.983	0.981	-	0.791	-0.973
GSK3β	-0.870	0.875	0.791	-	-0.808
β-catenin	0.979	-0.977	-0.973	-0.808	-

Note: The data correlation analysis has statistical differences between different indicators ($P < 0.05$).

Discussions

This study suggests that Wnt/β-catenin signaling pathways may be significant to the activity of cultured and preserved arthrodial cartilage tissues [10, 11]. The specific mechanism of action of Wnt/β-catenin signaling pathways is shown as follows: when Wnt signaling pathways are activated, Wnt may activate CKIε in cells by combining with Frizzled receptor. CKIε may lead to phosphorylation of Dsh, thereby releasing GBP. GBP combined with GSK-3β linked with Axin may inhibit the degradation of GSK-3β in the phosphorylation of β-catenin, thus causing the unphosphorylated β-catenin to accumulate excessively and transport to the cell nuclei. As a result, the compound protein formed by GRO, GBP, and LEF/TCF family is undermined, the transcription factor is activated, and the cell biological functions are regulated [12, 13]. Once Wnt pathways are not activated, Axin/APC/GSK-3β degradation complex is degraded by promoting the combination of the phosphate group and the serine/threonine residues at the N-terminal of β-catenin [14]. The present study is based on the mechanism of action. Kenpaullone (Wnt/β-catenin signaling pathway inhibitor) and SB-216763 (Wnt/β-catenin signaling pathway activator) are adopted for regulatory intervention of Wnt/β-catenin signaling pathways, and the expressions of β-catenin and GSK3β (key proteins) in Wnt/β-catenin signaling pathways are measured.

Combined with the indicators for the activity of the cultured and preserved arthrodial cartilage tissues, the pairwise correlation is analyzed to verify whether Wnt/β-catenin signaling pathways have regulatory intervention on the cultured and preserved arthrodial cartilage tissues. Experiment results show that after the Wnt/β-catenin signaling pathways are activated, the survival rate of chondrocytes increases compared with the control group, but the apoptosis decreases, and the apoptotic protein Caspase-3 content is reduced. The GSK3β content in the pathways is higher than that in the control group, while the β-catenin content is lower than that in the control group. Pure protein changes are not enough to interpret the relationships between the activity of arthrodial cartilages and Wnt/β-catenin signaling pathways. Therefore, correlation analysis in the changes of the above indicators is conducted. Survival rate and apoptosis rate are found to be highly negatively correlated with the Caspase-3 content and is also related to the GSK3β and β-catenin contents in the signaling pathways. On the whole, the GSK3β and β-catenin contents increase gradually with the extension of the preservation and have significant statistical differences. The GSK3β content of Group B, which has a high activity of arthrodial cartilage tissues, is the highest, whereas the β-catenin content is lowest. However, the GSK3β content of Group C, which has a low activity of arthrodial cartilage tissues, is the lowest, and its β-catenin content is the highest. An increase in the GSK3β content of Group B is related to the addition of the Wnt/β-catenin signaling pathway inhibitor, and a large amount of GSK3β is accumulated because it is not phosphorylated under the inhibitory actions on Wnt/β-catenin signaling pathways. However, β-catenin is phosphorylated under the actions of massive compounds of GSK3β, Axin, and APC. Thus, its content decreases, with less entering the nuclei and combining with downstream genes that regulate the apoptosis, thereby maintaining the activity of the cultured and preserved arthrodial cartilage tissues. For Group C, which has activated Wnt/β-catenin signaling pathways, β-catenin increases because it is not phosphorylated or degraded, with more entering the cell nuclei and combining with downstream genes that regulate the apoptosis. Thus, the activity of arthrodial cartilage tissues decreases greatly, but the apoptosis of chondrocytes increases. The results are consistent with the referenced literature, that is to say, activating Wnt/β-catenin signaling pathways may denature the chondrocytes and lead to an increase in the β-catenin content [15]. It is conducive to maintaining the activity of arthrodial cartilages by appropriately inhibiting the expression of Wnt/β-catenin signaling pathways [16].

This study, which is based on previous in vivo tests on Wnt/β-catenin signaling pathways, analyzes the changes in the expression of Wnt/β-catenin signaling pathways through in vitro culture and preservation of arthrodial cartilage tissues and through regulating Wnt/β-catenin signaling pathways. The survival rate of the cultured and preserved arthrodial cartilage tissues is improved, and the apoptosis rate is reduced by regulating the Wnt/β-catenin signaling pathways. This condition is the crux of the present experiment; few reports have focused on this subject. In future studies, in vivo tests may be conducted to further validate the regulatory interference, thereby forming a complete theoretical system and providing improved support for scientific and clinical studies.

Acknowledgements

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Disclosure of conflict of interest

None.

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