Involvement of the RhoA/ROCK signaling pathway in the pathogenesis of knee osteoarthritis

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Research Article

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Abstract

Purpose

This study compared the expression of the Ras homolog gene family member A (RhoA) and Rho-associated coiled-coil kinase (ROCK) signaling pathway members in knee joint synovial tissue from patients with primary knee osteoarthritis (KOA) who underwent lower limb disarticulation.

Methods

The specimens were separated into observation (synovial tissue removed during total knee arthroplasty of 30 patients with primary KOA) and control groups (synovial tissue removed during lower limb disarticulation of 25 patients).

Results

The observation group specimens showed high proliferation, congestion, edema, unorganized cellular arrangement, and presence of vacuolated cells. The control group specimens were white with a smooth surface and non-edematous texture, and showed organized and homogenous cell/stroma arrangement. The observation group showed higher expression of RhoA and ROCK transcripts and proteins than the control.

Conclusion

Patients with KOA expressed high levels of RhoA and ROCK transcripts and proteins in synovial tissues, suggesting a relationship between RhoA/ROCK and KOA. Our findings provide a potential therapeutic target for delaying synovial lesions in KOA.

Background

Osteoarthritis (OA) mostly occurs in older adults. Its incidence rate in China is approximately 17% in the middle-aged population, with higher prevalence in women [1, 2]. This disease commonly affects the weight-bearing joints, with knee osteoarthritis (KOA) accounting for > 80% of all OA cases [3, 4]. KOA manifests as sclerosis of the subchondral bone and periarticular cartilage, in addition to the wear and tear, degeneration, softness, deformation, and disappearance of the articular cartilage. Sclerosis of the subchondral bone, periarticular synovial hyperplasia, degeneration and atrophy of the joint capsule, peripheral ligaments, and other tissues, destruction of the integrity of the joint surface, and narrowing of the joint space can lead to knee deformity [5, 6]. Pathological changes mainly occur in the synovial membrane, cartilage, meniscus, and patella in the early stages of KOA [7], while knee joint pain, stiffness, swelling, limited activity, popping, and deformity appear gradually. KOA can involve the muscles, seriously

affecting patients' physical and mental health and daily activities [8]. Therefore, early diagnosis, intervention, and treatment have become the focus of joint surgeries.

Cartilage wear and tear is the core pathological change in KOA; however, pathological cartilage changes occur in the middle and late stages [9]. Synovial inflammation, an important early pathological change in KOA, can result in knee pain and structural changes, and its incidence has been increasing continuously [10]. Normal synovial and cartilage tissues are loose connective tissues that secrete synovial fluid to nourish the cartilage, reduce friction, maintain intra-articular metabolism, and provide nutrition to the articular cartilage [11, 12]. Intra-articular lesions lead to the loss of synovial nutritional function and production of many proteases and cytokines, damaging the chondrocytes. The pro-inflammatory factors released from synovial and cartilaginous tissues stimulate the production of protein hydrolyzing enzymes, thereby aggravating articular cartilage degeneration, which causes joint pain and deterioration [13–16].

Ras homolog gene family member A (RhoA) proteins are expressed in all tissues and affect cell growth, transformation, cytoskeletal regulation, actin stress fiber formation, and actin-myosin contraction [17–19]. Rho-associated protein kinase (ROCK), a downstream effector of RhoA, exists as two isoforms, ROCK1 and ROCK2. The RhoA/ROCK signaling pathway is involved in cell growth, differentiation, migration, and development [20, 21], and is required for neuronal synaptic growth, bone formation, dorsal closure, and myogenesis [22]. Its aberrant activation is associated with various cancer types and diseases affecting the neurological, endocrine, and cardiovascular systems [23–28]. Previous studies have shown that the RhoA/ROCK signaling pathway is closely related to cartilage degeneration in KOA, but such association has not been studied in synovial lesions in KOA.

The aim of this study was to investigate the correlation between the RhoA/ROCK signaling pathway and the pathogenesis of KOA by comparing the expression of RhoA and ROCK in the synovial tissue of patients with primary KOA as a way to potentially identify a therapeutic target for delaying synovial lesions in KOA.

Methods

Patients

Observation group: The observation group comprised synovial tissue samples removed during total knee arthroplasty (TKA) from 30 patients with primary KOA between January 2020 and December 2022. The patients were 57–82 years of age (average age, 66.40 ± 6.05 years), and included 11 men and 19 women.

The inclusion criteria were as follows: primary KOA and fulfillment of the diagnostic criteria of the Chinese Osteoarthritis Diagnostic and Treatment Guidelines (2018 edition) (Table 1); poor outcomes after receiving conservative treatment; unilateral knee arthroplasty for the first time; and provision of informed consent by patients or their family members.

Table 1
Diagnostic criteria for knee osteoarthritis (2018 version).

No.	Symptoms or signs
1	Recurrent knee pain within the last 1 month
2	Radiography (standing or weight-bearing position) showing narrowing of the joint space, subchondral osteosclerosis and/or cystic degeneration, and formation of bone capillaries at the joint margins
3	Examination of the synovial fluid in the knee joint consistent with osteoarthritis (clear, viscous, white blood cell count < 2.0×10^9/L)
4	Age ≥ 50 years
5	Morning stiffness ≤ 30 min
6	Sound of bone friction (sensation) during activity
Knee osteoarthritis can be diagnosed by meeting at least diagnostic criteria 1 and 2; 1, 3, 5, and 6; 1, 4, 5, and 6.	

The exclusion criteria were as follows: serious blood-related diseases or receipt of prolonged anticoagulant drug treatment in preoperative manner; combined joint tuberculosis, tumor, and other serious diseases; serious knee deformity or trauma preventing cooperation while receiving treatment; history of drug injection into the joint cavity within half a year; and receipt of oral treatment for OA within the previous 2 weeks.

Control group: Twenty-five control specimens were obtained from the synovial tissue of knee joints removed during “lower limb disarticulation” during the same period at our hospital. The patients were 19–39 years of age (average age, 31.07 ± 6.10 years), including 12 men and 13 women.

The inclusion criteria for the control group were as follows: age, 18–40 years; non-KOA; “lower limb disarticulation” because of malignant tumors of the lower limbs or lower limb disfigurement injuries; and voluntary study participation.

The exclusion criteria were as follows: arthritis on preoperative imaging; rheumatoid arthritis; knee joint infection; autoimmune disease; tuberculosis and other serious systemic diseases; and serious blood-related diseases.

This study was approved by the responsible ethics review board (No. 2020 – 974). Informed consent was obtained from the patients before enrollment in the study.

Reagents

The chemicals and reagents used included anti-ROCK1 (ab134181; Abcam, Cambridge, UK)/anti-ROCK2 (ab228000; Abcam) and anti-RhoA (ab54835; Abcam) antibodies, RNA solids (Wuhan Xavier Biotechnology Co., Ltd., Wuhan, China), and polymerase chain reaction (PCR) primers (Shanghai

Sangong Bioengineering Service Co., Ltd., Shanghai, China). All the other reagents were obtained from Yinchuan Runyan Biotechnology Co., Ltd. (Yinchuan, China).

Hematoxylin and eosin (HE) staining

The tissue sections were soaked in xylene I and II for 15 min for dewaxing. Subsequently, they were immersed in the following gradients of alcohol for 3–5 min each: anhydrous ethanol and , 95% ethanol, 80% ethanol, and 70% ethanol; the sections were then removed and rinsed with distilled water for 5 min. Then, 1% hydrochloric acid in alcohol was used for differentiation for 3–5 s, followed by rinsing for 5 min. To dye them blue, the tissue sections were immersed in 0.2% ammonia for 1 min, followed by rinsing in distilled water for 5 min. Next, the tissue sections were stained with 0.6% eosin for 30 s to 1 min. After dehydration, the sections were soaked in xylene I and II for 10 min each and sealed with optical resin glue.

Immunohistochemistry

The slices were put in the oven at 60°C for > 6 h, followed by dewaxing and rehydration. The sections were rinsed thrice for 5 min. Then, they were boiled in citrate buffer (pH 6.0) for 15 min and placed in a mixture of ice and water at 0°C for 30 min. The residual citrate buffer was washed out thrice using distilled water for 5 min each. The washed tissue sections were soaked in 3% H_2O_2 solution for 15 min and rinsed thrice for 5 min. The synovial tissue was blocked with goat serum at 20–25 ℃ for 15 min. A drop of one antigen was added to the synovial tissue, and the tissue sections were incubated overnight in the refrigerator in a humid box. The sections were rinsed thrice using phosphate buffered saline (PBS) for 10 min. In total, 2–3 drops of horseradish peroxidase-labeled goat anti-rabbit IgG antibody were added to cover the synovial tissue, and the tissue sections were incubated for 1 h at 37°C. The slices were rinsed thrice with PBS (for 5 min at a time). The slices were counterstained using the following sequence of reagents: hematoxylin staining solution for 2 min and 1% hydrochloric acid in alcohol solution for 30 s. The sections were dehydrated for 10 min.

Western blotting

Approximately 500 mg of each specimen was ground and transferred to an Eppendorf tube. A mixture of the whole protein lysate, protease inhibitor cocktail, and phenylmethylsulfonyl fluoride were added to each tube, mixed well, and placed on a vortex oscillator for 30 s; it was then placed on ice for tissue lysis, vortexed, and centrifuged again after 15 min; this procedure was repeated thrice. The tissue lysate was centrifuged (12,000 rpm) at 4 °C for 20 min. The supernatant and 1/4th of the supernatant were aspirated into 5× loading buffer, placed into a new Eppendorf tube for mixing, and boiled in hot water for 10 min. Equal amounts of protein from each sample were subjected to electrophoresis after adding loading buffer to achieve equal volumes across samples. Electrophoresis was initiated at 80 V and 400 mA, and after 30 min, the voltage and current were adjusted to 120 V and 400 mA, respectively. The gel was transferred to a methanol-soaked polyvinylidene fluoride (PVDF) membrane at 250 V and 260 mA for 90 min. The PVDF membrane was washed thrice with PBS-Tween 20 for 45 min. After sealing the

membrane, the latter was washed with PBST) three times (10 min each time) and incubated overnight at 4°C on a refrigerator shaker with primary antibodies diluted at different ratios. Subsequently, the membrane was incubated with a secondary antibody (horseradish peroxidase-labeled goat anti-rabbit IgG; 1:5,000 dilution) for 60 min at 20–25 ℃ on a shaking bed. The membrane was then washed thrice prior to development using a developing solution and visualization.

Quantitative reverse transcription-PCR

Approximately 30–40 mg synovial tissue was weighed and ground with liquid nitrogen to a fine powder, followed by the addition of 1 ml Trizol lysis buffer. The tissue was ground until liquification in the Trizol buffer. Two hundred microliters of chloroform was added, mixed to emulsify, placed at room temperature for 5–10 min, and centrifuged for 20 min at 12,000 rpm, 4°C. Five hundred microliters of the supernatant and an equal amount of isopropanol were mixed in a new tube. The tube was vortexed to separate the DNA, left standing for 5 min, and centrifuged for 20 min under the same conditions, following which the supernatant was discarded. One milliliter of 75% ethanol was used to wash the pellet and centrifuged in a low-temperature ultracentrifuge for 5 min at 4°C (7,500 rpm). The upper ethanol layer was discarded and the precipitate was put on the ultra-clean bench for 5 min. Diethyl pyrocarbonate-treated water was added to dissolve the precipitate, followed by heating at 65°C for 10 min. The cDNA was synthesized via reverse transcription of mRNA using an Applied Biosystems (Waltham, MA, USA) instrument under the following conditions: 37°C (15 min), 85°C (5 s), and 4°C (∞). Primers were designed and synthesized using the sequences listed in Table 2. *GAPDH* was used as an internal reference, and the relative expression of the target mRNAs was calculated using the $2^{-\triangle\triangle Ct}$ method and a LightCyder480 instrument.

Table 2. Primer names and sequences.

Primer name	Primer sequences (5′-3′)
RhoA-Forward	TGG AGC TGG GCT AAG TAA A
RhoA-Reverse	CTC TGG GAG GGA ACC TG
ROCK1-Forward	CAA ACG ATA TGG CTG GAA G
ROCK1-Reverse	TGG ATT GGA TIG CTC CTT A
ROCK2-Forward	CAG TTG GAA CAC CGG ATT A
ROCK2-Reverse	COC ACT AGC ATC TCA TAA AGG
GAPDH-Forward	GCC AAC GTG TCA GTG GTG
GAPDH-Reverse	AAG GTG GAG GAG TGG GTG T

Data analysis

All data were analyzed using the SPSS software (v25.0; IBM Corp., Armonk, NY, USA) and were expressed as mean ± standard deviation; comparisons between groups were performed using the Student's *t*-test for independent samples. Statistical results were plotted using the GraphPad Prism software (v7.0; GraphPad Software, La Jolla, CA, USA), with a significance level of $\alpha = 0.05$.

Results

Appearance and morphology of the synovial tissue

In the observation group, the synovial tissue showed high levels of proliferation and edema, with obvious congestion and a yellowish appearance (Fig. 1a). The synovial membrane surface in the control group was smooth. There were no signs of edema or congestion, and the tissue appeared white (Fig. 1b).

HE staining

RhoA expression in the synovial tissues

RhoA protein was expressed widely in the synovial tissues of 30 patients with KOA compared with that in the control group. RhoA expression appeared as light yellow or brownish-yellow particles in the cell cytoplasm, cytosol, and interstitium (Fig. 3a, c). RhoA expression was low in the control group (Fig. 3b, d).

ROCK1 expression in the synovium

ROCK1 was expressed in the synovial tissues of 30 KOA joints, and the positively-stained particles were distributed in the cell membranes (Fig. 4a, c). ROCK1 expression was low in the control group (Fig. 4b, d).

ROCK2 expression in the synovial membrane

Anti-ROCK2 staining was observed in the synovial tissue of 30 KOA joints, and the positively-stained particles were distributed in the cytoplasm (Fig. 5a, c). Anti-ROCK2 staining was low in the control group (Fig. 5b, d). Compared with the control group, numerous samples in the observation group showed significant positive immunoreactivity for RhoA and ROCK, which were distributed widely ($P < 0.05$; Table 3). The observation group showed higher RhoA and ROCK protein levels than the control group ($P < 0.05$; Fig. 6). The synovial tissue in the observation group showed higher levels of *RHOA* and *ROCK* transcripts than that in the control group ($P < 0.05$; Fig. 7).

Table 3. Comparison between RhoA, ROCK1, and ROCK2 Expression Intensity in the Two Groups (Mean ± SD).

Group	Observation group	Control group	*t*-Value	*P*
RhoA	7.11 ± 1.61	2.43±0.78	14.328	<0.001
ROCK1	6.61 ± 1.45	2.92±0.54	13.043	<0.001
ROCK2	5.05 ± 1.14	3.20±0.53	8.063	<0.001

Discussion

Surgical management using artificial joint arthroplasty is considered in clinical practice for patients with severe KOA, which affects their normal life activities. TKA can reduce the clinical symptoms, restore knee joint function, and improve the quality of daily life [29]. However, ensuring the survival of TKA prostheses after wear and tear, and rehabilitation of patients after knee arthroplasty are associated with high social, medical, and economic burdens. Early diagnosis and treatment can reduce the pain experienced by the patients and the economic burden. Hence, the study of early synovial inflammation pathogenesis in patients with KOA is crucial for its prevention and treatment [30, 31].

In our study, the high expression of RhoA and ROCK transcripts and proteins during synovial inflammation was verified using immunohistochemistry, western blotting, and quantitative reverse transcription-PCR, confirming the relevance of the RhoA/ROCK signaling pathway with the development of KOA synovitis. Synovial inflammation in KOA is considered an early-stage lesion. The RhoA/ROCK signaling pathway plays a key role in lipopolysaccharide-induced inflammatory pain and has been associated with signaling pathway activation to cause nociceptive hypersensitivity and the concomitant release of tumor necrosis factor-α and interleukin-1β [32, 33]. Suppression of ROCK expression and articular cartilage degeneration have been observed after the injection of RhoA/ROCK signaling inhibitors in rats, while the production of inflammatory factors *in vivo* and joint pain scores were reduced and chronic neuropathic pain was alleviated, consistent with our results [34, 35]. Synovial inflammation can increase peripheral vascular permeability, lower pain thresholds, and increase nociceptive sensitivity. Activation of the RhoA/ROCK signaling pathway promotes the release of inflammatory factors and participates in sensory processing of spinal cord injury, which in turn aggravates inflammatory responses and increases knee pain.

This study had certain limitations. We only verified the correlation between the RhoA/ROCK signaling pathway and KOA synovitis but did not use serology, cytology, and gene knockdown technology to further understand the mechanisms underlying the correlation. Moreover, the sample size was small, and the same group of specimens could not be used for all the testing techniques. Therefore, a larger sample size and comprehensive analysis are necessary to clarify the contribution of the pathway.

Conclusions

The expression of RhoA and ROCK transcripts and proteins was higher in the synovial tissue of patients with KOA than in those of the control, indicating that the RhoA/ROCK signaling pathway was involved in KOA. This study shed light on novel approaches for screening target genes for early diagnosis of KOA

and development of preventive treatment strategies. In the future, it would be important to study the correlation between this signaling pathway and KOA synovitis in depth, and to develop drugs that slow down the progression of synovitis and improve synovitis-induced joint pain.

Abbreviations

KOA
knee osteoarthritis
TKA
total knee arthroplasty
RhoA
Ras homolog gene family member A
ROCK
Rho-associated coiled-coil kinase

Declarations

Ethics approval and consent to participate

This study was approved by the Medical Ethics Review Board of the People's Hospital of Ningxia Hui Autonomous Region (No. 2020-974).

Consent for publication

Informed consent was obtained from the patients before enrollment in the study.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

All authors declare that they have no conflicts of interest related to the research or writing of the manuscript.

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Authors' contributions

T.Y. was responsible for case data compilation and manuscript preparation. Y.W. was responsible for instructing the lab. J.Z. and B.Z. were responsible for statistical analysis and literature review. A.Y. and W.W. were responsible for case data collection. Z.L. and D.C. supervised the manuscript preparation.

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Not applicable.

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Figures

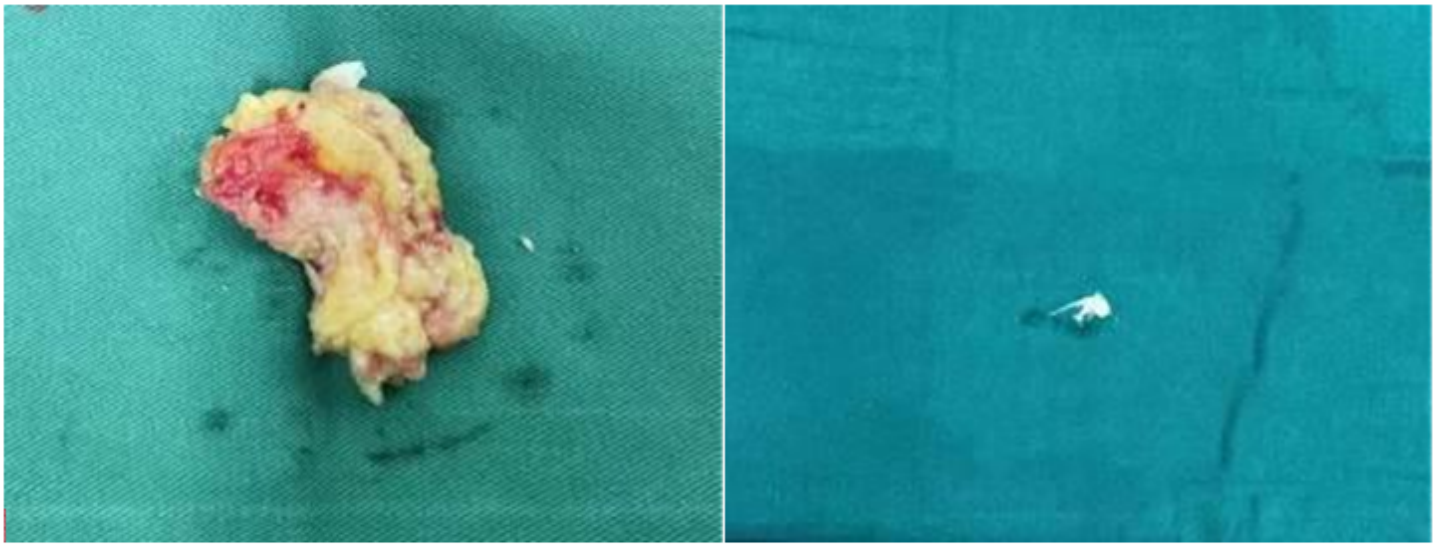

Figure 1

Appearance of synovial membrane specimens in the observation (a) and control (b) groups.

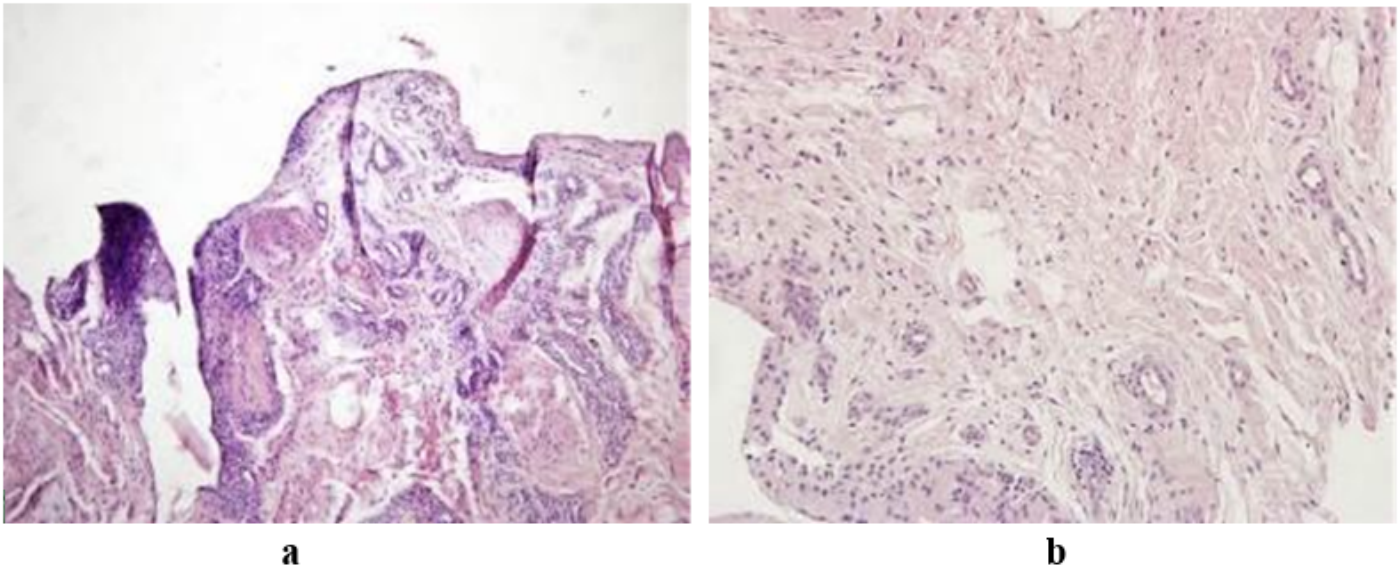

Figure 2

Hematoxylin and eosin staining of the synovial tissue. (a) Observation and (b) control groups (10× magnification).

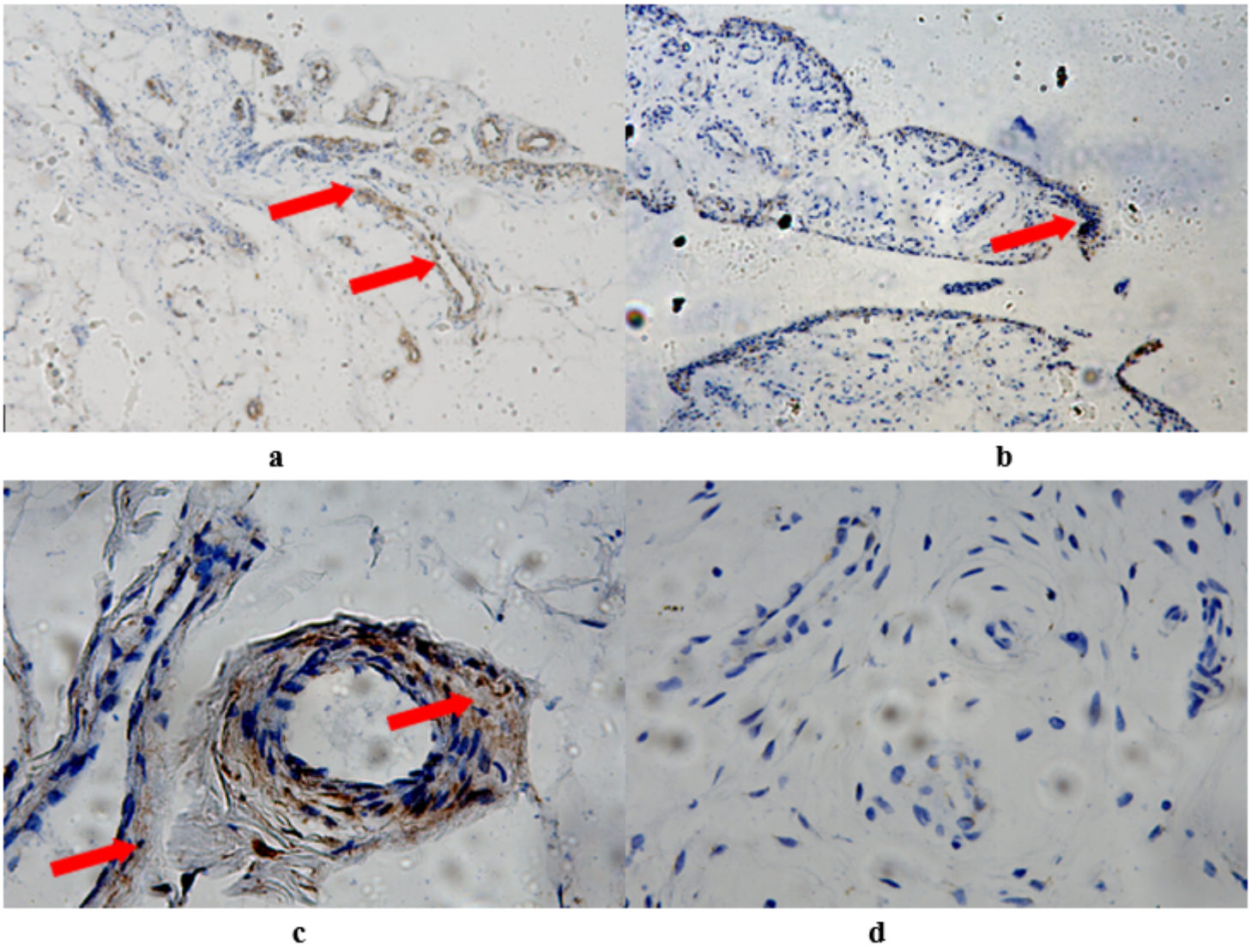

Figure 3

Immunohistochemistry of RhoA in the synovial tissues of the observation and control groups. (a) Observation group (10× magnification); (b) control group (10× magnification); (c) observation group (40× magnification); (d) control group (40× magnification); "↑" indicates the colored RhoA-positive particles.

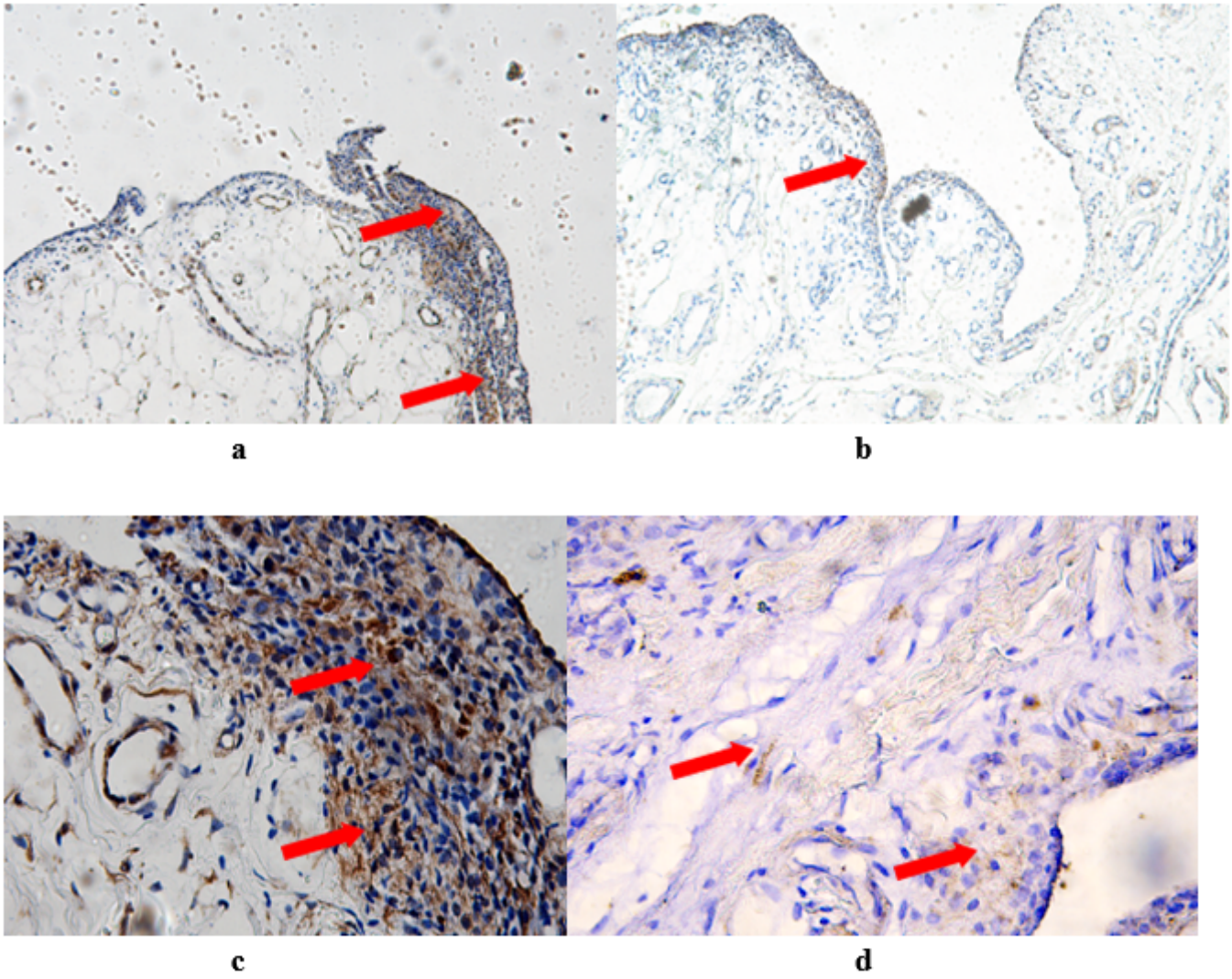

Figure 4

Immunohistochemistry of ROCK1 in the synovial tissues of the observation and control groups. (a) Observation group (10× magnification); (b) control group (10× magnification); (c) observation group (40× magnification); (d) control group (40× magnification); “↑” indicates the colored ROCK1-positive particles.

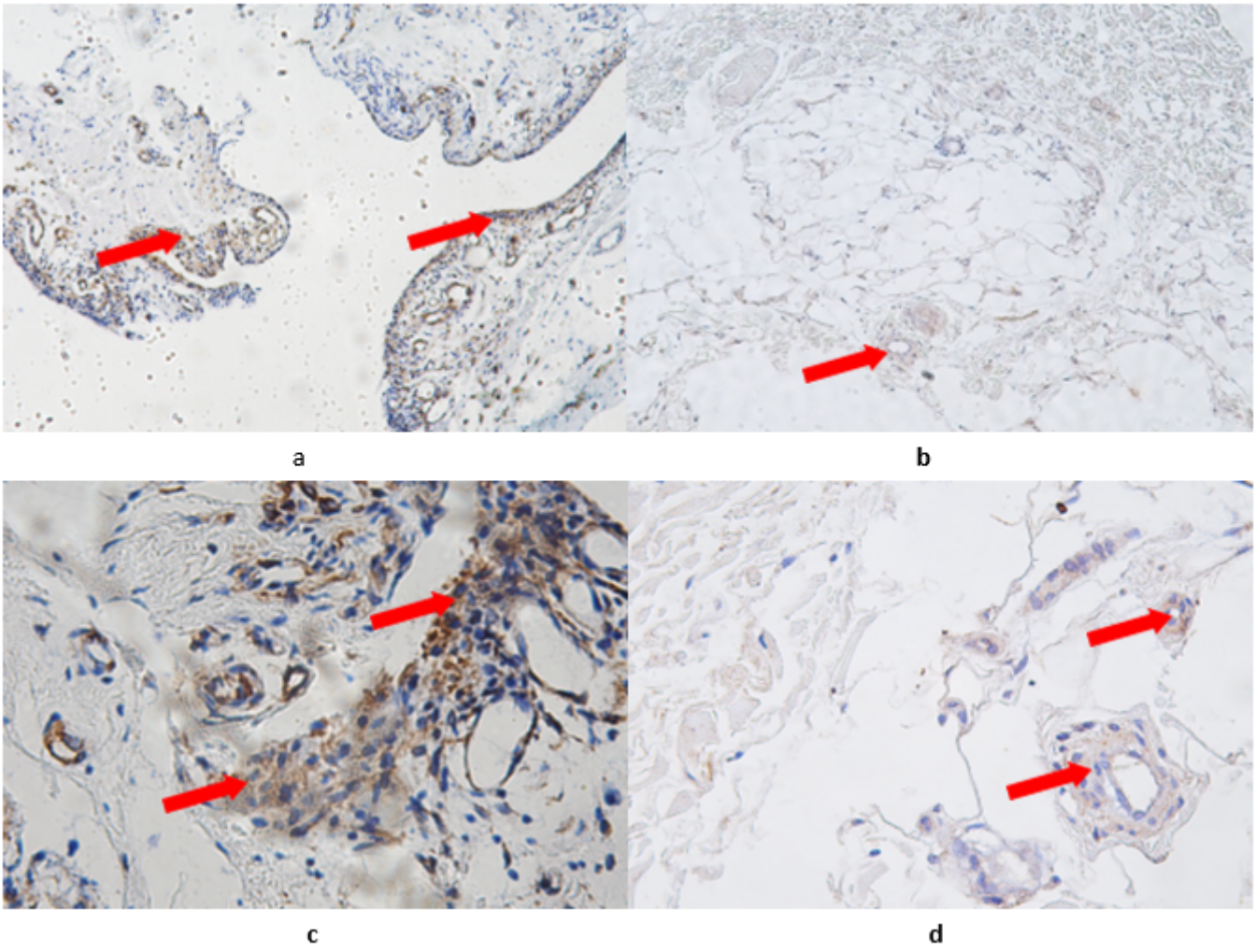

Figure 5

Immunohistochemistry of ROCK2 in the synovial tissue of the observation and control groups. (a) Observation group (10× magnification); (b) control group (10× magnification); (c) observation group (40× magnification); (d) control group (40× magnification); “↑” indicates the colored ROCK2-positive particles.

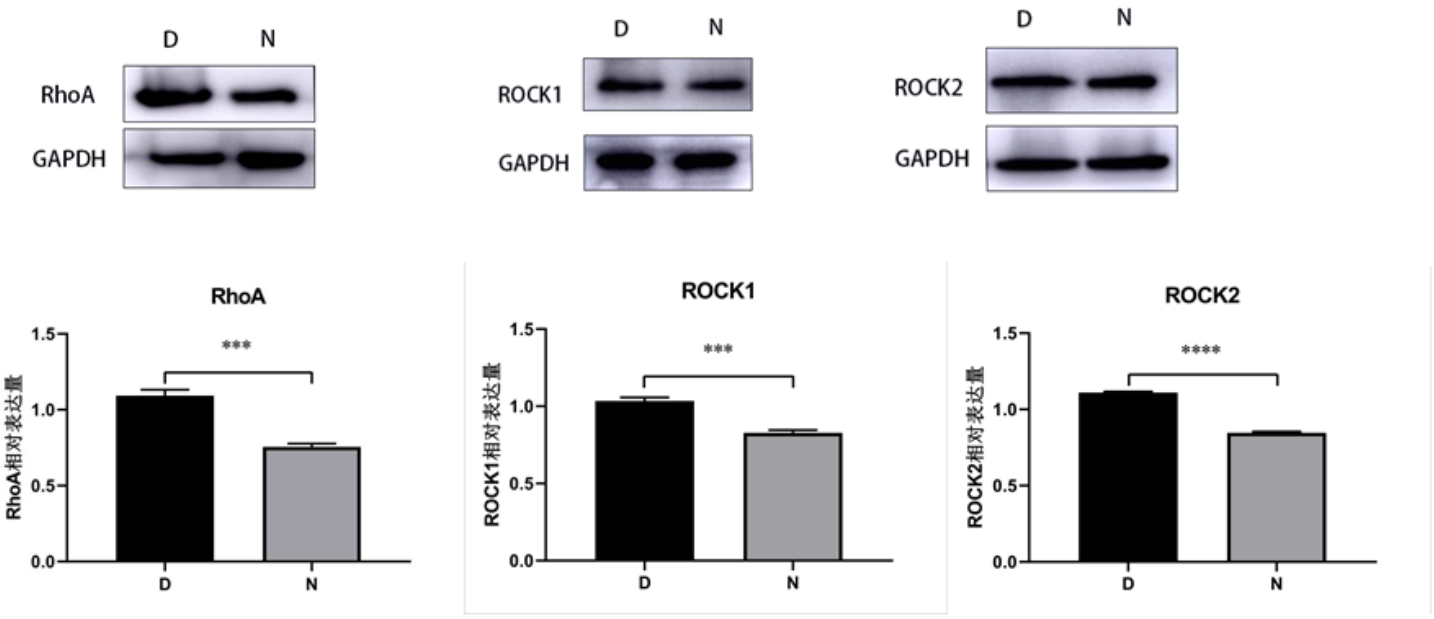

Figure 6

RhoA and ROCK protein expression in the synovial tissues of the observation (D) and control (N) groups. *P < 0.05, ** P < 0.01, ***P < 0.001, and ****P < 0.0001.

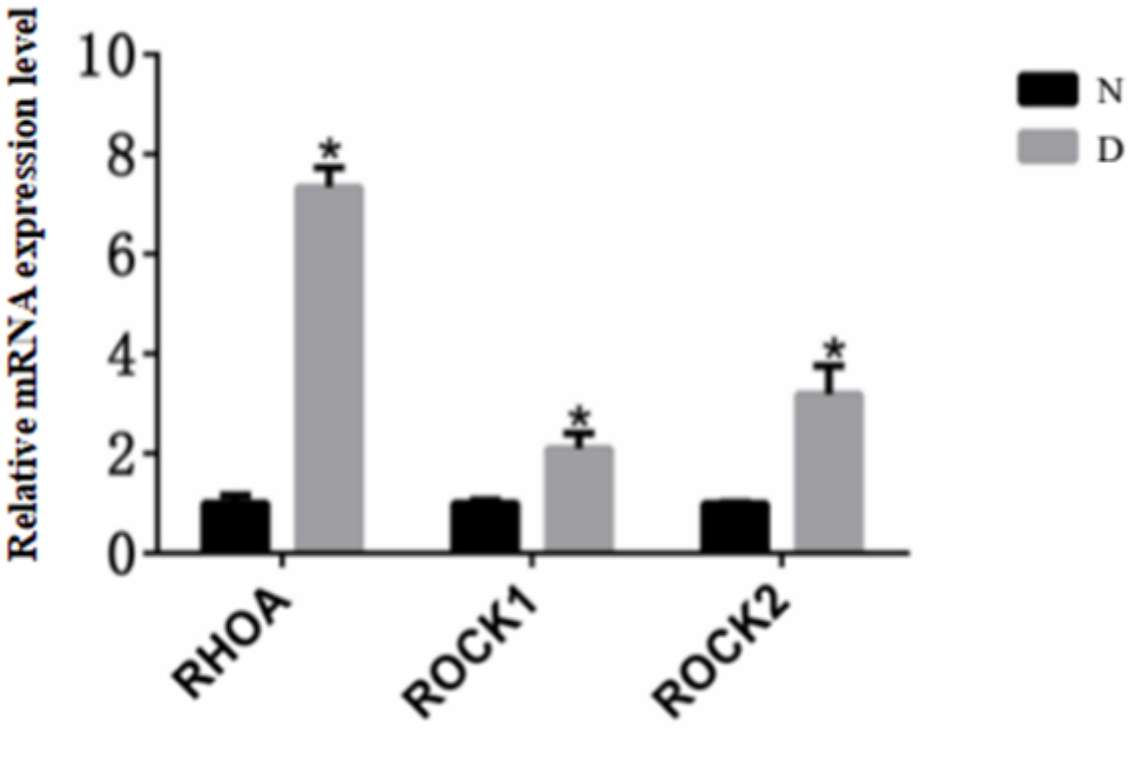

Figure 7

mRNA expression levels of *RHOA* and *ROCK*in the synovial tissues of the observation (D) and control (N) groups. *$P < 0.05$.