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PTH promotes rabbit tibial fracture healing *via* the Notch signaling pathway

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Abstract. – OBJECTIVE: To explore the effect of parathyroid hormone (PTH) on the expression of Jagged1 in the rabbit tibial fracture healing, and its function and mechanism in this process *via* the Notch signaling pathway.

MATERIALS AND METHODS: A total of 60 New Zealand white rabbits were randomly divided into control group (n=30) and experimental group (n=30). Then, a rabbit tibial fracture model was established. After surgery, the rabbits in experimental group were given 10 μg/kg PTH (1-34) once a day for 5 days a week, while those in control group were given an equal volume of normal saline. Six rabbits were randomly selected from each group at 1, 2, 3, 4, and 6 weeks after surgery to collect right tibia specimens. Next, X-ray examination, bone mineral density (BMD) test, histological detection, and serum biochemical test were performed. Additionally, the messenger ribonucleic acid (mRNA) expression levels of Notch1 and Jagged1 in the Notch signaling pathway were measured *via* polymerase chain reaction (PCR) assay. Their protein levels were detected through Western blotting analysis.

RESULTS: The healing and BMD in experimental group were better than those in control group since cortical and medullary bridging was observed in the rabbits of experimental group at the 6th week after surgery. Plasma level of alkaline phosphatase (ALP), P content, and the product of Ca and P significantly increased ($p<0.05$) in experimental group. The pathological morphology of the calluses stained with hematoxylin-eosin (HE) in experimental group was overtly superior to that in control group. The PCR results revealed that both mRNA and protein levels of Notch1 and Jagged1 were lower in control group than those in experimental group ($p<0.05$).

CONCLUSIONS: PTH (1-34) promotes the rabbit tibial fracture healing by regulating Jagged1 ligand molecules in the Notch signaling pathway.

Key Words:
PTH, Notch signaling pathway, Jagged1, Tibial fracture, Fracture healing, Rabbit.

Introduction

Fracture healing is a very important, complex, slow and long-term pathological process after fracture, involving chemotaxis and accumulation of mesenchymal stem cells (MSCs), differentiation and maturation of osteoblasts, and formation of extracellular matrix (ECM) and angiogenesis[1]. Besides, fracture healing is a self-repairing process of damaged tissues, and such a process may be slowed down or lead to bone nonunion by interventions with various factors[2]. Given this, clarifying the pathogenesis of fracture healing in depth is of great significance for the fracture treatment.

Parathyroid hormone (PTH), a hormone secreted by the parathyroid glands, regulates the Ca-P balance in the human body. Intermittent administration with PTH (1-34)[3] triggers endochondral bone formation and facilitates osteogenic differentiation of MSCs, thus accelerating fracture healing. At present, mechanisms of PTH underlying fracture healing remain to be clarified. The Notch signaling pathway plays a crucial role in cell survival and self-renewal. Jagged1, a Notch ligand, is an important participant in matrix interaction with hepatic stellate cells (HSCs)[4]. The activation of PTH1 receptor (PTH1R) in osteoblasts leads to matrix-mediated proliferation of HSCs. In mice with activated PTH1R only in osteoblasts (Col1-caPTH1R mice), the number of osteoblasts increases, and high-level Jagged1 is produced, while Notch is inactivated. *In vitro* HSC proliferation is blocked by γ-secretase inhibitor[5].

A rabbit tibial fracture model was established in this study to investigate the effects of PTH on Jagged1 and the Notch signaling pathway during the fracture healing, so as to make a contribution to the treatment of fracture healing.

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Materials and Methods

Materials

A total of 60 healthy New Zealand white rabbits (Laboratory Animal Center of Lanzhou University) aged 6 months old and weighing 2.8-3.4 kg were adaptively fed in separate cages (60×51×35 cm, W×D×H) at (22±2)°C and (40±20)% humidity for one week. Rabbits were given free access to water and specific granular food for laboratory animals. This investigation was approved by the Animal Ethics Committee of First People's Hospital of Fuyang District Animal Center.

Main Reagents and Instruments

Agarose and radio immunoprecipitation assay (RIPA; Yeasen, Shanghai, China); β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); automatic biochemical analyzer (Hitachi 7600-020, Hitachi, Tokyo, Japan); kirschner wire and triangular blade (1.2×230 mm; Kanghui Orthopedics, Changzhou, China); electronic balance (FA2004A, Shanghai, China); electric thermostatic water tank (DK-8AD, Shanghai, China); desktop centrifuge (TL-4, Hunan, China); internal cutting homogenizer (XHF-D, Ningbo, China); automatic microplate reader (EON, Landshut, Germany); rabbit anti-mouse Jagged1 polyclonal antibody β-actin, horseradish peroxidase (HRP)-goat anti-rabbit IgG, RIPA lysis buffer, bicinchoninic acid (BCA) protein assay kit and protein pre-stained marker (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China) and citicoline (Qilu Co., Ltd., Jinan, China).

Modeling of Laboratory Animals

The rabbits were anesthetized with urethane at 1.6 g/kg *via* subcutaneous administration and then placed in a lateral position on an operating table. Next, an incision (about 4 cm in length) was made on the skin close to the right tibia, followed by cutting of the fascia, separation of the muscles, and exposure of the posteromedial surface of the tibia. After that, the periosteum was longitudinally cut and retracted, and a transverse fracture in the middle part of the tibia was made using an oscillating saw. Thereafter, the fractured end was fixed with a 7-hole micro-anatomical plate and 6 screws (with 3 screws above and below the fracture line, respectively). Afterwards, the incision was washed with normal saline and sutured layer by layer. Subsequently, X-ray films were taken under anesthesia to observe the postoperative fixation of fracture. The rabbits recovered from anesthesia were placed in cages for feeding. They intramuscularly injected with 200,000 U of penicillin for 3 d, and then randomly divided into 2 groups (experimental group and control group, n=30 in each group). The rabbits in experimental group were injected with 10 μg/kg PTH (1-34) once a day for 5 days every week, while those in control group were subcutaneously injected with the same volume of normal saline every day. The injection was continued until the rabbits were executed.

Evaluation Via X-ray Examination

At 6 weeks after surgery, 6 rats in each group were randomly selected and anesthetized. Then, the right tibia of the rabbits was examined by X-ray for small animals under the same conditions to evaluate the callus and fracture line.

Determination of Bone Mineral Density (BMD)

After anesthesia with urethane at 0.8 g/kg, rabbit right tibia was scanned at a standard position using a dual-energy X-ray absorptiometry scanner, with the lateral surface of the bone facing the scanner plate. At 2, 4 and 6 weeks, BMD value was measured, including that in proximal and distal old bones and calluses.

Detection of Serum Alkaline Phosphatase (ALP), Ca, and P Levels

Blood was collected from rabbit ear vein at 1, 2, 3, 4, and 6 weeks after surgery and centrifuged at 10,000×g for 10 min. Next, plasma levels of ALP, Ca, and P were determined using the corresponding kits.

Hematoxylin-Eosin (HE) Staining

Rabbit right tibia was placed in 10% formalin for 48 h, decalcified with 5% nitric acid solution for 24 h, routinely dehydrated, hyalinized, and soaked and embedded in paraffin. Thereafter, an ultra-thin semi-automatic microtome was employed to slice the sagittal plane of the tibia, and 10 serial sections (3 μm in thickness) were obtained after baking at 45°C for 1 h. Afterwards, the sections were stained with HE and mounted with neutral resin, followed by observation of the morphology of tissues at the fracture site using a microscope.

Detection of Messenger Ribonucleic Acid (mRNA) Expressions of Notch1 and Jagged1 Via Polymerase Chain Reaction (PCR) Assay

The total RNAs were extracted from tissues using TRIzol (Invitrogen, Carlsbad, CA, USA), followed by determination of RNA concentration and purity using a Thermo NanoDrop 2000. Afterwards, complementary deoxyribonucleic acids (cDNAs) were synthesized using 1 μg of total RNAs, reverse transcriptase (Fermentas, Waltham, MA, USA) and Oligo-dT primers. A 50 μL of PCR system containing reaction buffer, Taq DNA polymerase, dNTPs, 1 μL of forward and 1 μL of reverse primers, and 3 μL of cDNAs (resulting products of reverse transcription reaction) was prepared for amplification. The samples were loaded, shaken for blending, and transiently centrifuged, followed by amplification using a PCR instrument under the following recommended reaction conditions: denaturation at 94°C for 20 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, for a total of 30 cycles, and extension at 72°C for 30 s. PCR was performed using a 7900 qPCR system (Applied Biosystems, Foster City, CA, USA), with the following cycle parameters: 95°C for 5 min, followed by 40 cycles at 95°C for 10 s and 60°C for 20 s. The sequences were shown in Table I.

Measurement of Protein Expression Levels of Notch1 and Jagged1 Through Western Blotting Analysis

The tissues were thawed, cut into pieces and lysed with radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris pH 8, 1% NP40, 0.5% DOC, and 0.1% SDS) containing protease inhibitors [Sigma-Aldrich, St. Louis, MO, USA, catalog number (Cat. No.): 11836153001] for 2 h. Tissue lysates were centrifuged at 15,800 RCF and quantified by Bradford assay (Bio-Rad protein assay, Hercules, CA, USA, Cat. No.: 500-0006). Thereafter, 25 μg (or above indicated on the specific blot) of total proteins were loaded onto NuPage 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA, Cat. No.: NP0322BOX) for electrophoresis in MES running buffer (Invitrogen, Carlsbad, CA, USA, Cat. No.: NP0002-02) and transferred onto a 0.2 μm nitrocellulose membrane (Invitrogen, Carlsbad, CA, USA, Cat. No.: LC2000). After that, membranes were blocked in 5% milk /0.1 M Tris (pH 7.4) /0.15 M NaCl /0.1% Tween 20. After incubation with primary antibody and HRP-conjugated secondary antibody (Jackson Immuno Research, West Grove, PA, USA), enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific, Waltham, MA, USA, Cat. No.: 34078) was used for color development.

Statistical Analysis

All data were expressed as mean ± standard deviation ($\chi \pm s$) and analyzed by Statistical Product and Service Solutions (SPSS) 11.0 software (SPSS Inc., Chicago, IL, USA). Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by the post-hoc test (Least Significant Difference). $p<0.05$ suggested that the difference was significant.

Results

X-ray Examination Results

No significant differences were observed in body weight and appetite in rabbits between two groups throughout the experimental period. Compared with control group, rabbits in experimental group had better healing displayed as cortical and medullary bridging, in which cortical bridging was manifested as disappeared dense gaps between the callus and the debris (Figure 1).

Table I. Primer sequences.

Gene	Primer sequence	Tm	Length
Notch1	GCAGGAGGGAGGTTTGTGA GGAGTATAACCTGACCATAGCAT	56°C	423 bp
Jagged1	CTTCACGGGCACCATTCA TCCGCAAAGTACACTGCACTAG	55°C	453 bp
GAPDH	GTGCTGAGTATGTCGTGGAG GTCTTCTGAGTGGCAGTGAT	57°C	301 bp

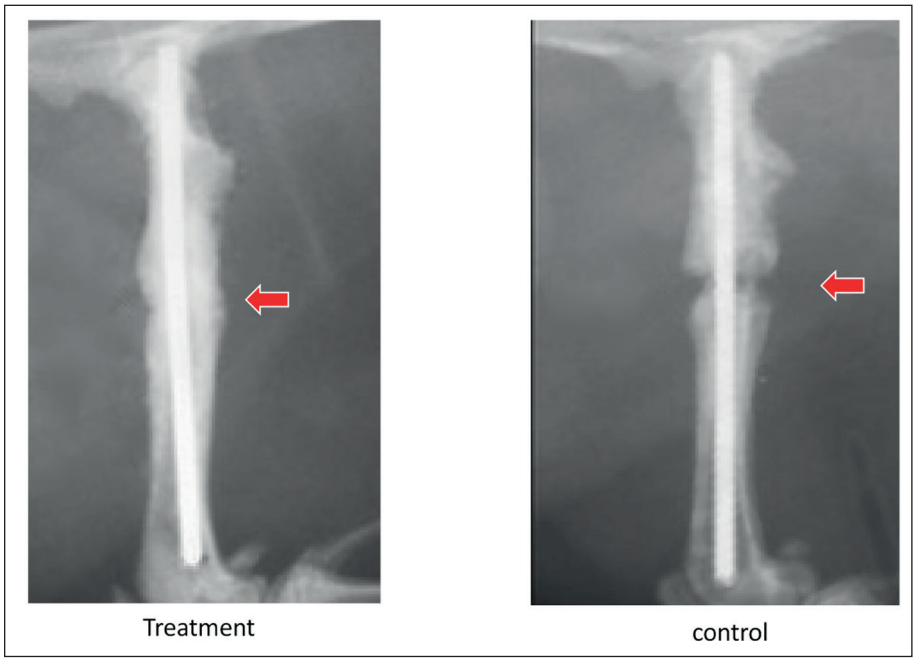

Figure 1. Imaging evidences of fracture healing in control group *(right)* and experimental group *(left)* at 6 weeks after fracture. Representative X-ray films include the fracture site *(red arrow)*.

BMD

The BMD displayed the same change tendency between experimental group and control group, which was the highest at 4 weeks and then declined. The BMD was higher in experimental group than that in control group, and the difference was statistically significant at 4 weeks (p<0.05) (Table II).

Comparisons of Serum Ca and P

At 6 weeks after surgery, serum content of Ca in experimental group was statistically different from that in control group (p<0.05) (Table III).

Serum AKP Level

In the same period, the AKP level was higher in experimental group than that in control group, showing a statistically significant difference (p<0.05) (Table IV).

HE Staining Results

At 6 weeks after surgery, in control group, calluses were formed at the fracture site, while in experimental group, non-hypertrophic chondrocytes were observed around the fracture site. Meanwhile, a small quantity of osteoblasts was found in the tibial space. In comparison with con-

Table II. Changes in BMD at the fractured end in each group of rabbits during fracture healing (g/cm^3, χ±s).

Group	At 2 weeks	At 3 weeks	At 4 weeks	At 6 weeks
Experimental group	0.3078±0.0114	0.3478±0.0847	0.3501±0.0108Δ	0..326±0.0321
Control group	0.2946±0.0276	0.3452±0.0769	0.3069±0.0534	0.2945±0.0765

Note: $^{\Delta}p$<0.05 *vs.* control group.

Table III. Comparisons of serum Ca and P at 6 weeks after surgery (mmol/L, χ±s).

Group	n	Ca	P	Product of Ca and P
Experimental group	6	7.3±0.35	4.76±0.26$^{\Delta}$	35.77±1.76$^{\Delta}$
Control group	6	8.20±0.36	3.87±0.26	30.66±1.66

Note: $^{\Delta}p$<0.05 *vs.* control group.

Table IV. Comparison of serum ALP level (U/L, $\chi\pm s$).

Group	At 2 weeks	At 4 weeks	At 5 weeks
Experimental group	126.72±19.38	168.15±20.45$^{\Delta}$	167.55±25.48$^{\Delta}$
Control group	126.09±18.27	136.22±26.11	134.84±22.74

Note: $^{\Delta}p<0.05$ *vs.* control group.

trol group, rabbits in experimental group exhibited increased calluses and dense periphery at the fracture site, hypertrophic chondrocytes, locally expressed new trabeculae and a large number of osteoblasts in the peripheral area (Figure 2).

Results of PCR Assay

The mRNA expressions of Notch1 and Jagged1 were lowly expressed in control group, while they were upregulated in experimental group compared with those in control group ($p<0.05$) (Figure 3).

Western Blotting Results

Only slight protein expressions of Notch1 and Jagged1 were detected in control group, while those in experimental group were remarkably upregulated ($p<0.05$) (Figure 4).

Discussion

Fracture healing is the continuous function restoration of the damaged bones. Most fractures could be completely healed. However, 10-20% develop into bone nonunion[6]. Risk factors for nonunion of fractures include malnutrition, infection, metabolic disease, dysvascularization or vascular disease, comminution at the fracture site, and improper fixation or stabilization of the fracture site (the most common factor)[7,8]. However, potential involvement of systemic factors or impaired cellular function in the pathogenesis of fracture nonunion is still doubtful. In the past few decades, researchers have attempted to facilitate fracture healing by applying drugs or growth factors (i.e., bisphosphonates and PTH)[9].

PTH (1-34), also known as teriparatide, has been proven to stimulate the formation and activity of osteoblasts, thus promoting the growth of bone tissues. In addition to direct stimulation on bone formation, it can also facilitate fracture healing compared with anti-bone resorption drugs[10,11]. Accumulating evidence[12] has shown that intermittent daily treatment with PTH is able to enhance fracture healing in several animal models and clinical trials. Johansson et al[13] proved the efficacy of intermittent treatment with PTH (1-34) at 60 or 200 μg/kg/day in the rat model of tibial fracture, and that the strength and callus quality at the fracture site are increased in two dose groups. Moreover, animal experiments have demonstrated that intermittent administration with PTH (1-34) accelerates fracture healing by triggering endochondral bone formation and promoting osteogenic differentiation of MSCs[14]. However, the potential mechanism of PTH (1-34) in affecting bone healing is rarely reported so far.

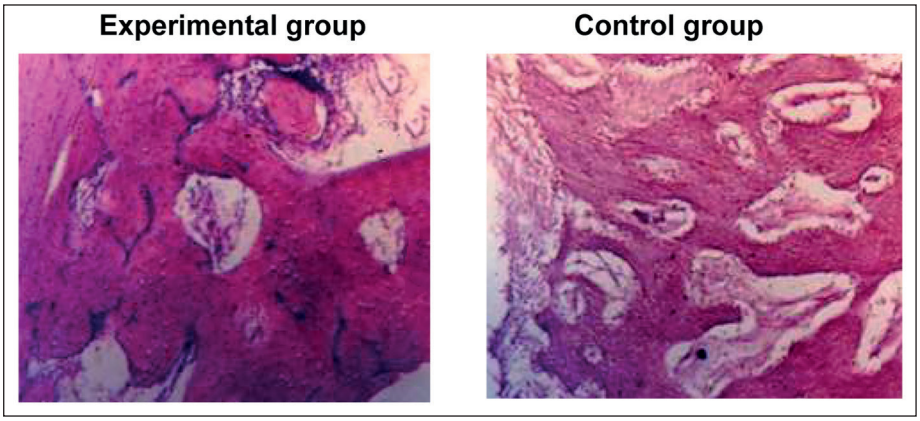

Figure 2. Pathological morphology of calluses at 6 weeks after surgery (HE staining, magnification ×100).

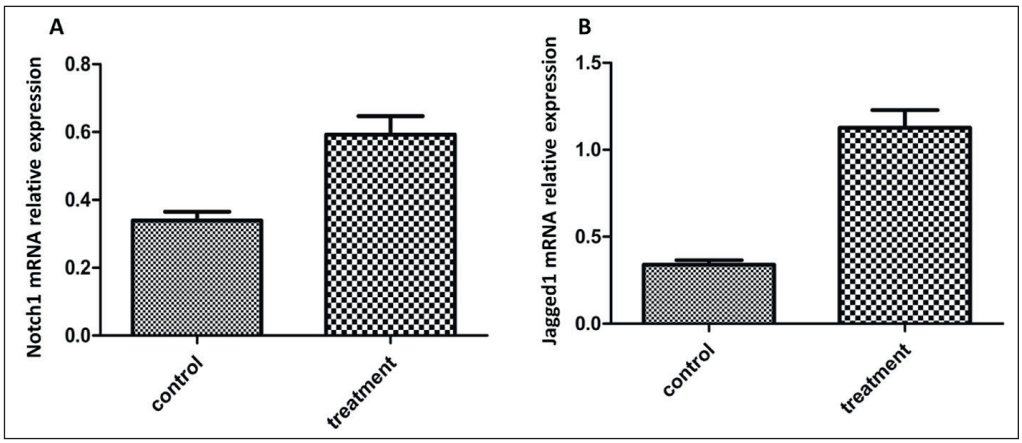

Figure 3. MRNA expressions of Notch1 and Jagged1 in each group detected via PCR assay; **A**, MRNA expressions of Notch1. Compared with that in control group, the mRNA expression of Notch1 is elevated in experimental group ($p<0.05$). **B**, MRNA expressions of Jagged1. The mRNA expression of Jagged1 is higher in experimental group than that in control group ($p<0.05$).

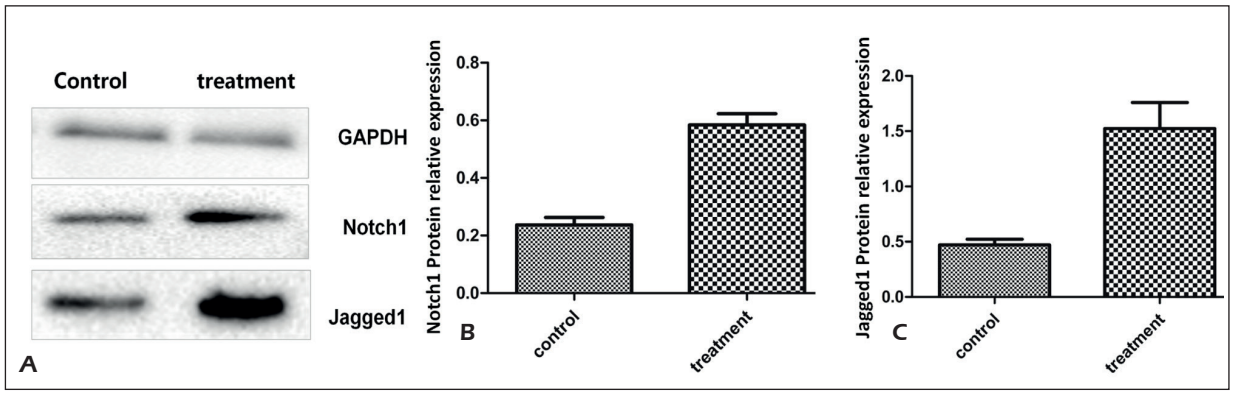

Figure 4. Protein expressions of Notch1 and Jagged1 in each group detected via Western blotting analysis. **A**, Protein expressions of Notch1 and Jagged1 are barely expressed. **B**, Protein expressions of Notch1. Compared with control group, experimental group has an increased protein expression of Notch1 ($p<0.05$). **C**, Protein expressions of Jagged1. The protein expression of Jagged1 rises in experimental group compared with that in control group ($p<0.05$).

The Notch signaling pathway is a vital pathway for skeletal development and disease in animals and humans[15]. Fracture repair mechanism is considered to outline a series of signal transduction events occurring during skeletal development, implying the potential involvement of the Notch signal transduction[16]. The following evidence further proves the role of Notch in fracture healing: (a) up-regulation of Notch-associated genes in mouse calluses during fracture healing, (b) inactivation of the Notch signal transduction in skeletal progenitor cells during early fracture repair in specific mice, and (c) systemically inactivation of the Notch signal transduction before fractures prolongs the inflammatory phase and alters fracture healing in mice[17-19]. The above research findings indicated that Notch has a correlation with fracture repair, but the exact role of the Notch signaling pathway in fracture healing remains unknown. It is confirmed[20] that Jagged1 is a Notch ligand with the highest up-regulation during fracture repair, which is expressed in osteoblasts at several stages of differentiation. Besides, Youngstrom et al[21] testified that Jagged1 induces osteogenic differentiation in primary human bones. These results suggested that Jagged1 may be osteoinductive, which can be used to promote the formation of bone tissues. However, these experiments have been conducted in a polystyrene (TCPS) cell culture model[22]. Therefore, this study further explored the mechanism between Jagged1-induced Notch activity and PTH through an *in vivo* model.

In our study, a rabbit tibial fracture model was constructed. PTH (1-34) was injected into the rabbits to observe potential effect of PTH (1-34) on fracture healing through the Notch pathway. The results showed that the BMD and X-ray score of rabbits treated with PTH (1-34) were significantly raised. Furthermore, HE staining results also revealed that the volume of calluses and the number of osteoblasts were remarkably elevated. In addition, PTH (1-34) could up-regulate the mRNA expressions of Notch1 and Jagged1, the osteogenesis-related genes, in rabbit fractures. The detection of plasma contents of Ca, P, and AKP manifested that Ca and P interacted with each other in the blood. At a certain level, the increased products of Ca and P were beneficial to the deposition of Ca and P in bones, which was conducive to the tibial fracture healing in rabbits. Raised serum content of ALP indicated active osteoblasts and increased bone formation ability. Yoon et al[23] found that runt-related transcription factor 2 (Runx2), a key transcription factor for osteogenic differentiation, plays an indispensable role in accelerating the osteogenic differentiation of stem cells and differentiation of osteoblasts and chondrocytes. The results of this study suggested that endogenous PTH increased the expression of Jagged1 in osteoblasts through the Notch pathway and promoted the expression of Runx2 at the same time. The above study results suggested that PTH may affect the function of osteoblasts at the fracture site *via* the above pathways, ultimately promoting fracture healing.

One limitation of the present study is that the HE staining results did not include the overall condition of the fracture site. Hence, HE staining results shall be more accurately assessed in future studies to evaluate the morphological changes of fractures. Despite the limitations, this study reveals that PTH (1-34) activates the Notch pathway to promote the expressions of osteogenesis-related genes, thereby facilitating fracture healing in rabbits.

Conclusions

In summary, we first clarified that PTH (1-34) promotes the rabbit tibial fracture healing by regulating Jagged1 ligand molecules in the Notch signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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