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ORIGINAL RESEARCH

Exploring the Analgesic Effect of Acupuncture on Knee Osteoarthritis Based on MLT/cAMP/PKA/CREB Signaling Pathway

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Background: Acupuncture is an effective treatment for knee osteoarthritis (KOA), reducing pain and improving function. While melatonin (MLT) has notable pain relief benefits, the analgesic mechanism of acupuncture in KOA and its relationship with melatonin are still unknown. This study aims to explore this mechanism.

Methods: In this work, the KOA rabbit model was constructed using the traditional Hulth method, and the therapeutic effect was assessed by the Lequesne MG score and Pain assessment by hot plate test. The pathological alterations of cartilage tissue were observed using hematoxylin and eosin (H&E) staining, Safranin O-fast green and MASSON staining to observe the pathological changes in cartilage tissue, and the efficacy was evaluated according to the principles of Mankin score and Osteoarthritis Research Society International (OARSI) score. Meanwhile, MLT in serum, cyclic adenosine monophosphate (cAMP) in cartilage, and matrix metalloproteinase-3 (MMP-3) in joint fluid were detected by enzyme-linked immunosorbent assay. In addition, the expression of aromatic L-amino acid N-acetyltransferase (AANAT), melatonin receptor 1 (MT1) and 2 (MT2) mRNAs in cartilage was determined by real-time quantitative reverse transcription-polymerase chain reaction, and the levels of proteins related to PKA/CREB signaling pathway were detected by Western blotting.

Results: Based on the results of Lequesne MG score and Pain assessment by hot plate test experimental data, the treatment group presented significant improvements in knee pain and overall function relative to OA (Osteoarthritis) group. Besides, according to results of histologic staining, Mankin and OARSI scores, articular cartilage degeneration of treatment group remarkably improved. In addition, acupuncture significantly reduced the expression of the inflammatory factor MMP-3 in knee joint fluid and significantly increased the levels of MLT, AANAT, MT1, MT2, cAMP, PKA and CREB.

Conclusion: By regulating sympathetic excitability, acupuncture may activate the MLT/cAMP/PKA/CREB signaling pathway, decrease inflammatory factor expression and slow down degradation of articular cartilage, resulting in the relief of knee pain.

Keywords: acupuncture, knee osteoarthritis, MLT/cAMP/PKA/CREB pathway, melatonin

Introduction

Knee osteoarthritis (KOA) represents a chronic degenerative disease that involves the articular cartilage, leading to subchondral bone remodeling, meniscal degeneration, inflammatory fibrosis of the infrapatellar fat pad, and damage to the surrounding muscles, which results in biomechanical changes in the lower extremity.[1,2] Its typical clinical symptoms include progressively worsening stiffness, swelling, and pain of the knee, with a lower prevalence in men than in women.[3] Nowadays, injury, age and obesity are its major causative factors, and prevalence and disability rates of KOA

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are rising year by year as obesity rate and population aging increase worldwide.[4] KOA is now the fourth most disabling disease in the world[5] and brings substantial economic burdens on the families and the society.[6,7] Currently, there are various treatments available for managing the pain of KOA, but the disease cannot be completely cured. Knee replacement surgery is considered the last resort for advanced KOA, yet it is unaffordable for many patients due to the high cost of surgery, postoperative rehabilitation complications and risks.[8] Acupuncture is the treatment that inserts fine needles in specific body points to cause a systemic multiorgan response through local stimulation, thus achieving the analgesic effect and disease treatment.[9] Acupuncture has been demonstrated with significant therapeutic effects on a wide range of systemic diseases,[10] especially in analgesia and the promotion of organ function recovery.[9] As reported in multiple randomized controlled trials, acupuncture attains a favorable therapeutic effect on patients with KOA.[11,12] However, the molecular biological mechanism in KOA pain remains unclear, and the analgesic mechanism of acupuncture in KOA has not been fully elucidated.

Melatonin (MLT), an endogenously active indole-like hormone, is generated in pineal gland in vivo and can be secreted to systemic circulation.[13] Recent studies indicate that melatonin receptors (MT) are widely present in mammalian tissues, predominantly concentrated in the central nervous system and also extensively distributed in peripheral tissues.[14] The production of MLT is regulated by aromatic L-amino acid N-acetyltransferase (AANAT), which is in turn affected by sympathetic nerves.[15] In some studies, MLT is found to be involved in pain regulation and has a protective effect on bone and joints.[16–19] Cyclic adenosine monophosphate (cAMP), as a second messenger in the body, can directly regulate a wide range of cell biological processes, like metabolism, gene expression, cell growth, apoptosis and differentiation.[20] cAMP functions as a second messenger by binding to and activating protein kinase A (PKA), as well as stimulating exchange proteins (Epac) and ion-gated channels.[21] The cAMP response element-binding protein (CREB) is a key PKA target protein. As the key transcription factor, CREB exerts a key effect on pain relief and inflammatory factor inhibition.[22] Nonetheless, no study has reported the effects of MLT and cAMP/PKA/CREB signaling pathway on KOA pain and the underlying regulatory mechanisms.

Consequently, the present work focused on investigating how acupuncture affected the MLT level and the MLT/cAMP/PKA/CREB pathway in a rabbit model of KOA, so as to provide a more in-depth scientific foundation for applying acupuncture in treating KOA pain.

Materials and Methods

Experimental Animals

All animal experiments were in accordance with the Guidelines for the Care of Medical Laboratory Animals in Tianjin and ARRIVE guidelines,[23] China and were completed within the premises of Yi Shengyuan Gene Technology (Tianjin) Co. The experimental protocol was approved by the Animal Care and Use Committee of Yi Shengyuan Gene Technology (Tianjin) Co., Ltd. (protocol number YSY-DWLL-2023430). The present work utilized fifteen healthy male 6-month-old New Zealand white rabbits (weight, 2 ± 0.2 kg). Prior to experiments, the rabbits were domesticated for a period of 7 days in a controlled environment, where the ambient temperature was kept under 24 ± 0.5°C, humidity was between 40% and 70%, and an alternating light-dark cycle was used, and the rabbits could take water and food freely. The behavioral experiment was carried out at daytime from 9:00 am to 5:00 pm.

Establishment of the KOA Animal Model

Prior to the start of the experiment, 15 numbered rabbits were randomly divided into three groups—control, OA, and acupuncture treatment—using the random number table method, with five rabbits in each group. In OA and acupuncture groups, a KOA model was established according to the Hulth method.[24] Under general anesthesia, the inhalant isoflurane (Sinopharm, Beijing, China) was used for anesthesia (4 L/min for induction anesthesia; 1.5 L/min for maintenance anesthesia). Thereafter, the rabbit was immobilized in the supine position, and a 10-mm longitudinal incision was made on medial right posterior knee to peel off the medial collateral ligament and expose the joint cavity for the resection of the medial meniscus. In addition, both anterior and posterior cruciate ligaments were also incised, and then the anterior drawer test was conducted to confirm ligament rupture. Careful maneuvering was required during the procedure for

avoiding damage to the articular cartilage, and the resection was closed layer by layer after resection. Rabbits in the control group only underwent skin incision at the same location. Perioperative antibiotic prophylaxis with intramuscular penicillin (400 000 U/d) (Sinopharm, Beijing, China) was given to rabbits for 3 days.

Animal Treatments

In accordance with *Experimental Acupuncture Science*,[25] the rabbits in the treatment group received treatment at six specific acupoints in the right knee joint, including Neixiyan (EX-LE4), Dubi (ST35), Xuehai (SP10), Zusanli (ST36), Hexiang (LI4) and Yanglingquan (GB34). After strict sterilization, the needles (length: 25 mm, diameter: 0.25 mm; Jiangxi Huatuo Medical Equipment Co., Ltd, China) were inserted into each point and left in place for 20 min. Treatment was performed once daily for four weeks. While rabbits in the control and OA groups did not receive any treatment. After the treatment was completed, euthanasia was performed in accordance with animal welfare and ethical guidelines. The procedure involved the induction of anesthesia using ketamine and xylazine, followed by intravenous administration of 1 mL of potassium chloride (15% w/v).[23] Subsequently, we harvested blood, pineal gland and knee joint samples in later analysis and evaluation.

Behavioral Assessment

Lequesne MG

According to the experimental design, each rabbit was scored once four weeks after surgery and once four weeks after treatment. The behavioral performance of each rabbit was subdivided into four key dimensions of pain, joint mobility, gait, and joint swelling according to the assessment criteria of the Lequesne MG scoring scale.[26] Based on the four dimensions mentioned above, specific scores were assigned according to the Lequesne scale, and the scores for each item were recorded for a comprehensive assessment. To be specific, animal behaviourists unrelated to this study were invited to score all rabbits on the Lequesne MG. After the scoring was completed, the scores of the three groups were averaged.

Pain Assessment by Hot Plate

The rabbits were placed in a Plexiglas cage for half an hour each day for 1 week before the experiment, so that they were acclimatized to the experimental environment. On days 28, 35, 42 and 56, the pain threshold was determined by observing the latency of the rabbit's injurious response. In brief, the rabbits were placed on the surface of a metal hot plate at a set temperature of 50°C. Notably, it should be ensured that the Plexiglas cage was suitable for the hot metal surface. A timer was started at the same time when the rabbit was put onto the hot plate, with first hind paw lick latency being recorded to the nearest 0.01 second. The above steps were repeated thrice for each rabbit to take the average, with no less than 40 min between two measurements for the same test subject.[27] Rabbits that exhibited unsettling behaviors, such as avoidance and jumping, during the course of the experiment were removed. Before starting the experiment for each rabbit, it must be ensured that the temperature of the metal plate matched that of the test chamber, and the surface of the test plate should be cleaned to minimize the influence of biosignals and urine on the experimental results. To avoid any type of tissue damage, a maximum cut-off time of 35 seconds was set for all experimental groups.

Hematoxylin and Eosin (H&E), Safranin O-Fast Green and MASSON Staining

First of all, the knee tissues of rabbits were immersed in 4% paraformaldehyde (Sinopharm Chemical Reagent Co., Ltd., Beijing, China) for a 24-h. After fixation, samples underwent decalcification with the 10% ethylenediaminetetraacetic acid (EDTA) (Sinopharm Chemical Reagent Co., Ltd., Beijing, China) decalcification solution. Next, the samples were subjected to dehydration, wax immersion and sagittal embedding, and later sliced to the 3-μm sections for H&E, Safranin O-fast green and MASSON staining.[28] At last, knee cartilage section staining was visualized using the light microscope (Olympus, Japan) at the 200x magnification.

Osteoarthritis Research Society International (OARSI) Score and Mankin Score

After routine rabbit knee joint embedding, the samples were cut into coronal sections. Following H&E staining treatment, the sections were observed and scored by a microscope. The OARIS scoring system evaluates articular cartilage damage

and osteophyte generation and covers four key articular surfaces: the medial femoral condyle, the lateral femoral condyle, the medial tibial plateau and the lateral tibial plateau (Supplementary Table 1).[29] Every section was scored and summed to obtain an overall score. Moreover, the Mankin score was processed in a similar manner to OARSI score, but the evaluation criteria for the Mankin score focused on cartilage structure, chondrocytes, matrix staining, and the severity of the tidal line injury.[30] The scoring process was carried out independently by professionals unaffiliated with the present experiments, and the final results from three measurements were averaged as the final score.

Chondrocytes Isolation

In the isolation of articular cartilage, it was first rinsed with PBS buffer containing 1% antibiotic mixture (Corning, USA) and then the articular cartilage was cut into small pieces. Next, the cartilage pieces were digested using type II collagenase (Sigma-Aldrich, USA) for 6 hours. At the end of digestion, the supernatant was filtered and centrifuged for 5 min, rinsed twice with ice-cold PBS buffer, and finally the cells were resuspended in DMEM F12 medium containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA) and 1% antibiotic mixture.[31]

Enzyme-Linked Immunosorbent Assay (ELISA)

Blood was sampled in abdominal aorta from five rabbits in each group and stood under ambient temperature for 20 min to allow for natural blood clotting. Subsequently, blood was subjected to 10 min of centrifugation at 3000 rpm to collect the upper serum layer for assay. Corresponding ELISA kits (Rabbit cAMP: F4108-A, Spec. 96 Test; Rabbit MT: F0015-A, Spec. 96 Test; Rabbit MMP-3: F4090-A, Spec. 96 Test; Vankyo, Shanghai, China) were utilized to detect MLT and matrix metalloproteinase-3 (MMP-3) levels in rabbit serum, MLT and cAMP levels in cartilage, and MMP-3 level in knee joint fluid. Absorbance was read with the plate reader (Prang DNM-9602, China) at 450 nm for quantitative analysis.

Real-Time Polymerase Chain Reaction

Chondrocyte and pinealocyte RNA were separated with the Animal Tissue RNA Extraction Kit (Foregene, Beijing, China) in line with specific instructions. Later, complementary DNA (cDNA) synthesis was completed through reverse transcription with the PrimeScrip RT kit (TaKaRa, Beijing, China). Then, using GAPDH as the reference quantification standard, the mRNA expression levels of MT1, MT2, and AANAT were determined using MagicSYBR Mix (Kangwei Century, Beijing, China) on the Q7 Fluorescence Quantitative PCR Instrument (Bio-Rad Life Sciences Products (Shanghai) Co., Ltd., Singapore). All primers were prepared in Shanghai Bioengineering Co., Ltd. and purified with ULTRAPAGE. Table 1 displays primer sequences. The $2^{-\Delta\Delta CT}$ approach was employed for data analysis.[32]

Western Blotting (WB) Assay

One hundred milligrams of chondrocytes were taken and ground with liquid nitrogen, then placed on ice and lysed using the RIPA lysis buffer (Biyuntian, Nanjing, China) for 20 min. Later, the cells underwent 10 min of centrifugation at 12,000 rpm under low temperature to collect supernatants. Protein contents were quantified by bicinchoninic acid (BCA) approach. In brief, 30 μg proteins were taken prior to heating within the 100°C metal water bath for 10 min, then separated using 10% sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE), followed by transfer

Table 1 Primer Sequence

Gene	Forward	Reverse
GAPDH (165bp)	5′-CGAGACACGATGGTGAAGGT-3′	5′- GCCGTGGGTGGAATCATACT-3′
AANAT (90bp)	5′-TGAGATTGAGCGAGAAGCCTT-3′	5′-TCTGGACACAGGGTGAGGAAG-3′
MT1(107bp)	5′- ACGAGCCTTGCGGAGTCTT-3′	5′- TGGCACAGGTGCAGGAGTT-3′
MT2(84bp)	5′- CGGGCTCTTTGCCGCTATA-3′	5′- CCGCTGAAGAGATCGCTAGG-3′

onto the 0.45-μm polyvinylidene fluoride (PVDF) membrane (Ebendor, USA). Subsequently, after blocking with 5% milk powder, the membrane was incubated with primary antibodies including β-tubulin (Zhongsui Jinqiao TA-10, China, 1:2000), PKA (BIOSS (bs-0520R), China, 1:1000), and CREB (Proteintech (67927-1-1G), China, 1:20,000) under 4°C overnight. Later, PVDF membrane was rinsed using Tris buffered saline Tween (TBST, Zs-BIO, China) (5 min each time, 3 washes in total), then incubated with secondary antibodies including Goat Anti-Rabbit IgG (ZB-2301, China, 1:5000) and Goat Anti-Mouse IgG (ZB-2305, China, 1:5000) for 2 h, and washed with TBST (5 min each time, 3 washes in total). Finally, the images were developed with the ultrasensitive ECL (Thermo Fisher Scientific, MA, USA) luminescent solution, while protein band images were collected and analyzed by an ImageQuant LAS4000mini image system. Relative grayscale values were calculated with Image J software and target protein levels were measured with β-tubulin being an internal reference.[33]

Statistical Analysis

IBM SPSS 26.0 (IBMCorp., Armonk, N.Y., USA) was employed for statistical analysis, whereas graph drawing was implemented with GraphPad Prism 8 software (GraphPad Software, San Diego, CA). Experimental results were indicated by mean ± standard deviation (SD). Every experiment was repeated strictly thrice independently. Normality and variance chi-square tests were performed on all datasets. When the data met the conditions of variance chi-square and normal distribution, we conducted one-way ANOVA and Least Significant Difference (LSD) tests for comparing between-group differences. Otherwise, Kruskal–Wallis independent sample test was chosen if the data failed the variance chi-square and normality tests. $P < 0.05$ stood for statistical significance. For the full data of the experiment, see Supplementary Tables 2–12.

Results

Model Validation

On day 28 during modeling, the Lequesne MG scores of OA and control groups were markedly different from control group ($P < 0.001$), but OA group was not significant from treatment group ($P = 1$), validating the successful model construction. After 28 days of treatment, the cartilage tissues of rabbit knee joints were analyzed by H&E staining, MASSON staining and immunohistochemistry. Consequently, articular surface in OA group was severely worn out, part of the articular surface was bare, and the collagen deposition was significantly reduced, consistent with the pathological characteristics of KOA. In contrast, cartilage damage of treatment group was mitigated relative to KOA group, accompanied by increased collagen deposition.

Acupuncture Improves the Behaviors of KOA Rabbits

The behaviors of KOA rabbits were evaluated by Lequesne MG score and pain assessment through hot plate, so as to investigate the effect of acupuncture on pain and overall condition of KOA rabbits. As revealed by the results of Lequesne MG score, the Lequesne MG score in OA group apparently increased relative to control group ($P < 0.001$). After intervention for 4 weeks, Lequesne MG score in treatment group significantly decreased ($P < 0.001$) compared with OA group (Figure 1a). From the results of the pain assessment by hot plate the pain threshold of the OA group slowly decreased over time, whereas that of treatment group elevated. At 14 days following treatment, the score was significantly different between treatment and OA groups ($P < 0.001$) (Figure 1b). Based on these above findings, acupuncture intervention significantly elevates the pain threshold, reduces pain and further improves the walking ability and localized swelling of the knee joints in rabbits.

Acupuncture Improves the Pathological Characteristics and Mankin and OARSI Scores of Knee Cartilages in Rabbits with KOA

KOA mainly exhibits corresponding clinical symptoms by accumulating alterations of subchondral bone and articular cartilage. Therefore, the degrees of cartilage wear and tear and subchondral bone exposure can be effectively assessed by the knee joint degeneration degree evaluated through staining. The results of H&E, Safranin O-fast green and MASSON staining revealed no apparent defects on cartilage surface of control group; meanwhile, the chondrocytes were neatly arranged

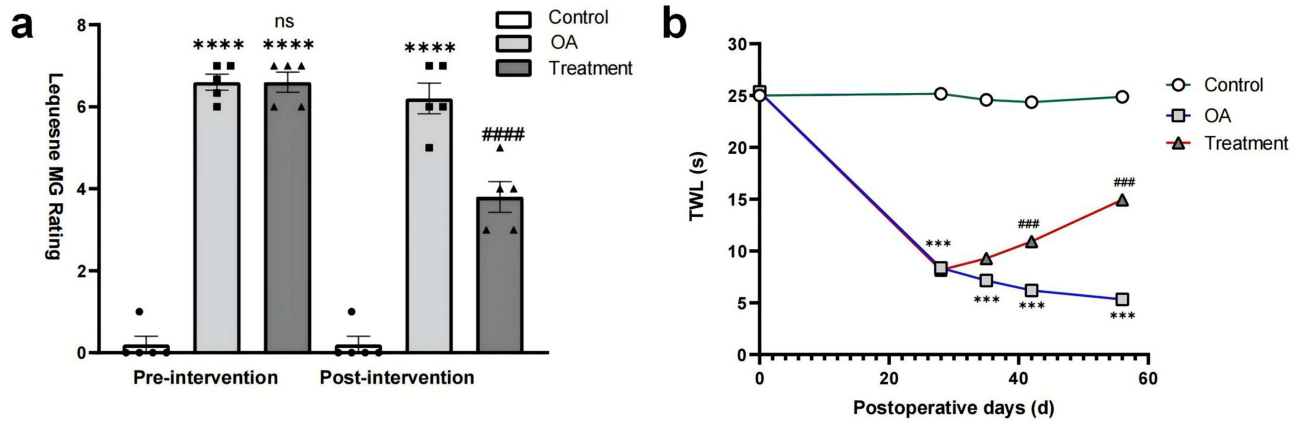

Figure 1 Improvement of behavioral performance of KOA rabbits by acupuncture treatment. (**a**) The effect of acupuncture treatment was assessed by the Lequesne MG Rating Scale (N=5 per group); (**b**) the improvement of pain thresholds by acupuncture was assessed by the hot plate method (N=5 per group); All data are expressed as mean ± standard deviation (mean ± SD). # represents comparison with the control group and * represents comparison with the OA group. *** or ### means p<0.001, **** or ##### means p<0.0001.

and undisturbed, and the cartilage structure was clear with normal cell morphology. In the OA group, the chondrocytes were aggregated, cracked, irregularly arranged, the cell morphology was incomplete, with blurred tide lines and partial exposure of subchondral bone (Figure 2). In comparison, cartilage surface of treatment group was smooth compared with OA group, the cells were more neatly arranged in columnar arrays, the overall cellular morphology was mostly normal although some chondrocytes appeared to be defective, with repetitive tidemarks, and the subchondral bone was not obviously exposed. According to the results of the Mankin scores, OA group was significantly different from control group after treatment ($P < 0.001$). In addition, OA group was apparently different from treatment group ($P < 0.001$) (Figure 3a). OARSI score came to the same results as those of Mankin score ($p < 0.001$; $p < 0.001$) (Figure 3b), suggesting that acupuncture treatment significantly improves the structure and function of knee cartilages and effectively delays the further progression of KOA.

Acupuncture Promotes the Expression of MLT/cAMP/PKA/CREB Pathway in KOA Rabbits

For investigating analgesic mechanism in acupuncture, we hypothesized that it might be mediated through the MLT/cAMP/PKA/CREB pathway. To verify this hypothesis, immunohistochemistry, RT-PCR and Western blotting assays were carried out for examining MLT level in serum, the level of AANAT in the pineal gland, and MT1, MT2, cAMP, PKA, and CREB levels in chondrocytes. From RT-PCR assay, AANAT, MT1 and MT2 mRNA expression were up-regulated in OA group relative to control group ($P = 0.559$; $P < 0.001$; $P = 0.010$). Meanwhile, AANAT, MT1 and MT2 mRNA expression also increased in treatment group relative to OA group ($P = 0.065$; $P = 0.003$; $P = 0.014$) (Figure 4a, c and d). Based on the ELISA results, relative to control group, OA group showed significantly lower ($P < 0.001$) MLT and cAMP ($P < 0.001$) contents. Besides, MLT and cAMP content markedly increased in treatment group relative to OA group ($P < 0.001$; $P = 0.011$) (Figures 4b and 5c). Western blotting results revealed that the PKA and CREB protein expression dramatically decreased in OA group compared with control group ($P < 0.001$; $P = 0.004$) but remarkably increased in treatment group relative to OA group ($P = 0.049$; $P = 0.024$) (Figure 5a, d and e). These findings illustrate that acupuncture may relieve knee pain and inhibit the expression of inflammatory factor by up-regulating MT1, MT2, cAMP, PKA and CREB in KOA chondrocytes.

The Effect of Acupuncture on the Inflammatory Factor MMP-3 in the Knee Joints of Rabbits with KOA

As shown in Figure 5b, the content of MMP3 in knee joint fluid markedly elevated in OA and treatment groups compared with control group ($P < 0.001$). Besides, its content declined in treatment group compared with OA group ($P < 0.001$). These results indicate that acupuncture therapy effectively reduces inflammatory response that affects the progression of KOA.

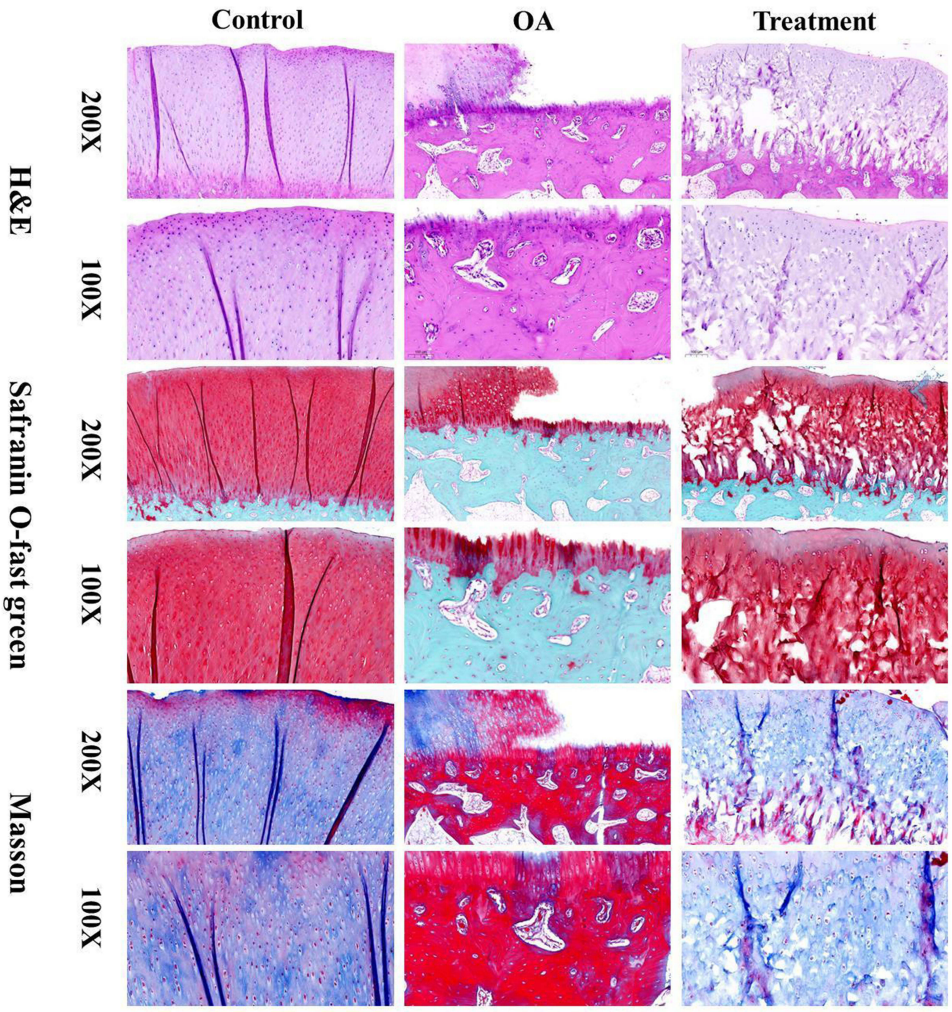

Figure 2 Effect of acupuncture on the pathology of the knee joints of rabbits with KOA induced by the traditional Hulth method. The pathological characteristics of the knee joints of rabbits with KOA were observed by H&E, Safranin O-fast green and MASSON staining (scale bar = 200 μm and 100 μm). H&E, Safranin O-fast green and MASSON staining showed that the articular cartilage in the OA group was severely worn out, glycosaminoglycan was severely lost, collagen fibers were lost, and the subchondral bone was exposed, and the treatment group significantly improved the above phenomena.

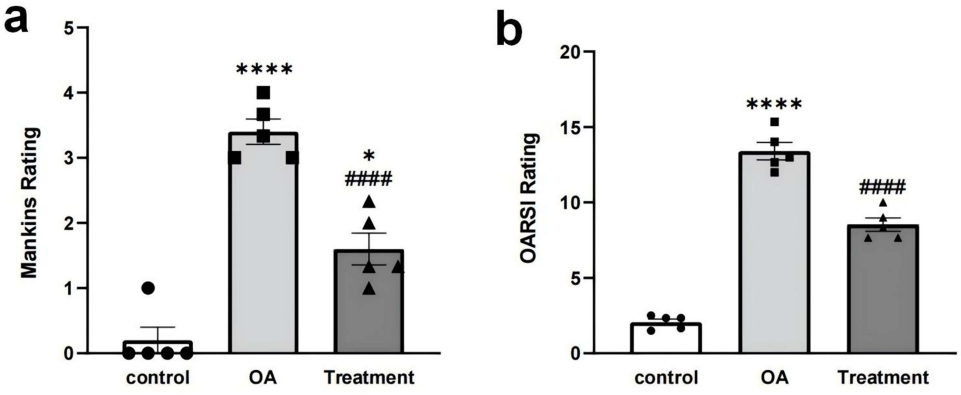

Figure 3 Improvement of histologic manifestations in KOA rabbits by acupuncture treatment. (**a**) The effect of acupuncture treatment was evaluated at the micro level based on the Mankin score (N=5 per group); and (**b**) the effect of acupuncture was evaluated at the micro level by the OARSI score (N=5 per group). # represents comparison with the control group and * represents comparison with the OA group. **** or #### means p<0.0001.

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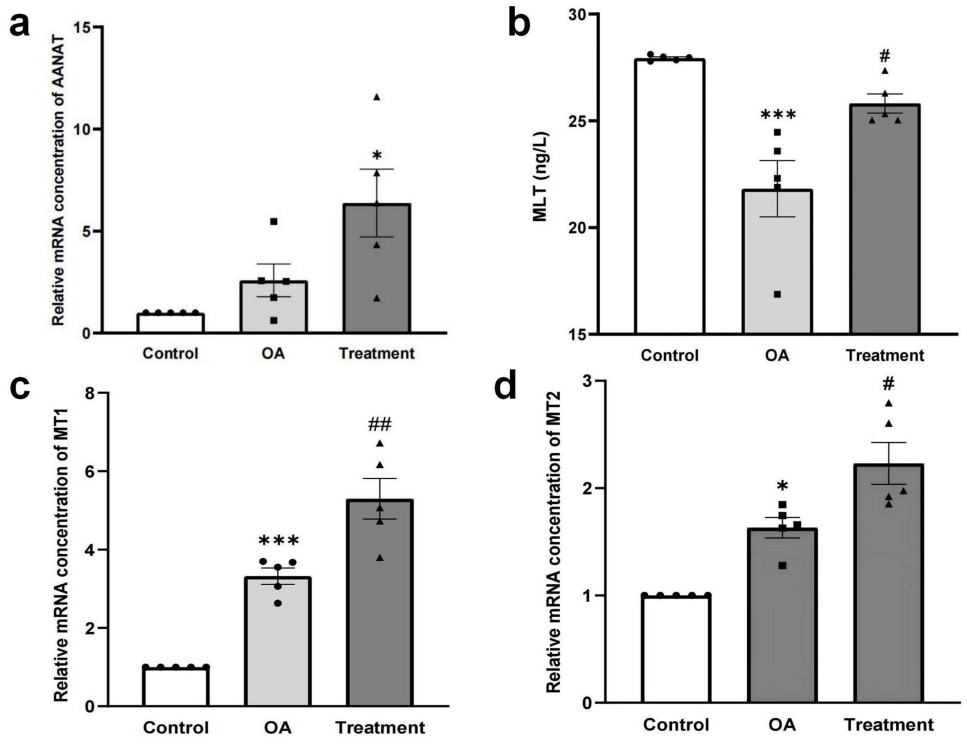

Figure 4 Effect of acupuncture on MLT synthesis. (**a**) mRNA expression of AANAT in rabbit pineal gland was evaluated using RT-qPCR (N=5 per group); (**b**) MLT content in serum was determined using ELISA (N=5 per group); (**c** and **d**) mRNA expression of MT1 and MT2 in knee cartilage tissue was evaluated using RT-qPCR (N=5 per group). # represents comparison with the control group and * represents comparison with the OA group. * or # means p<0.05, ## means p<0.01, *** or ### means p<0.001.

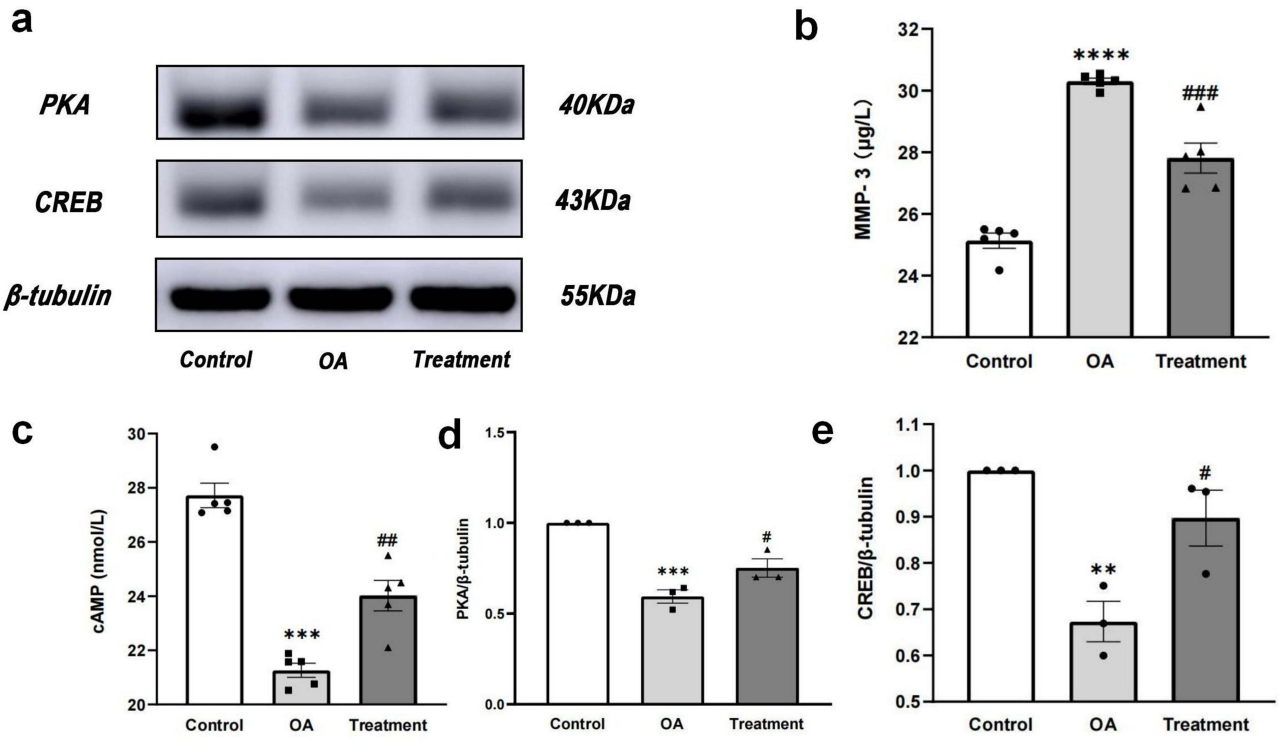

Figure 5 Effect of acupuncture on MLT/cAMP/PKA/CREB signaling pathway induced by traditional Hulth method in KOA rabbits. (**a**) Western blot detection of PKA, CREB protein expression in cartilage tissues; (**b**) Determination of MMP-3 in joint fluid using ELISA (N=5 per group); (**c**) Determination of cAMP in chondrocytes by enzyme-linked immunosorbent assay (N=5 per group); (**d** and **e**) Data analysis of PKA, CREB protein expression in cartilage tissues by Western blot testing (N=3 per group). # represents comparison with the control group and * represents comparison with the OA group. * or # means p<0.05, ** or ## means p<0.01, *** or ### means p<0.001, **** or ##### means p<0.0001.

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Discussion

The analgesic effects of acupuncture have been widely recognized, but the molecular mechanisms underlying pain control remain unclear. In the present study, the KOA model was induced using the Hulth method, and it was found that after acupuncture treatment, AANAT content in the rabbit pineal gland, MLT level in serum, and MT1 and MT2 protein expression levels in cartilage significantly increased. In addition, the PKA/CREB signaling pathway was up-regulated, while the expression of MMP-3, a protein associated with inflammation, was down-regulated. These results suggest that acupuncture may ultimately produce the biological effect of reducing KOA pain by affecting the excitability of sympathetic nerves, activating AANAT, regulating the synthesis and secretion of MLT, and promoting the protein and gene expression of factors associated with the MLT/cAMP/PKA/CREB signaling pathway.

KOA is a disease characterized by degenerative changes in the joints, resulting in damage to the articular cartilage. Pain is the main clinical manifestation of the disease, while inflammation of the synovial membrane and the infrapatellar fat pad and biomechanical instability are the main factors leading to medial knee pain.[2,34] With the continuous progress of modern medicine, more and more treatments are available for KOA.[35,36] Notably, acupuncture is widely recognized and applied in the treatment of KOA pain due to its significant efficacy, non-invasiveness, and minimal side effects, but the underlying molecular mechanisms remain unclear. Several studies have reported that acupuncture has favorable anti-inflammatory and analgesic effects, which may be related to its ability to reduce MMP-3 expression.[37,38] It is suggested that reducing the expression of local inflammatory factors can effectively reduce the pain response. Specifically, large amounts of inflammatory cytokines are present in the knee joints of KOA patients. These factors not only lead to tissue damage and cell death, and release endogenous pain mediators such as ATP and uric acid to exacerbate pain,[39,40] but also effectively activate MMP-3 expression. Overexpression of MMP-3 can directly affect the structure of articular cartilage and may contribute to the degradation of the major structural proteins (like proteoglycan and type II collagen (Col 2a1)) in the articular cartilage, which in turn aggravates the progression of KOA and causes more severe cartilage degradation and pain.[41,42] In this experiment, the medial knee cartilage in the OA group showed significant wear and tear, accompanied by overexpression of MMP-3, similar to the structure of the joint microenvironment in patients with KOA.[43,44] In addition, the MMP-3 expression within joint fluid in treatment group remarkably decreased compared with OA group, and these results were consistent with previous experimental observations.[45]

MLT is a key biological rhythm-related molecule, and it exerts an important effect on regulating seasonal oestrus and circadian rhythms in mammals.[46] Its secretion is not only regulated by the photoperiod, but also by sympathetic influences that are involved in pain regulation. MLT synthesis begins with the conversion of tryptophan, which first produces 5-hydroxytryptophan, and then 5-hydroxytryptamine. Catalyzed by AANAT, 5-hydroxytryptamin is further converted to N-acetyl-5-hydroxytryptamine, and finally MLT is synthesized. Sympathetic nerve impulses and the synthase AANAT exert crucial regulatory effects on the synthesis of MLT.[47] As suggested by domestic and foreign studies on the biological function of MLT, MLT, as a pain mediator, has a close relationship with nociceptive sensitization, and possesses a significant analgesic effect.[48] In this paper, we examined whether acupuncture affected the biological function of MLT. To verify whether acupuncture affected the synthesis of MLT, we tested AANAT, cAMP, MLT, MT1 and MT2 expression. As a result, acupuncture was able to effectively promote MLT generation and MT1 and MT2 gene levels. This may be due to the continuous activation of the sympathetic excitatory state after acupuncture at specific acupoints, leading to the release of norepinephrine (NE) from the postganglionic nerves of the sympathetic nerves. NE can stimulate the activity of adenylate acylase and promote the conversion of ATP into cAMP through activating the β-adrenergic receptor, which then binds to GS proteins. In the presence of cAMP, AANAT is activated to catalyze the generation of N-acetyl-5-hydroxytryptamine from 5-hydroxytryptophan, which ultimately synthesizes MLT.[49,50] Thereafter, MLT binds to MT1 and MT2 receptors on the membranes of knee joint chondrocytes, thus inhibiting the release of the excitatory neurotransmitter glutamate and promoting the activation of the endogenous analgesic endorphin system.[51] This results in the alleviation of knee joint pain. This phenomenon also explains the persistent increase in pain threshold of the treatment group and the opposite trend of the OA group in the thermal pain sensitization experiment. Recently, MLT is found to significantly protect the articular cartilage.[52,53] On the one hand, MLT can directly affect cartilage tissue and regulate chondrocyte proliferation, apoptosis, and endochondral bone activity; on the other hand, it can co-incubate with bone marrow-derived mesenchymal stromal stem cells (MSCs), leading to up-regulation of basic fibroblast growth factor and hepatocyte growth factor.[54] The adult fibroblast

growth factor not only promotes chondrocyte proliferation, but also induces MSCs to repair cartilage defects. This accounts for the lower degree of cartilage damage in treatment group compared with OA group in this experiment. Although molecular mechanisms of MLT in analgesia and cartilage protection are still under intensive investigation, the PKA/CREB pathway and its related active components may be the main mechanisms of acupuncture in relieving KOA pain and delaying cartilage degeneration in the knee joint. Previously, the cAMP/PKA/CREB pathway has been commonly adopted in the study of neurodegenerative diseases and cancer, and previous studies generally conclude that this pathway promotes the expression of inflammatory factors and aggravates the disease process. However, cAMP/PKA/CREB pathway has a complex effect and may even exhibit pro-inflammatory or anti-inflammatory effects on different types of tumors.[21] According to our results, cAMP, PKA, and CREB levels remarkably increased in treatment group compared with OA group, accompanied by a decrease in MMP-3 expression, conforming to the previously reported trend.[55] Further studies show that overexpression of MT1/2 gene within Bactrian camel ovarian granulosa cells activates cAMP/PKA/CREB pathway.[56] When the MT1 gene is overexpressed, GNB2 protein onto cell membrane is activated, leading to down-regulation of the inhibitory housekeeping gene GNB2, which can thus activate adenylyl cyclase on cell membrane for catalyzing pyrophosphate dissociation from ATP, thus producing cAMP. However, in our experiments, MT1 and MT2 were overexpressed in rabbits after acupuncture, and their downstream genes including cAMP, PKA, and CREB were also up-regulated. This may be due to the fact that acupuncture stimulation promotes cAMP expression, and it can bind to four sites on the PKA regulatory subunit and in turn activates PKA. The activated PKA catalytic subunit can enter the nucleus to phosphorylate CREB.[57] CREB, as the regulatory protein, can bind to CREB-binding protein (CBP) to cause chromatin and shift from a closed state to an open state,[58] a process that is usually followed by an increase in RNA polymerase chain reaction (RPCR). This process typically promotes the binding of RNA polymerase II and basic transcription factors to open DNA to initiate transcription, often involving anti-inflammatory factor expression like interleukin-10 (IL-10).[59] The activation of IL-10 inhibits p38 MAPK and c-Jun N-terminal kinase (JNK) phosphorylation, thereby restricting MyD88 signaling pathway activation while decreasing MMP-3 expression.[60]

Collectively, we believe that the favorable efficacy of acupuncture in treating KOA pain may be associated with its effect on activating MLT/cAMP/PKA/CREB signaling pathway. During acupuncture, the acupuncture feature points stimulate the sympathetic nerves, triggering the release of NE from the postganglionic nerves of the sympathetic nerves. Thereafter, NE activates the β-adrenergic receptor, which facilitates the conversion of ATP to cAMP. cAMP affects physiological processes via two main pathways: for one thing, cAMP activates AANAT, which catalyzes the conversion of 5-hydroxytryptophan to N-acetyl-5-hydroxytryptophan; for the other thing, the elevated expression of cAMP activates PKA, prompting its catalytic subunit to enter the nucleus and phosphorylate CREB, which, when combined with the coactivator CBP, promotes the expression of IL-10 and reduces that of MMP-3.

However, this research still has some limitations, especially in the bidirectional modulation effect of acupuncture on the MLT/cAMP/PKA/CREB signaling pathway and its reciprocal effects with other signaling pathways in KOA, which warrants deeper investigation.

Conclusion

According to the results in this study, by regulating the sympathetic excitability, acupuncture may activate the MLT/cAMP/PKA/CREB signaling pathway, decrease inflammatory factor levels and slow down articular cartilage degradation, finally relieving knee pain.

Abbreviations

KOA, knee osteoarthritis; MLT, melatonin; MT, melatonin receptor; cAMP, cyclic adenosine monophosphate; MMP-3, matrix metalloproteinase-3; AANAT, aromatic L-amino acid N-acetyltransferase; MTI, melatonin receptor 1; MT2, melatonin receptor 2; OA, Osteoarthritis; OARSI, Osteoarthritis Research Society International; H&E, Hematoxylin and eosin; ELISA, Enzyme-linked immunosorbent assay; RT-PCR, Real-time polymerase chain reaction; WB, western-blot; LSD, Least Significant Difference tests; NE, norepinephrine; IL-10, interleukin-10.

Data Sharing Statement

All data generated or analyzed during this study are included in this article and the Supplementary Information.

Ethics Approval

Animal studies were approved by the Institutional Animal Care and Use Committee of YSY Genentech (Tianjin) Co., Ltd. (Program No. YSY-DWLL-2023430). All the experiment methods were in accordance with ARRIVE guidelines.

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Disclosure

The authors declare no conflict of interest.

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