Selection and Investigation of a Primate Model of Spontaneous Degenerative Knee Osteoarthritis, the Cynomolgus Monkey (*Macaca Fascicularis*)

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The aim of this study was to identify a primate model of degenerative knee osteoarthritis (KOA) that may be more relevant for research studies on degenerative KOA in humans.

Material/Methods: Sixteen specific-pathogen-free (SPF) male cynomolgus monkeys (*Macaca fascicularis*) were divided into group A (n=8), an old group (22.0–25.3 years of age), and group B (n=8), a young group (3.0–5.2 years of age). For each primate, the behavior was observed, knee circumference was measured, knee joint X-rays were performed, and peripheral blood white blood cell (WBC) counts were measured, and the Kellgren and Lawrence (K-L) system was used for the classification of osteoarthritis. An enzyme-linked immunoassay (ELISA) was performed on knee joint fluid to measure levels of interleukin (IL)-1β, transforming growth factor (TGF)-β1, and matrix metalloproteinase (MMP)13. Changes in articular cartilage were evaluated using the Brittberg score and the Mankin histopathology grading score, respectively. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) and Western blot were used to measure the expression of the *NOTCH3*, *JAG1*, and *ACAN* genes in knee cartilage specimens, and the findings in the two groups of primates were compared.

Results: Seven old aged primates in group A were compared with group B, and showed significant differences in WBC count, synovial fluid IL-1β, TGF-β1, and MMP13 levels, expression levels of the *NOTCH3*, *JAG1*, and *ACAN* genes in knee cartilage specimens, and in the Brittberg and Mankin scores (all, P<0.05).

Conclusions: Cynomolgus monkeys (*Macaca fascicularis*) might be a model for age-related degenerative KOA.

MeSH Keywords: Cartilage, Articular • *Macaca Fascicularis* • Osteoarthritis • Receptors, Notch

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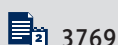
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Background

Worldwide, osteoarthritis (OA), including degenerative knee osteoarthritis (KOA), is a chronic progressive joint disease, which involves inflammation, pain, and degenerative structural changes in the joint, resulting in functional disability [1,2]. Osteoarthritis commonly affects the weight-bearing joints, particularly the knee [3,4]. In the US, by the year 2040, the prevalence of diagnosis of osteoarthritis is expected to rise to 78.4 million, or 25.9% of all adults [5]. The 2010 Global Burden of Disease study reported that the global age-standardized prevalence of KOA was 3.8% and hip OA was 0.85%, without any obvious change between 1990–2010 [6,7]. Degenerative OA, including degenerative KOA, is a serious global problem affecting the quality of life, and continued research is needed, both in clinical studies and in animal models.

Choosing an animal model for degenerative KOA is very important, to allow for basic research studies to be undertaken. Currently, OA research models can be classified into two major groups, the experimentally-induced models, and the spontaneous disease models. However, there are many problems in deciding which animal model may be most appropriate for studies on degenerative KOA. In recent decades, research studies have used animals such as mice, New Zealand white rabbits, sheep, pigs, and canines, which do not show the same patterns of disease as humans, particularly chronic degenerative disease [8–11]. Also, previous animal model studies have used trauma or injury models, including for models of KOA [12,13]. However, in human, OA is characterized as a long-term or chronic degenerative joint disease of the elderly [14].

The aim of this study was to evaluate a potential animal model for degenerative KOA, the cynomolgus monkey (*Macaca fascicularis*), because of its long life-span and because primates develop spontaneous degenerative diseases, including OA, which could be evaluated by simple scoring systems used in human [15,16]. Also, in this animal model, molecular changes were evaluated, including the Notch signaling pathway, and were compared between young and old-aged cynomolgus monkeys (*Macaca fascicularis*) [17,18]. This preliminary study was undertaken to determine the relevance of this animal model and to evaluate to changes associated with KOA, before planning further and more detailed studies.

Material and Methods

Ethical statement

This study was approved by the Experimental Animal Ethics Committee (No. 2016001) of Yunnan Yingmao Biotechnology Co. Ltd. (SYXK2009-0003) and also followed the Guidelines from Science and Health Reports CPRR/NIH 1996.

Animals: specific-pathogen-free (SPF) male cynomolgus monkeys (*Macaca fascicularis*)

Sixteen specific-pathogen-free (SPF) male cynomolgus monkeys (*Macaca fascicularis*) were divided into group A (n=8), an old-aged group (22.0–25.3 years of age; weight for 5.2–7.1 kg), and group B (n=8), a young group (3.0–5.2 years of age; weight for 5.5–6.9 kg). Osteoarthritis in cynomolgus monkeys (*Macaca fascicularis*) develops from the age of 5 to 15 years, which is an accelerated process, compared with humans [19–21]. This study was assisted by The Association for Assessment and Accreditation of Laboratory Animal Care International (AAALACI). Because the cynomolgus monkey (*Macaca fascicularis*) is naturally a forest-dweller, in this study, they were housed in several stable cages during periods of activity, feeding, resting, and sleeping.

Basic-line measurements

At the beginning of the study, the age, weight, crown, and left or right knee circumference were measured. Peripheral blood white blood cell (WBC) counts were measured.

X-ray investigations of the knee joints of the cynomolgus monkeys (*Macaca fascicularis*)

The knee joint was randomly selected for X-ray, but the young cynomolgus monkeys (*Macaca fascicularis*) were difficult to keep still enough for this procedure, and some required sedation with intravenous (IV) diazepam (10 mg) to perform the test. The monkeys were identified by having their group (A or B) and their number (1 to 8) written on their left arm. Kellgren and Lawrence (K-L) system were used for the classification of osteoarthritis [22].

Enzyme-linked immunosorbent assay (ELISA)

The fluid was aspirated from the knee joint of all monkeys, using a 5 ml syringe and samples were tested using an ELISA kit (SignaGen, USA) specific for each chemokine (R&D Systems) according to the manufacturer's protocol. All 16 fluid samples were centrifuged for 15 min at 1500 rpm. ELISA was performed on knee joint fluid to measure levels of interleukin (IL)-1β, transforming growth factor (TGF)-β1, and matrix metalloproteinase (MMP)13 and the results were read at 450nm with 2010 enzyme marker (Anthos2010, Austria) and the findings were compared with a standard curve to read the IL-1β, TGF-β1, and MMP13 results in the sample.

Morphological examination of the knee joints in group A and group B cynomolgus monkeys (*Macaca fascicularis*)

Intravenous anesthesia with 5 mg/kg intramuscular (i.m.) Zoletil 50 (Virbac, Carros, France) was given to the 16 cynomolgus monkeys (*Macaca fascicularis*). The monkeys were

placed in the supine position, and the sites of the surgical areas were shaved. Incisions over the knee joint, measuring between 4–6 cm, were made to remove the knee joint. The articular cartilage Brittberg score [23] was used to measure both young and old-aged cynomolgus monkeys (*Macaca fascicularis*) and articular cartilage were cut into pieces from the excised knee joints. Specimens were stored in frozen 1.4% paraformaldehyde (Biomics Laibo, Beijing, China). Surgical incisions were closed with sutures (ZSGB-BIO). An aseptic-bandage was wrapped around the wound for one week or more and the monkeys were given time to recover. On the third postoperative day, levofloxacin hydrochloride and sodium chloride (8 mg/kg intravenously) (Heng Ao, Zhejiang, China) were given once every 12 hours, to prevent infection. The cynomolgus monkeys (*Macaca fascicularis*) were monitored daily and tramadol hydrochloride i.m. was injected (2 mg/kg, once/day) (QiMaiTe, China) to relieve pain. The incisions healed between by between 7–10 days (mean, 8.5 days).

Histology and histochemistry (including Masson staining)

Knee joint bone and cartilage specimens were decalcified with EDTA (Merck, Germany) embedded in paraffin wax (KUNHUA, China), and cut into 5 µm sections onto glass slides. Sealed with neutral gum after staining with Masson histochemical stain. Light microscopy (Nikon, Japan) was used to observe the specimen according to the manufacturer's protocol. The Mankin histological score was also used to diagnose and score the knee osteoarthritis (KOA) [24].

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

RNA extraction and identification were performed on 50 mg samples of cartilage to which were added 1 ml TRIZOL, and the samples were homogenized on the ice following the manufacturer's protocol of the total RNA Extraction Kit (SignaGen, USA). Detection of the extracted RNA (A260 nm/A280 nm) (between 1.8–2.0 nm) using the Nucleic Acid Protein Detector (ScanDrop, Jena, Germany). The remaining extracted RNA was stored at –80°C. Premier 5.0 software (Shanghai, China) was employed to design the primers:
R-β-actin-F: 5'-GATCAAGATCATTGCTCCTCCTG-3', 58.93.
R-β-actin-R: 5'-GTCACAGTCCGCCTAGAAGC-3', 60.46; 163 bp.
R-*NOTCH*-3-F: 5'-GAT CAA GAT CAT TGC TCC TCC TG-3',59.3.
R-*NOTCH*-3-R: 5'-GGTGTGTTTCGTGCAGTCAT-3', 59.97; 180 bp.
R-*JAG*-1-F: 5'-TAA CAT AGC CCG AAA CAG TAG C-3', 58.21.
R-*JAG*-1-R: 5'-ACC GGT ACC AGT TGT CTC CA-3', 60.47; 197 bp.
R- *ACAN* –F: 5'-ACTGGCGAGCACTGTAACAT-3', 59.68.
R- *ACAN* –R: 5'-AGTCTTGGGCATTGTTGTTGAC-3', 59.64; 179 bp.

For the reverse transcription reaction, 1 µg RNA was used with the cDNA synthesis kit (SignaGen, USA) for the total

extraction RNA reversed transcription to generate cDNA, as follows: PrimeScript RT Master Mix, 2 µl; sample corresponding to RNA, 500 ng; double-distilled (dd)H$_2$O, 10 µl. The reaction conditions were 37°C for 15 min and 85°C for 5 seconds.

A fluorescent marker was used to test each gene following RT-qPCR, SYBR Green (QIAGEN, Germany). β-actin was used for internal control with 50 cycle times, and the following conditions: SYBR Premix Ex TaqII, 10 µl; Primer F, 0.3 µl; Primer R, 0.3 µl; cDNA, 1 µl; ddH$_2$O, 8.4 µl. The reaction conditions were as follows: 95°C, for 35 secs; 95°C for 5 secs; and 62°C for 35 secs. The samples were then amplified with RT-qPCR (Thermo, Arktik, USA) in triplicate, with the relative quantification of $2^{-\Delta\Delta Ct}$ to analyze the results.

Western blot

Western blot was used with the Total Protein Extraction Kit (Besebio, Shanghai, China) to extract proteins and it was tested using the Thermo Scientific Pierce BCA Protein Assay Kit, a separating gel and stacking gel, following of manufacturer's protocol. A constant volt (80 V stacking gel, 200 V separating gel) was used to test the samples and included staining with bromophenol blue (BPB). When separation of the gel was complete, Western transfer buffer was used with an ultra-thick filter paper and polyvinylidene difluoride (PVDF) membrane for 3 mins. The PVDF membrane was covered in transfer buffer at 200 mA for 2 hours, and the PVDF was separated and moved to a plate containing tris-buffered saline with Tween 20 (TBST) solution. The PVDF membrane was mixed with buffer, which included an equal volume of the substrate for the enhanced chemiluminescence (ECL) kit for 5 min.

Statistical analysis

Statistical analysis was performed by the SPSS version 19.0 statistical software package (IBM). The data were expressed as the mean ± standard deviation (SD) analyzed by a paired test, and one-way analysis of variance (ANOVA). A statistical P-value of $P<0.05$ was viewed as a statistically significant difference, and the test level was α=0.05.

Results

Base-line findings in old-age and young cynomolgus monkeys (*Macaca fascicularis*) with and without knee osteoarthritis (KOA) (group A and group B)

Sixteen male cynomolgus monkeys (*Macaca fascicularis*) were divided into group A (n=8), an old-age group (22–25.3 years of age), and group B (n=8), a young group (3–5.2 years of age). There were statistically significant differences between group

Table 1. The basic line comparison of the groups A and B ($\bar{\chi}\pm$s).

Groups	Age	Weight (kg)	Knee circumference (cm)	Crown (cm)
Group A	24.13±0.24	6.43±0.19	15.44±0.17	47.12±2.47
Group B	4.16±0.25[a]	6.36±0.24[b]	10.80±0.15[c]	41.62±1.92[d]
t	58.65	0.54	20.59	4.43
p	0.000	0.600	0.000	0.003

[a] P<0.05 *vs.* Group A; [b] P>0.05 *vs.* Group A; [c] P<0.05 *vs.* Group A; [d] P<0.05 *vs.* Group A.

A and group B in age, knee circumference, and crown measurement (P<0.05). The crown measurement was an important sign in old-aged cynomolgus monkeys (*Macaca fascicularis*), but there was no significant difference in their weight (kg) (P>0.05) (Table 1).

X-Ray results of knee joint analysis in old-age and young cynomolgus monkeys (*Macaca fascicularis*) with and without knee osteoarthritis (KOA) (group A and group B)

There were 7 old-aged cynomolgus monkeys (*Macaca fascicularis*) with a reduced knee joint space and other changes of KOA, as shown in Figure 1A 1B, and 8 young monkeys with a normal knee joint space, as shown in Figure 1C. However, one (number 7) old-aged monkey showed changes as in Figure 1C. The Kellgren and Lawrence (K-L) grades for KOA showed 4 cases (4/8) of K-L grade 2, 2 cases (2/8) of K-L grade 3, and 1 case (1/8) of K-L grade 4.

Enzyme-linked immunosorbent assay (ELISA) of synovial fluid in old-age and young cynomolgus monkeys (*Macaca fascicularis*) with and without knee osteoarthritis (KOA) (group A and group B)

There were statistical differences in white blood cell (WBC) count, interleukin (IL)-1β, transforming growth factor (TGF)-β1, and matrix metalloproteinase (MMP)13 between group A and group B (P<0.05). However, in group A, monkey number 7 showed a level of 0.99 ng/ml for IL-1β, 2.41 ng/ml for TGF-β1, and 0.36 ng/ml for MMP13,which was similar for group B (Table 2, Figure 2).

Morphological findings in old-age and young cynomolgus monkeys (*Macaca fascicularis*) with and without knee osteoarthritis (KOA) (group A and group B)

Comparison of the Brittberg scores of group A and group B showed a significant difference (P<0.05) (Table 2) (Figures 3, 4D) but monkeys number 1 and 7 in group A had similar scores, indicating that the two old-aged monkeys might have had less osteoarthritic knee joints.

Histology and histochemistry (including Masson staining) in old-age and young cynomolgus monkeys (*Macaca fascicularis*) with and without knee osteoarthritis (KOA) (group A and group B)

The knee joint cartilage of cynomolgus monkeys (*Macaca fascicularis*) in both group A and B were histologically evaluated by light microscopy using Masson staining (Figure 5). The Mankin scores are shown in (Table 3). In group A (old-aged), 7 monkeys had severely osteoarthritic damage (Figure 5A, 5B). The reduced thickness of the cartilaginous bone joint surface, the transition layer, the radiative layer, and calcification were damaged, and the 'tidal zone' was indistinct, and there was few or no cartilage cells on the joint surface. In group B (young), the cartilaginous joint surface, transition layer, radiative layer and calcification were normal. There were many cartilage cells on the joint surface which were regularly arranged (Figure 5C). The surface of knee joint bone cartilage was smooth with a clear 'tidal line.' However, in group A (old-aged), there was one monkey (number 7) with similar changes to the young monkeys in group B (Figure 5D).

Expression of *NOTCH3*, *JAG1*, and *ACAN* mRNA in the knee cartilage in old-age and young cynomolgus monkeys (*Macaca fascicularis*) with and without knee osteoarthritis (KOA) (group A and group B)

This study used quantitative reverse transcription polymerase chain reaction (RT-qPCR) to examine the expression of the *NOTCH3*, *JAG1*, and *ACAN* genes in knee cartilage specimens. There was a significant increase in expression of these genes in the monkeys in group A compared with group B (P<0.05) (Figure 4A–4C). However, monkey number 7 in group A showed similar results with group B.

Western blot for *NOTCH3*, *JAG 1*, and *ACAN* in old-age and young cynomolgus monkeys (*Macaca fascicularis*) with and without knee osteoarthritis (KOA) (group A and group B)

Western-blot analysis was used to examine the expression of the *NOTCH3*, *JAG1*, and *ACAN* genes by measuring the optical density (OD) value, and β-actin was used as a reference with OD

Figure 1. Knee joint X-ray findings in old-age and young cynomolgus monkeys (*Macaca fascicularis*) with and without knee osteoarthritis (KOA) (group A and group B). (**A**) Anterior-posterior (AP) X-ray view of the knee joint of an old-aged cynomolgus monkey (*Macaca fascicularis*), shows a reduced joint space and osteophytes in the joint space, consistent with knee osteoarthritis (KOA). (**B**) Lateral X-ray view the knee joint of an old cynomolgus monkey (*Macaca fascicularis*). (**C**) Normal anteroposterior (AP) X-ray view of a young cynomolgus monkey (*Macaca fascicularis*). (**D**) Lateral X-ray view of the knee joint of a young cynomolgus monkey (*Macaca fascicularis*).

Table 2. The comparison of Brittberg score between group A and group B ($\overline{\chi}\pm s$).

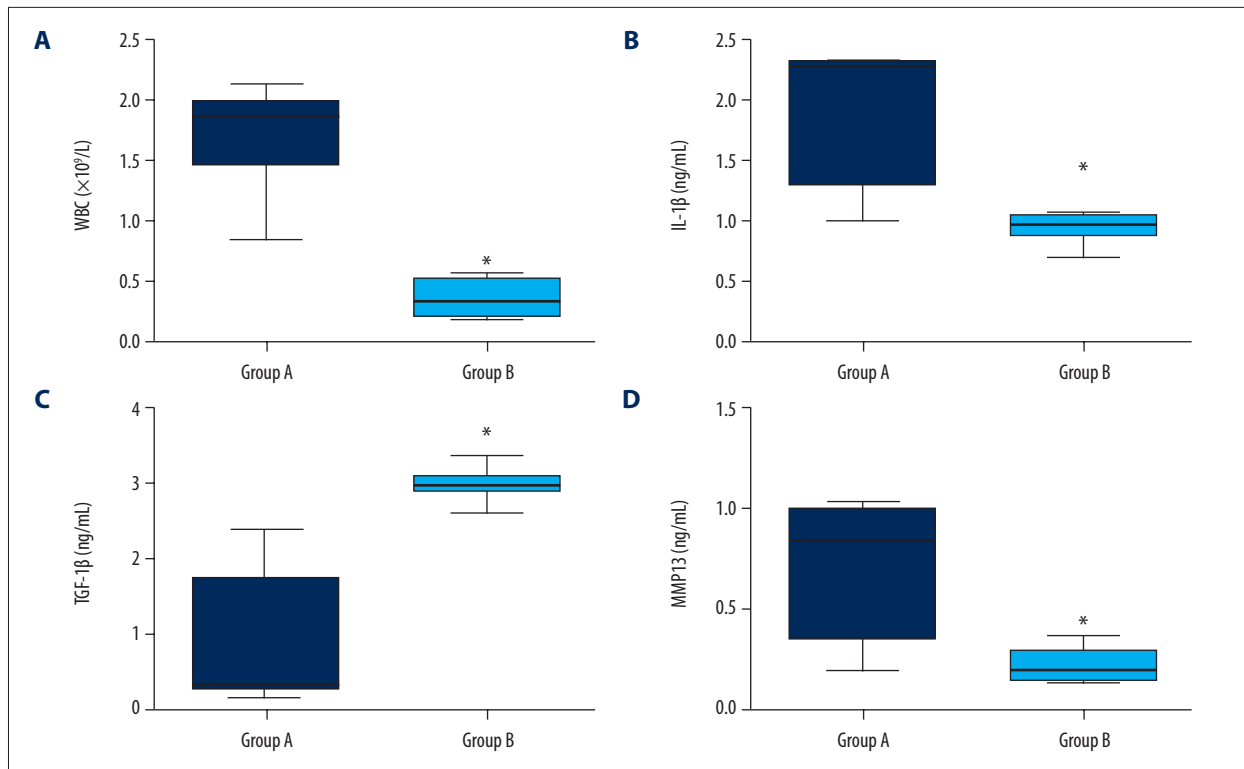| Groups	0	1	2	3	4	t	p
Group A	0	n=2	n=4	n=2	0	8.89	0.001
Group B	n=5	n=3	0	0	0		

Figure 2. Enzyme-linked immunosorbent assay (ELISA) of the synovial fluid from old-age and young cynomolgus monkeys (*Macaca fascicularis*) with and without knee osteoarthritis (KOA) (group A and group B). (**A**) Differences in white blood cell (WBC) count (×10⁹/L). (**B**) Differences in interleukin (IL)-1β (ng/ml). (**C**) Differences in transforming growth factor (TGF)-β1 (ng/ml). (**D**) Differences in matrix metalloproteinase (MMP)13 (ng/ml). * P<0.01 *vs.* A group.

analysis software used to analyze the membrane protein band (OD protein/OD β-actin ratio). There were statistical differences between group A and group B (P<0.05) (Figures 6, 7). However, monkey number 7 in group A showed similar results with group B.

Discussion

The aim of this study was to identify a primate model of degenerative knee osteoarthritis (KOA) that may be more relevant for research studies on degenerative KOA in humans. For this study cynomolgus monkeys (*Macaca fascicularis*) were divided into group A (n=8), an old-aged group (22–25.3 years of age), and group B (n=8), a young group (3–5.2 years of age). The findings of this preliminary study supported a role for the use of cynomolgus monkeys (*Macaca fascicularis*) as a model for age-related degenerative KOA.

There were statistically significant differences in old-aged cynomolgus monkeys (*Macaca fascicularis*) for X-ray findings, peripheral blood white blood cell (WBC) counts enzyme-linked immunoassay (ELISA) on knee joint fluid levels of interleukin (IL)-1β, transforming growth factor (TGF)-β1, and matrix metalloproteinase (MMP). Quantitative reverse transcription polymerase

chain reaction (RT-qPCR) and Western blot confirmed significantly increased expression of the *NOTCH3*, *JAG1*, and *ACAN* genes in knee cartilage specimens in group A, old-age cynomolgus monkeys (*Macaca fascicularis*), These findings support the possible role for older cynomolgus monkeys (*Macaca fascicularis*) as an animal model for KOA.

A limitation of this animal model for the study of KOA might be that the cynomolgus monkey (*Macaca fascicularis*) walks with on all fours, but rarely upright, which is different from human beings. In humans, the population who are at most risk of KOA are >60 years old [25]. This age group is similar to, but not the same as, the cynomolgus monkey (*Macaca fascicularis*) used in group A of this study. However, previously published research has shown that wild cynomolgus monkeys (*Macaca fascicularis*) at 12 years and 13 years-of-age were considered to be old-aged [26]. However, other published studies have indicated that osteoarthritis in these monkeys can develop from the age of between 5 years to 15 years [19–21]. Therefore, in this study, the choice of cynomolgus monkeys (*Macaca fascicularis*) aged from 22 years to 25.3 years, with a weight of between 5.2 kg to 7.1 kg, even though they were raised in a laboratory, might still have represented a model for human
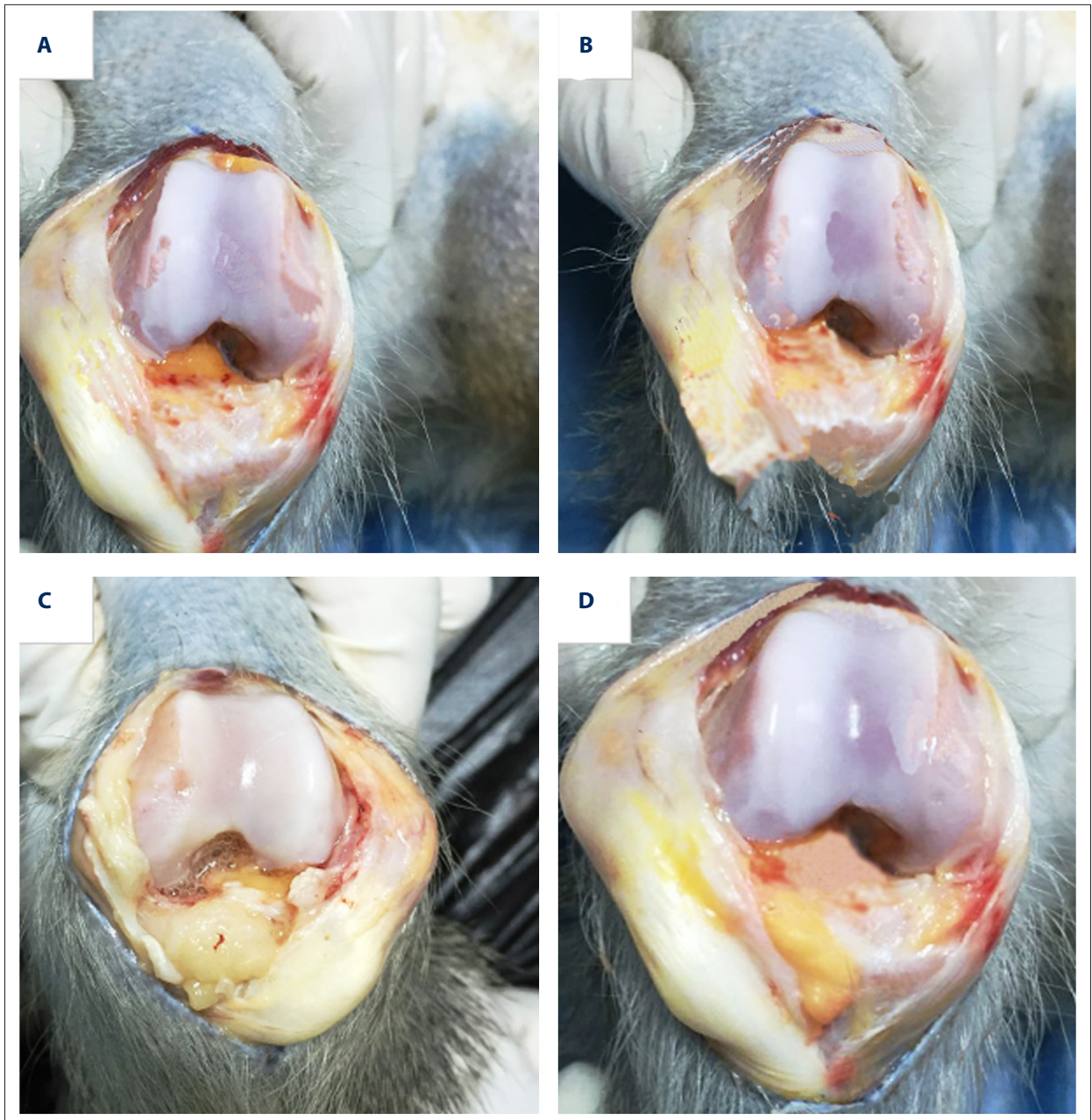
Figure 3. Macroscopic appearance of the knee joint cartilage in old-age and young cynomolgus monkeys (*Macaca fascicularis*) with and without knee osteoarthritis (KOA) (group A and group B). (**A, B**) Show the appearance of the knee joint surface in old cynomolgus monkeys (*Macaca fascicularis*). The surface of the articular cartilage is damaged and eroded and dull. (**C, D**) The appearance of the knee joint surface in young cynomolgus monkeys (*Macaca fascicularis*). The surface of articular cartilage is normal with a smooth and glossy appearance and with clear joint fluid.

KOA. In this study, only one old-aged monkey was found to have many of the same characteristics as the young monkeys.

In this study, the NOTCH signaling pathway in KOA was studied by the use of quantitative reverse transcription polymerase chain reaction (RT-qPCR) and Western blot to measure the expression of the *NOTCH3*, *JAG1*, and *ACAN* genes in knee cartilage specimens, with a comparison between group A and B. Although there were no definitive findings from this preliminary study on the role of the NOTCH signaling pathway in a primate model of KOA, the preliminary findings support the need for further and more in-depth comparative studies with larger samples size. However, there have been previously published studies that have reported the relationships between
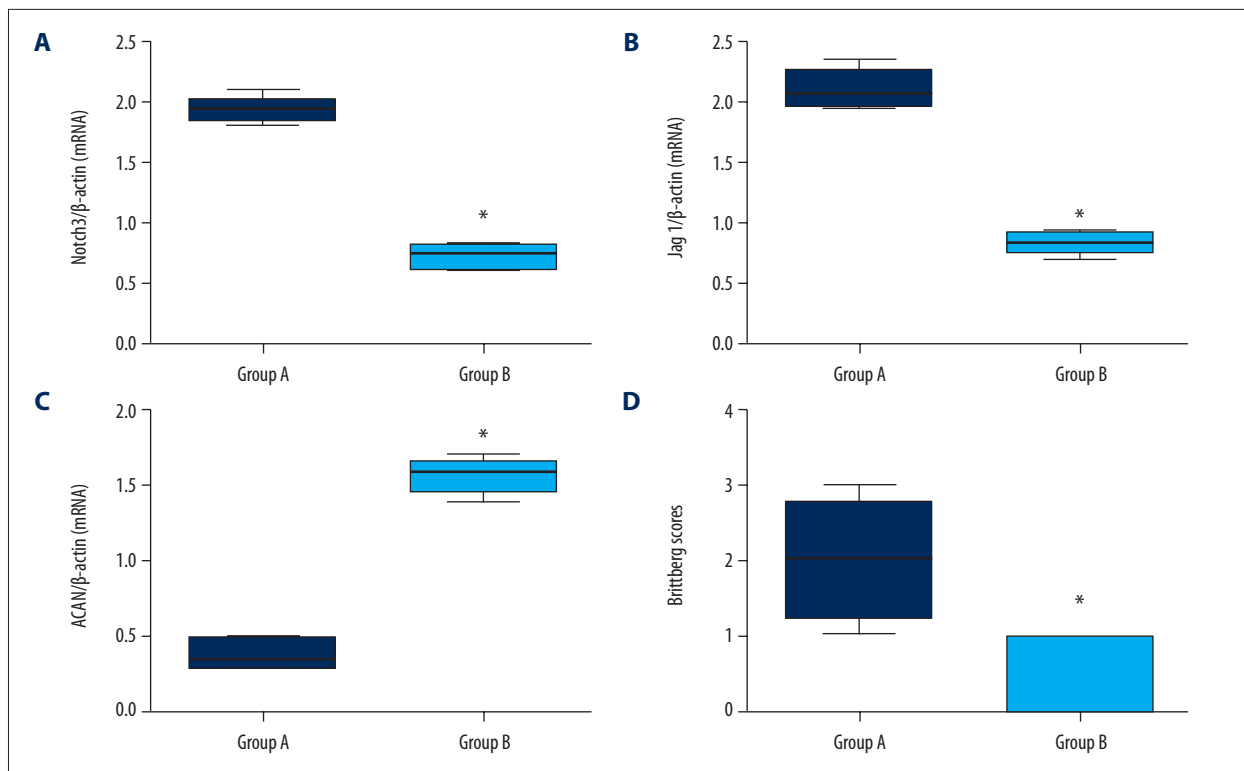
Figure 4. The *NOTCH3*, *JAG1*, and *ACAN* mRNA in knee joint cartilage and the Brittberg score in old-age and young cynomolgus monkeys (*Macaca fascicularis*) with and without knee osteoarthritis (KOA) (group A and group B). Comparison between group A and B. (**A**) Differences in *NOTCH3*/β-actin (mRNA). (**B**) Differences in *JAG1*/β-actin (mRNA). (**C**) Differences in *ACAN*/β-actin (mRNA). (**D**) Differences in the Brittberg score. * P<0.01 group A *vs.* group B.

osteophyte size and risk factors of OA and the composition of subchondral bone in OA [27,28].

Stiffness and pain were the important reported symptoms of KOA, but these symptoms could not be evaluated in the cynomolgus monkey (*Macaca fascicularis*) model [29]. The knee circumference was measured and compared between groups A and B, and the results showed that old-aged cynomolgus monkeys (*Macaca fascicularis*) had swollen knees. X-ray was a simple and useful method to make a preliminary diagnosis of KOA as well as the Kellgren and Lawrence (K-L) system, which was used for the classification of grade and severity of KOA [22].

From the findings of this study, one of the old-aged monkeys, diagnosed with K-L grade 1 also had some similar knee joint changes with the younger population, with no KOA. It has previously been reported that there was a moderate relationship between the OA imaging features and the symptoms of KOA [30]. However, some patients can also have serious symptoms of OA, such as pain and stiffness, while there were no obvious radiological features and of OA [31,32]. Therefore, it is possible that the identification of serum or joint biomarkers could reflect the state and symptoms of joint function and predict the progress of KOA, and may be more sensitive than

traditional imaging tests [33]. Also, according to the pathophysiological processes of osteoarthritis, a reliable biomarker might more accurately predict the severity of the disease [34–36].

Rheumatoid disease and infection should be excluded from the diagnosis of patients with symptoms of KOA and so, clinically, synovial fluid, blood and serum markers play an important role in excluding knee joint infection [37,38]. Interleukin (IL)-1β) and tumor necrosis factors were considered to be the most important pro-inflammatory mediators in OA [39]. IL-1 and other cytokines have been shown to have an important role in the pathogenesis of KOA, which could inhibit the synthesis of hyaline cartilage, collagen type IX, and promote the synthesis of type III collagen [40]. However, transforming growth factor (TGF)-β is mainly involved in the protection of cartilage, while the deficiency of TGF-β1 might be one of the important factors for the development of KOA [41]. In the pathophysiology of osteoarthritis, these cytokines could induce extracellular and extracellular matrix degradation by regulating the expression of the matrix metalloproteinase (MMP) family of catabolic enzymes, including MMP-1, MMP-3 and MMP-13 and plays an important role in OA [42–44]. Therefore, these biomarkers were investigated in knee joint synovial fluid in this study, and the findings showed that there were significant differences in
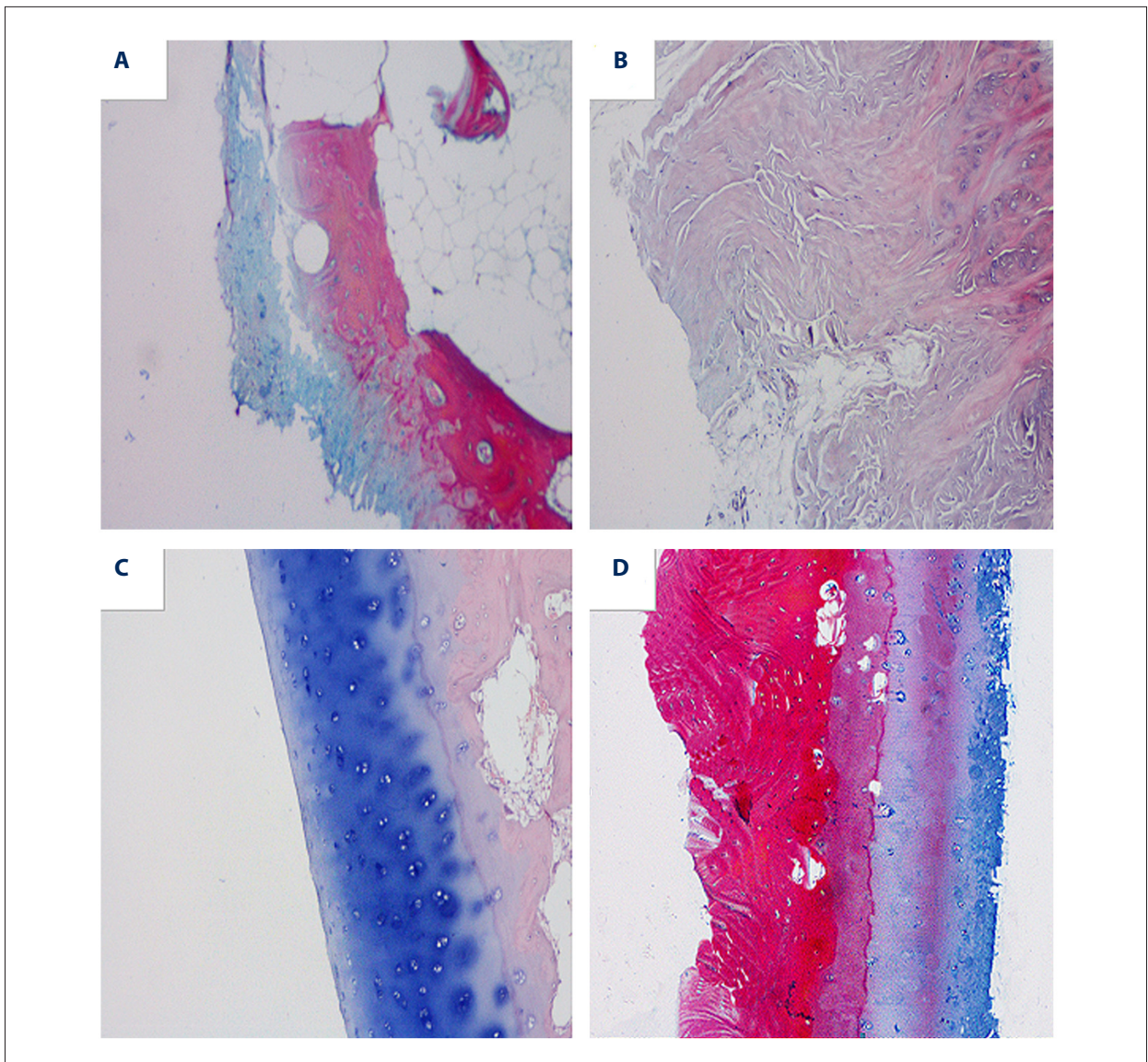
Figure 5. Photomicrographs of the histology of the knee joint cartilage (Masson staining) in old-age and young cynomolgus monkeys (*Macaca fascicularis*) with and without knee osteoarthritis (KOA) (group A and group B). (**A, B**) Show the histology of the knee joint cartilage in old cynomolgus monkeys (*Macaca fascicularis*).The cartilaginous surface is thinned. The transition layer, and calcified layer are completely damaged or disordered. The line of separation ('tide mark') has disappeared and there are no cartilage cells on the shallow surface of the cartilage. (**C**) Shows the histology of the knee joint cartilage a young cynomolgus monkey (*Macaca fascicularis*), The cartilage surface shows normal calcification. There are many cartilage cells in the cartilage surface. The articular chondrocytes are arranged with a columnar orientation, perpendicular to the joint line, and a clear 'tide mark' distinguishes the deep zone from the calcified cartilage, which are arranged in an orderly way. (**D**) A group A (number 7) cynomolgus monkey (*Macaca fascicularis*) shows changes similar to (**C**). There are some cartilage cells in the shallow cartilage surface, but the 'tide mark' is clear.

Table 3. The Mankin score comparison of the groups A and B ($\overline{\chi}$±s).

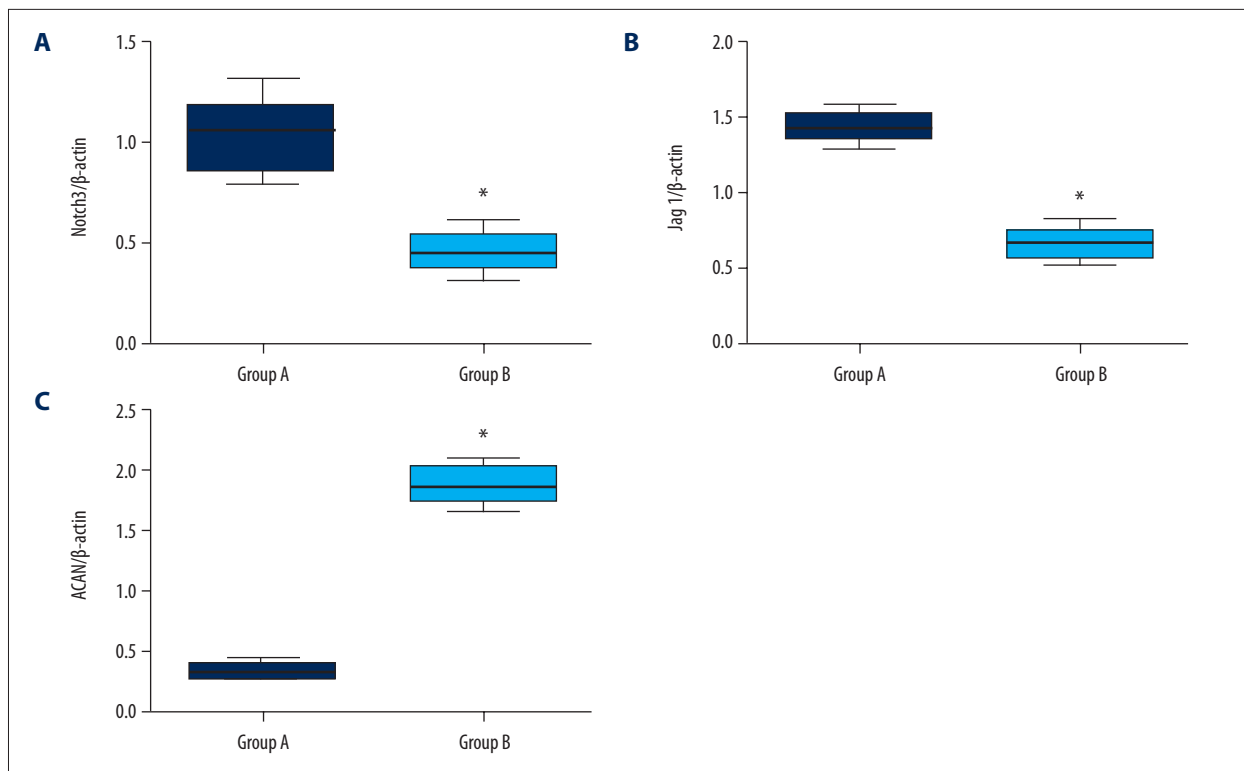| Contents	Group A	Group B	t	p
Mankin score	9.86±1.25	0.87±0.99[a]	15.06±0.59	0.0001

[a] P<0.05 *vs.* Group A.

Figure 6. Western blot measurements of the expression of the *NOTCH3*, *JAG1*, and *ACAN* in old-age and young cynomolgus monkeys (*Macaca fascicularis*) with and without knee osteoarthritis (KOA) (group A and group B). Comparison between group A and group B. (**A**) Differences in *NOTCH3*/β-actin. (**B**) Differences in *JAG1*/β-actin. (**C**) Differences in *ACAN*/β-actin. ***** P<0.01 group A *vs.* group B.
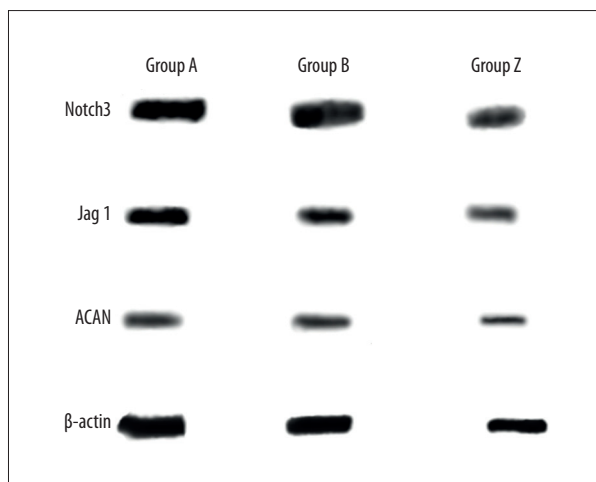

Figure 7. Western blot measurements of the expression of the *NOTCH3*, *JAG1*, and *ACAN* in old-age and young cynomolgus monkeys (*Macaca fascicularis*) with and without knee osteoarthritis (KOA) (group A and group B) Comparison between group A and group B. Group Z is a frame of reference.

the white blood cell (WBC) count, levels of IL-1β, TGF-β1, and MMP13 between group A (old-age) and B (young) cynomolgus monkeys (*Macaca fascicularis*) in this study (P<0.05).

Changes in articular cartilage were evaluated using the Brittberg score, which was found to be another simple way to detect KOA [23]. Compared with Group B, the articular cartilage in the old-aged monkeys macroscopically showed a rough surface, cartilage defects, dull color, and lack of subchondral bone exposure (Figure 3A), compared with normal articular cartilage (Figure 3C). From the findings of this preliminary study, it was possible to determine that the choice of a suitable primate model of human KOA was old-aged (>22 years old) cynomolgus monkeys (*Macaca fascicularis*) with KOA, a Kellgren and Lawrence (K-L) score >grade 2 and WBC count <2×10^9/L, and increased levels of IL-1β (1.95±0.59 34 ng/ml), MMP1 (0.72±0.34 ng/ml), a reduced level of TGF-β1 (0.80±0.92 ng/ml), which were significantly different between primate groups A and B. The reliability of the use of the Mankin histopathology grading score for OA has been shown since 1992 and a score of 6 or more is diagnostic for OA [24]. In the present study, the mean Mankin score of group A primates was found to be 9.86 ± 1.25, which was diagnostic for KOA. A modified Mankin score has also been developed to assess the degree of OA [45].

The NOTCH signaling pathway was first described by Mohr in 1917 [17]. This signaling pathway has been shown to have an important role in the formation of cartilage [46]. Some researchers had highlighted the specific mechanism of NOTCH signaling in chondrocyte differentiation and found that it could inhibit the development of chondrocytes [47]. Therefore, in this present study, the expression of the NOTCH signaling pathway components was compared in the cartilage between group A and group B (old-age and young) cynomolgus monkeys (*Macaca fascicularis*) in the KOA model. Also, previously published studies have shown that the NOTCH3 receptor was located in the deep layer of articular cartilage and the bone growth plate [48,49]. The *NOTCH* gene signaling molecules have been shown to be present in human mesenchymal stem cells in human cartilage, and the JAG1 ligand expression increases rapidly in early NOTCH-driven cell differentiation, which supports the role of JAG1 as a key ligand in the activation of NOTCH signaling and the effective catalyst of bone marrow mesenchymal stem cell differentiation to chondrocytes [50,51]. Therefore, the findings of the present study have support from those of previous studies. The use of cynomolgus monkeys (*Macaca fascicularis*), investigation of the *NOTCH3* and *JAG1* genes and pathways, chosen in this preliminary study, require further investigation.

This study had several limitations. Because of the high costs of using primates as experimental animals, there were only 16 cynomolgus monkeys (*Macaca fascicularis*) used in this study, and all were male. Micro-arthroscopy was not used in this study, which meant that open surgery on the knee was required to observe and sample the knee joint cartilage. Also,

all experimental animals used in this study were chosen from same animal experiment center.

Conclusions

The aim of this study was to identify a primate model of degenerative knee osteoarthritis (KOA) that may be more relevant for research studies on degenerative KOA in humans. Young and old-age male cynomolgus monkeys (*Macaca fascicularis*) were chosen, and the study included current methods of diagnosing and grading KOA. Investigation of the *NOTCH3* and *JAG1* genes and pathways were chosen in this preliminary study, which showed that age, joint X-ray findings, and the use of an enzyme-linked immunosorbent assay (ELISA) to evaluate synovial fluid were simple, but useful, methods to use in old-aged spontaneous degenerative KOA in the cynomolgus monkey (*Macaca fascicularis*) animal model. Also, when evaluating the osteoarthritic changes in articular cartilage, the Brittberg score and the Mankin histopathology grading score, and measurement of components of the NOTCH signaling pathway were shown to be of use, and require further study.

Conflict of interest

None.

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