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Posttraumatic Osteoarthritis of Temporomandibular Joint in Miniature Pigs

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Abstract: The present study investigated the changes in Indian hedgehog (Ihh), periarticular cell-derived parathyroid hormone-related protein (PTHrP), and runt-related transcription factor 2 (Runx2) in the temporomandibular joint (TMJ) cartilage with posttraumatic osteoarthritis (PTOA). The miniature pigs were randomly divided into two groups: the experimental group (EG) (n=8) and the control group (CG) (n=4). The left side of EG had type B intracapsular fractures with anterior disc displacement (ICF+DD), while the right side only had type B intracapsular fractures (ICF), and the CG was a blank control. The production of Ihh, PTHrP, and Runx2 was detected by immunohistochemistry staining and real-time polymerase chain reaction (PCR) at weeks 4 and 12 post-surgery. The expression of Ihh, PTHLH/PTHrP, and Runx2 in the EG was significantly lower than that in the CG at 4 and 12 weeks after the operation (P<0.05). Moreover, significant differences were detected between ICF+DD and ICF (P<0.05). Ihh, PTHLH/PTHrP, and Runx2 proteins affect the endochondral osteogenesis of TMJ and play a significant role in PTOA. Our findings suggested that the interaction mechanism among Ihh, PTHLH/PTHrP, and Runx2 is activated when posttraumatic osteoarthritis (PTOA) occurs, but how they regulate each other remains to be investigated.

Key words: Indian hedgehog, Osteoarthritis, Periarticular cell-derived parathyroid hormone-related protein, Runt-related transcription factor 2, Temporomandibular joint

Introduction

The molecular biological mechanism of posttraumatic osteoarthritis (PTOA) is yet unclear. The studies on joint pathological changes have shown that damaging factors may impact local cartilage, resulting in decreased cell function and an imbalance of synthesis and decomposition. The cell differentiation ability declines and eventually leads to apoptosis. After decomposition, some substances are released and act on the cartilage matrix, causing degradation. The mandible is one of the critical anatomical structures of the human body. For instance, condylar cartilage is the central load-bearing part of the mechanical load with a key role in the ossification in the cartilage. Endochondral osteogenesis is essential for repair after fracture and is the primary source of callus. When a fracture occurs, mesenchymal stem cells become active and further differentiate to form cartilage membranes and chondrocytes. The latter have a proliferative effect and can secrete the cartilage matrix rich in type II collagen and proteoglycans. These chondrocytes cease to proliferate under stimulation signals and develop hypertrophy. The secreted matrix components are also altered, especially type X collagen. As the secretion of this matrix increases, the surrounding cartilage matrix continues to degrade, blood vessels proliferate and invade into the matrix, the chondrocytes begin to differentiate continuously, and a large number of osteoblasts appear. Finally, bone tissue completely replaces the cartilage. This complicated and precisely regulated process involves a variety of hormones and negative and positive regulators. Positive regulators include runt-related transcription factor-2 (Runx2), and negative regulators are parathyroid hormone-related proteins.

PTOA causes a series of pathological manifestations but currently lacks effective treatment methods. Most of these focus on symptomatic treatment. The application of non-steroidal anti-inflammatory drugs alleviate the patients’ pain symptoms but induce a range of side effects. Gastrointestinal reactions occur frequently. Also, it can cause cardiovascular disease. With the continuous development of the disease, the degree of temporomandibular joint (TMJ) damage has gradually developed and deformed, and the movement is restricted. If the effect of conservative treatment is not satisfactory, joint replacement surgery can be performed. Although surgery can improve symptoms, there are some problems, such as enormous surgical trauma, high requirements for artificial joints, and increased financial burden. Thus, PTOA may bring long-term irreversible pain to patients. If it is discovered promptly and reasonable measures are taken, the therapeutic effect can be improved significantly to curb the development of the disease and promote recovery. It has important clinical significance.

The present study detected the expression of Ihh, PTHLH/PTHrP, and Runx2 proteins in PTOA cartilage and quantitatively analyzed their
interaction in chondrogenesis and osteogenesis.

Materials and Methods

Experimental design

In this study, 12 miniature pigs, aged 2-3 months were selected, without restriction on gender. They were raised in separate cages 7 days before the start of the experiment. Those who had no prominent systemic diseases, no teeth loss, and abnormal eating were included in this study and randomly divided into two groups: experimental group (n=8) and control group (n=4). The left TMJ of the experimental group had intracapsular type B fractures with anterior disc displacement (ICF+DD), while the right side only had intracapsular type B fractures (ICF), and the control group did not do any treatment. The experimental protocol was approved by the Medical Ethics Committee of Stomatology Hospital Affiliated to China Medical University (K202009).

Surgical procedures

The pigs were anesthetized with 2.5% sodium pentobarbital before making a “crutch” incision in front of the ear. Then, the masseter muscle attachment along the base of the zygomatic arch was peeled off to fully expose the joint capsule that was cut open to enter the upper and lower joint cavities. The condyle and disc were exposed, and an osteotome was used to split the condyle from the lateral to the medial obliquely. The fracture line was located in the middle 1/3rd of the condyle, forming a type B intracapsular condylar fracture. Then, the anterior and posterior attachment of the disc on the left side was cut off, and the fracture block and disc forward anterior were pushed to establish a disc displacement model without reduction. The anterior attachment of the disc was retained on the right side, and the fracture fragment was only pushed forward anterior. The wound was rinsed with 0.25% chloramphenicol and saline, and the wound closure was sutured in layers. Computed tomography (CT) and magnetic resonance imaging (MRI) was performed immediately after the operation to confirm that the model was established successfully. Penicillin was injected intramuscularly for three days after surgery, and soft pellets were given. After seven days, the pigs were given regular feed.

Specimen collection and processing

A total of 24 joints were collected at 4 and 12 weeks postoperatively. The condyle was incised in the coronal position when the specimens were collected. The half of the condylar cartilage was placed in a 10% neutral formaldehyde solution for 48 h and then in ethylenediaminetetraacetic acid for decalcification, and the other half was stored in liquid nitrogen. Serial sections (5-μm-thick sections) were made at the coronal position and numbered for immunohistochemistry (IHC) staining. Finally, the condylar cartilage was stored in liquid nitrogen, total RNA was extracted, its concentration determined, and reverse transcribed into complementary DNA (cDNA) using a PrimeScript™ RT reagent Kit (TAKARA Bio, Inc., RR037A).

IHC

The sections were placed in an incubator (60℃) for 180 min and de-waxed in xylene and gradient ethanol sequentially. Subsequently, the treated sections were subjected to antigen retrieval by boiling at high heat for 20 min. Then, the sections were sealed with 3% H2O2-methanol treatment sections were subjected to antigen retrieval by boiling at high temperature for 20 min at room temperature. Then, 100 ml of the primary antibodies, Ihh (1:100; Antibodies-online Inc., ABIN2782160), PTHLH/PTHrP (1:200; Antibodies-online Inc., ABIN641289), and Runx2 (1:200; Abcam, ab76956) was added to the slices, respectively and incubate overnight at 4℃, followed by the addition of the enhancer at room temperature for 30 min. The universal IgG antibody-Fab segment-HRP polymer (Fuzhou Maixin Biotechnology Development Co., Ltd, KIT-9902) was dropped onto the slices and incubated at 37℃ for 30 min. The color was developed using DAB (Dako, Agilent Technologies Inc., K346711), counter-stained with hematoxylin, followed by dehydration and mounting. The protein expression in the tissue cells was observed under a microscope; three high-expression regions were selected to capture the images.

Real-time polymerase chain reaction (PCR) detection

Three representative marker genes (Ihh, PTHLH/PTHrP, and Runx2) were selected to assess the mandibular condyle endochondral ossification. Primer sequences are shown in Table 1. The specific detection steps of real-time PCR were as follows: 1) dilute the primers to 10 μM; 2) reaction system: cDNA template 1 μl, upstream primers (10 μM) 0.5 μl, and downstream primers (10 μM) 0.5 μl; 3) SYBR GREEN master mix 10 μl, make up the volume to 20 μl with ddH2O; 4) reaction conditions: 95℃ for 10 min, then 40 cycles of 95℃ for 10 s, 60℃ for 20 s, and 72℃ for 30 s, followed by 5 min incubation at 4℃. The fold-change in gene expression of interest was calculated using the ∆∆Ct method and normalized against that of GAPDH as the reference gene.

Statistical analysis

SPSS20.0 software was used for statistical analysis, and the measurement data were recorded as mean±SD. A one-way analysis of variance was used to compare indicators among the three groups, and LSD method was used for multiple comparisons between groups. Paired t-test was used to compare the two-time points within groups. P<0.05 indicates statistical significance.

Results

Expression of Ihh, PTHLH/PTHrP, and Runx2 proteins in condylar cartilage

Firstly, we used IHC staining to investigate the distribution and expression of Ihh, PTHLH/PTHrP, and Runx2 in the condylar cartilage of PTOA (Figs. 1–3). At 4 weeks after the operation, Ihh and Pthlh/Phtrp were uniformly distributed in the cytoplasm and expressed in the chondrocytes of each layer of condylar cartilage in the experimental and blank control groups. Then, the expression in the blank control group was more substantial than that in the experimental group. At 12 weeks after surgery, Ihh and PTHLH/PTHRP were mainly expressed in the proliferative layer in the experimental group, with a small amount was ex-

| Oligo Name | Sequence          |
|-----------|------------------|
| GAPDH-F   | ATCAAGAAGGTGGTGAGGAG |
| GAPDH-R   | CACGATCAGGATGAGGAGG |
| Ihh-F     | TCACAGCGCAACTCAACACA |
| Ihh-R     | CACGACCGACGACCATACT |
| PTHLH/PTHrP-F | CCTGCCGATTTGGTGCTG |
| PTHLH/PTHrP-R | GTCTCTTAGCTGGCTCTTT |
| Runx2-F   | GTTAGGCGATCCACCTTGAC |
| Runx2-R   | GTTCTGTGAGCCGATAGG |

Table 1. Primer sequences
pressed in the hypertrophic layer, while in the blank control group, these proteins were expressed in the proliferative and hypertrophic layers. Strikingly, the expression was weaker at 12 weeks than at 4 weeks after surgery.

Furthermore, at 4 weeks after the operation, Runx2 in the experimental and blank control groups was evenly distributed in the cytoplasm and expressed in the proliferative and hypertrophic chondrocytes of the condyle cartilage. Also, the expression in the control group was more potent than that in the experimental group. After 12 weeks, Runx2 was expressed in each layer of the experimental group but not in the hypertrophic layer of the control group. This expression was weaker than that observed at 4 weeks postoperatively.

**Relative quantitative analysis of Ihh, PTHLH/PTHrP, and Runx2 genes in condyle cartilage**

Next, we used real-time PCR to detect the content of Ihh, PTHLH/PTHrP, and Runx2 in the condylar cartilage (three replicate holes for each sample and each gene in parallel experiments, and data were selected according to the deviation, and average taken). The results showed that the expression of Ihh, PTHLH/PTHrP, and Runx2 mRNA in the experimental group was significantly lower than that in the control group at 4 and 12 weeks after the operation ($P<0.05$). However, no significant differences were detected between ICF+DD and ICF groups ($P>0.05$) (Figs. 4–6).

![Figure 1. Expression of Ihh in the condylar cartilage (Scale bars: 100 μm)](image1)

![Figure 2. Expression of PTHLH/PTHrP in the condylar cartilage (Scale bars: 100 μm)](image2)
Discussion

Posttraumatic osteoarthritis (PTOA) is one of the common types of osteoarthritis. According to statistics, >10–12% of the individuals experience cartilage damage. Intraarticular fractures, sports injuries, and osteoarthritis cause joint cartilage damage. Cartilage damage is challenging to recover naturally, and cartilage damage may be challenging. Therefore, it has drawn extensive attention from scholars to explore its natural evolution process. However, a consistent conclusion has not yet been obtained, and the pathogenesis needs to be further clarified. Typically, PTOA secondary to intraarticular fractures occurs due to the failure of anatomical reduction of the fracture fragments and postoperative joint instability. Although with the development and improvement of surgical operation and internal fixation technology, intraarticular fractures have been anatomically reduced, and the postoperative joints have also been stabilized, the incidence of PTOA is still high. One reasonable explanation is that the internal fractures are accompanied by damage to the cartilage. Consequently, many in vitro and in vivo cartilage injury models have been established to clarify the pathogenesis of PTOA. The
data suggested that a series of changes occur in the cartilage after injury, proteoglycan synthesis is affected, and the overall level of the proteoglycan is decreased, resulting in the changes in cartilage matrix, while the water content increases, which affect permeability. These changes can deform or rupture the connection between collagen fiber network and collagen-proteoglycan, causing matrix edema, cartilage cell damage, or cell death. However, the initiating factor leading to PTOA after cartilage injury is yet unknown. Also, the various structural and mechanical changes that occur after an injury need to be investigated further. If early detection and timely intervention are possible, it will inevitably delay the disease’s progression, terminate the evolutionary process, and even reverse the cartilage damage of TMJ(20). PTOA caused by an intracapsular fracture in children might influence the growth and development of mandible. Thus, it is essential to investigate the underlying pathogenesis to identify valid therapeutic targets.

Mandibular condyle cartilage is secondary fibrocartilage, which is structurally different from the growth plate and articular cartilage of long bones. Thus, it is susceptible to many factors, and various biological stimulations effectuate the changes resulting in different consequences. If appropriate, they will have a positive effect, promote cartilage development, and maintain typical structure and function; if not appropriate, they will cause structural modifications, pathological changes, and even affect function, resulting in TMJ degenerative disease. Kurio et al(10) found that conditional knockout of the Ihh gene in juvenile/adult mice damages the structure and function of cartilage progenitor cells and decreases the proliferation of cartilage progenitor cells and chondrocytes. Their findings indicate that cartilage progenitor cells continue to participate in the condyle’s growth after birth, and their structure and function depend on the signal transduction of the Ihh. Furthermore, Ihh signaling stimulates chondrocyte differentiation and maturation in cultured meniscus cells, as assessed by enhanced chondrocyte proteoglycan synthesis and alkaline phosphatase activity, respectively, and prevents this maturation process when Ihh is antagonized using hedgehog protein inhibitors(21). Consistent with these results, Yang et al(20), pointed out that Phtr1a−/−, Smo−/− mice showed that inhibited Ihh signaling in osteoarthritis-like TMs prevents chondrocytes from undergoing terminal differentiation through a Phtr1-dependent mechanism. Thus, it is inevitable to conduct further research to ascertain the potential pathophysiology activation of Ihh and PTHrP signals in osteoarthritic TMs.

Parathyroid hormone-related protein (Pthrp) is critical to maintaining the microenvironment and is strictly associated with calcium and phosphorous metabolism. As a vital calcium-regulating hormone, Pthrp plays an irreplaceable role and directly determines the growth capacity of the bone(15,16). In addition, Pthrp can promote calcium deposition and accelerate bone formation, while on the other hand, it exerts an inhibitory role and creates a counterbalance between osteoblast and osteoclast activity. If PTH is injected in the body, it will affect osteoblasts. Different injection methods provide different results. Previous studies have found that intermittent injection of PTH significantly increases the degree of mineralization, while continuous injection has an inhibitory effect, resulting in slow growth and bone resorption(15,16). The condylar process is the mandible growth and development center promotes bone growth via endochondral osteogenesis. At the time of development, chondrocyte components differentiate, cell number increases, and cells mature, resulting in hypertrophic chondrocyte, and gradually forming osteoblasts and osteocytes during the process of continuous transformation. The structure of mandible changes and the mandibular branch grows continuously. Ihh plays a crucial role in this process, elevates the expression of PTHrP, supports cell proliferation, inhibits hypertrophy, maintains the proliferation state, and inhibits terminal differentiation of cells. If the stimulation of PTHrP is weakened, the secretion of Ihh increases, the feedback effect is restored, and the expression of PTHrP increased. Consequently, the chondrocytes are coordinated to maintain the balance between proliferation and maturation(18).

Runx2 is a member of the Runt family of transcription factors. As a cytokine, Runx2 is derived from multifunctional mesenchymal stem cells, which has a regulatory effect and is closely related to bone formation. Runx2 exists as a transcriptional regulator, induces osteoblast differentiation, promotes chondrocyte maturation, plays an essential role in bone formation, and is an indispensable part of osteocyte differentiation. It also regulates mineralization-related protein genes, promotes gene transcription of pre-osteoblasts and chondrocytes, differentiates gradually, and effectuates bone formation. Hinoi et al(19) determined the transcription basis for the inhibition of chondrocyte maturation by perichondrium through experimentation and revealed an antagonistic role of Runx2 in chondrogenesis. In 2019, Liao et al(20) deleted Runx2 in chondrocytes in postnatal mice and assessed the consequences of TMJ cartilage growth and remodeling. The study also demonstrated that Runx2 is required for chondrocyte proliferation and hypertrophy in TMJ cartilage and postnatal TMJ cartilage growth and homeostasis as well as the regulation of chondrocyte-derived subchondral bone remodeling.

Herein, we found that the normal cartilage structure gradually becomes disordered and may disappear over a period after the fracture, and the degeneration of the subchondral bone becomes apparent. Then, the condyle is directly exposed to the glenoid fossa in the ICF+DD side at the advanced stage of PTOA, and the stress in the joint cavity changes, which in turn decreases the number of chondrocytes in the hypertrophic layer and thinning of this layer; simultaneously, the number of cells in the proliferative layer is also decreased considerably, and the proliferative layer becomes thinner(21). By analyzing the results of ICH, it was found that in the early stage of PTOA, Ihh and PTHLH/PTHrP were expressed in the cytoplasm of each layer of chondrocytes, but the expression was weaker than that in the control group. On the other hand, in the vicinity of the chondrocyte clusters, the expression of Ihh and PTHLH/PTHrP in the naive chondrocytes is slightly stronger. In the late stage of PTOA, Ihh and PTHLH/PTHrP are still expressed in the proliferative and hypertrophic layers; however, the expression is relatively weak. It can be observed that Ihh and PTHLH/PTHrP play a significant role in the process of TMJOA cartilage repair by regulating cartilage proliferation and promoting structure and function recovery; this function is similar to the regulatory role of these proteins in endochondral osteogenesis, i.e., promoting chondrocyte proliferation and inhibiting its differentiation and apoptosis(22). Furthermore, Runx2 is expressed in the cytoplasm of the proliferative and hypertrophic layers at the early stage of PTOA, albeit the expression is weak in each layer of the disordered cartilage, which might relate to the formation of late osteophytes. In addition, real-time PCR also showed that expressed Ihh, PTHLH/PTHrP, and Runx2 mRNA gradually decreased over time, which was evident in the experimental group (P<0.05). At 4 and 12 weeks after surgery, the expression of Ihh, PTHLH/PTHrP, and Runx2 was the lowest in the ICF-B+DD side, followed by the ICF-B side (P<0.05). The results were consistent with those of IHC.

Under normal circumstances, Ihh and PTHLH/PTHrP form a negative feedback loop to control the proliferation and differentiation of chondrocytes. Nevertheless, in this study, this loop is out of adjustment. One reason might be that after the condyle cartilage structure is disordered, the potential of chondrocytes to proliferate and the hypertrophy
are disrupted. The other reason may be that the regulation among these factors depends on other pathways or modes.

In conclusion, Ihh, PTHHL/PTHrP, and Runx2 proteins affect the endochondral osteogenesis by influencing the proliferation and maturation of TMJ condyle chondrocytes and play a critical role in the occurrence and development of PTOA, which has been rarely mentioned in previous literature on PTOA.

The expression of Ihh, PTHHL/PTHrP, and Runx2 was downregulated with the increase in PTOA degree and was notably downregulated in PTOA accompanied by anterior disc displacement. This could be attributed to the interaction mechanism among Ihh, PTHHL/PTHrP, and Runx2 simultaneously when PTOA occurs, but how they are regulated is yet to be investigated.

In recent years, the investigation on the subchondral bone of osteoarthritis has become a hot topic, which is not addressed in the present study. Therefore, the prospective research direction would focus on the mechanism of cartilage and subchondral bone interaction.

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Conflicts of Interest

The authors have declared that they have no conflict of interests.

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