ZJXG Decoction Promotes the Expression of Bone Morphogenetic Protein-7 to Enhance Fracture Healing In Rats

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Abstract
The aim is to investigate the effects of Zhuang Jin Xu Gu Decoction (ZJXG Decoction) on femoral fracture healing in rats. Femur fractures were generated in fifty male adult Wistar rats by cutting femur transversely at middle point. ZJXG Decoction was administered orally after surgery for 7-14 d. Radiological evaluation or X-ray imaging analysis indicated that the fibrous callus tissue at the femoral fracture-end increased and the fracture line became fuzzy at 7-14 d following treatment with ZJXG Decoction. Hematoxylin-Eosin (HE) staining showed that the fibrous-granular tissue at the fracture-end changed gradually to fibrous, cartilaginous and osseous callus tissues. Immunohistochemical staining and Enzyme Linked Immunosorbent Assay (ELISA) results showed that BMP-7 in the fibroblasts and osteoblasts of callus and its serum level increased significantly 7-14 d following treatment with ZJXG Decoction. It is concluded that ZJXG Decoction could enhance the fracture healing by up-regulating the expression of BMP-7 in fibroblasts and osteoblasts of callus in rats.

Keywords: ZJXG decoction; Fracture; Callus; Pathology; Bmps; Rats

Introduction
The fracture healing is an extremely complicated process of skeletal reconstruction. Many growth factors could promote osteoblast development, proliferation, differentiation and accelerate new bone formation in the process of fracture healing and remodeling [1]. Bone Morphogenetic Proteins (BMPs) and Neuropeptide Y (NPY) play important roles in bone fracture repair process [2]. BMP induces cartilage and bone formation [3] and been used to treat fracture models in rodents [4-6], and some experimental and clinical reports illustrated the effect of BMPs on fracture healing [7-9]. Current fracture care includes internal and external fixation with early mobilization to restore function earlier and more completely. But fracture fixation could cause serious trauma and mostly need the secondary operation. In addition, radiological imaging analysis indicated that the fibrous callus tissue at the femoral fracture-end increased and the fracture line became fuzzy at 7-14 d following treatment with ZJXG Decoction. Hematoxylin-Eosin (HE) staining showed that the fibrous-granular tissue at the fracture-end changed gradually to fibrous, cartilaginous and osseous callus tissues. Immunohistochemical staining and Enzyme Linked Immunosorbent Assay (ELISA) results showed that BMP-7 in the fibroblasts and osteoblasts of callus and its serum level increased significantly 7-14 d following treatment with ZJXG Decoction. It is concluded that ZJXG Decoction could enhance the fracture healing by up-regulating the expression of BMP-7 in fibroblasts and osteoblasts of callus in rats.

Materials and Methods

Animal model and grouping
Total of 50 male adult Wistar rats (Experiment Animal Center of Qingdao Drug Inspection Institute, SCXK (L) 20090010) weighting 190-210 g were used and all experimental procedures were approved by the Ethics Committee of Qingdao University Medical College (No.QUMC2011-09) in this study. The rats were anesthetized with intraperitoneal injection of 100 g/L chloral hydrate (300 mg/kg) and then restrained in a supine position for operation. The femoral fracture model was established by cutting the femur transversely at the middle section (about 1.0 cm below the great trochanter) from medial parapatellar incision [12]. After the manual reduction, the fractured femur was fixed with intramedullary Kirschner wires (diameter 1.0 mm, Shanghai Medical Apparatus Co. Ltd.). The sham group was allowed to drink and eat freely after surgery. The survival rate is 100%. The rats were divided randomly into five groups of 10 rats in each group. The low, medium and high dose group rats were treated with ZJXG Decoction of 1.25, 2.5 and 5 g/kg respectively while the vehicle was given at the same volume to sham and control group rats. At the time points of 7 d or 14 d, rats were subjected to X-ray image taking after chloral hydrate anesthesia and then euthanized for blood and tissue collection.

Preparation of ZJXG decoction
Zhuang Jin Xu Gu Decoction (ZJXG Decoction) was derived from the ZJXG Pellet recorded in “Shang Ke Da Cheng” written by Zhao Lian of the Qing Dynasty in China. It is composed of 12 constituents listed in table 1. The ZJXG Decoction was decocted according to the Standard of Decocting Herbal Medicine promulgated by Chinese Administration Department of Traditional Chinese Medicine. The mixture of all herbal plants were immersed in distilled water for 20-30 min at 20-25°C with relative humidity ≤ 85%, and then cooked on simmer for 10-15 min to concentrate the extracts, and then cooled to be liquid before use.

Radiological and gross observation
An initial X-ray examination was performed in all animals after the fracture. At 7 and 14 d following surgery, all the rats were anesthetized for X-ray evaluation (GE Revolution RE/d, USA). The anesthetized rats were then sacrificed and the femurs were taken out, washed normal saline for general observation.

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**Histological analysis**

For morphological analysis, the femur were cut and incubated in 40 g/L formaldehyde solution for 4 h and rinsed in distilled water for 4 h, and then decalcified for 10 days in 20% Ethylenediamine Tetracetic Acid (EDTA). The samples were then dehydrated using graded ethanol, immersed in dimethylbenzene for 2 h, embedded by paraaffin. The 7 μm thickness slices were made by mirotome (Leica RM2135, Shanghai Leica Instruments, China) and attached to poly-L-lysine processed microscope (manufacture). LEICA Qwin microgramme analytical system was used to analyze the expression of immunosignals, illustrated by absorbance values (A).

**Enzyme linked immunosorbent assay (ELISA)**

About 4 ml blood was aseptically collected from abdominal aorta of each rat and centrifuged for 10 minutes at 4000 rpm at 4°C to separate the serum which was then kept at -20°C until required for analysis. The serum level of BMP-7 measured using commercially available ELISA kits (Blue Gene Co. Ltd). The procedure was performed following manufacturer’s instruction. The OD was calculated with BioRad 550 microplate reader (USA) set to 450 nm to reflect the level of BMP-7. The assay sensitivity is 1.0 ng/L.

**Statistical analysis**

The data was expressed by mean ± standard deviation (X ± s) and analyzed with SPSS 11.5 statistical software. Analysis of variance was used to compare whether there are obvious differences among groups. P<0.05 was considered significantly.

**Results**

**X-Rays and gross observation**

X-rays revealed that the fracture-end of femur of the control group began forming fibrous callus at 7 day after surgery with the fracture line still clear; at 14 days, the fracture line became unclear. In the treated groups, the fibrous callus was more than that in the control group and the fracture line became fuzzy at 7 days and tended to disappear at 14 days following treatment. On day 7 in control group, granulation tissue in the fracture breaking-end was observed and fibrous callus at 14 days following surgery. In the treated groups, fibrous callus formed at 7 days and formation of cartilaginous and osseous callus was present on day 14 (Figure 1). There was no statistical difference among treatment groups.

**Immunohistochemistry**

Minimal expression of BMP-7 was detected in the sham group (P >0.05). BMP-7 positive cells were observed in callus tissues in control group on day 7 and the absorbance values on day 14 was greater compared to 7d control group (P<0.05). In paired comparisons of groups, the grade of values of absorbance (A) of BMP-7 was significantly higher in the treatment groups compared to control group (P<0.05). It was not significantly different among the high-dose, medium-dose and low-dose treated groups (P >0.05) (Table 2 and Figure 3).

**The serum levels of BMP-7**

There was no significant difference of serum level of BMP-7.
between day 7 and day 14 in sham operation group (P>0.05). It is significantly different between day 7 and day 14 within each group, with higher levels on day 14 (P<0.05). At same time points, the serum level of BMP-7 in the control group was significantly higher than those in sham operation group and significantly lower than those in the treated groups (P<0.05). No significant difference among the high-dose, medium-dose and low-dose groups was observed (P>0.05) (Table 2).

**Discussion**

Previous clinical studies have demonstrated that applications of ZJXG Decoction enhanced healing of fractured humerus and femur [13-15]. Consistent with these empirical observations, here we presented the first robust evidence of the effectiveness of ZJXG Decoction in the promotion of fracture healing in the experimental animal. Fracture healing is an extremely complex process which is reportedly influenced by multiple cytokines and growth factors [16]. In the present study, we hypothesized that the efficacy of ZJXG Decoction be mediated via the up-regulation of local and systematic BMP-7. Bone Morphogenetic Proteins (BMPs) are known to play critical roles in the formation of cartilage and bone during embryonic development, and the loss of certain BMP molecules leads to a severe impairment of osteogenesis [17]. The molecules of BMP-2, -4, -5, -6, -7, and -9 have effects on osteogenic action [18], though different members play specific roles in bone formation during different stages. BMP-7 induces osteogenic differentiation of mesenchymal stem cells by regulating the transcription factors Runx2 and Osterix [19].

In addition to the effects in skeletal development, BMP-7 has been well characterized for its involvement in the fracture healing. For example, Kloen et al. [20] showed the presence of BMP molecules including BMP-7 in human fracture callus. In another study, clinical application of BMP-7 could induce the osteoblastic activity and repair bony defects [21]. Furthermore, rhBMP-7 accelerated the healing in distal tibial fractures treated by external fixation [22]. The results of these studies suggest BMP-7 can be a positive modulator of fracture healing. This experiment showed that the BMP-7 expression in blood and in the femoral fracture callus tissue of rats significantly increased after treatment with ZJXG Decoction at different stages; meanwhile, HE staining and X-ray evaluation demonstrated the efficacy of ZJXG Decoction in enhancing the fracture healing. The current data suggested the involvement of BMP-7 be an important mediator in the accelerated healing by ZJXG Decoction. Other Chinese herbal medicines such as Mixture of Kidney-Tonifying also increased BMPs concentration in the area of implant-synostosis and promoted fracture healing of rats. It is therefore possible that up-regulation of MBP-7 could be a common therapeutic mechanism employed by different treatments for enhancing fracture healing.

**Conclusions**

The current data demonstrated that ZJXG Decoction significantly promotes the fracture healing in a fracture rat model, and might partially be due to its influence on the expression of growth factors of BMP-7. Future studies will be needed to investigate which signaling pathways are affected by ZJXG decoction.

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