Full Length Research Paper

Zhuang Jin Xu Gu Decoction improves fracture healing in rats by augmenting the expression of NPY

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Abstract

To investigate the effects of Zhuang Jin Xu Gu Decoction (ZJXG Decoction) on healing of femoral fracture in rats. Femur fratures were generated in fifty male adult *Wistar* rats by cutting femur transversely at middle point. ZJXG Decoction was administered orally after surgery for 7-14d. The healing process was analyzed by X-ray and hematoxtlin-eosin (HE) staining in rats. The expression of neuropeptide Y (NPY) in fibroblasts and osteoblasts in callus was evaluated by immunohistochemical assay. The serum level of NPY was assessed by enzyme linked immunobsorbent assay (ELISA). 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. HE staining showed that the fibrous-granular tissue at the fracture-end changed gradually to fibrous, cartilaginous and osseous callus tissues. Immunostaining and ELISA results showed that NPY in the fibroblasts and osteoblasts of callus and their 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 NPY in fibroblasts and osteoblasts of callus in rats.

Keywords: ZJXG Decoction, fracture, callus, X-ray, NPY, rats.

INTRODUCTION

The fracture healing is an extremely complicated process of skeletal reconstruction. Many growth factors could promote osteoblast differentiation, proliferation, development and accelerate new bone formation in the process of fracture healing and remodeling (Pogoda et al., 2005). Bone morphogenetic proteins (BMPs) and neuropeptide Y (NPY) play important roles in bone fracture repair process (Oreffo, 2004). NPY affects the activities of osteoblasts by inhibiting the synthesis of circle adenosine monophosphate (cAMP) during the fracture repair process (Lindblad et al., 1994). As a skeletal maintenance regulation-related factor, NPY improves skeletal synthetic metabolism, controls bone remodling, regulates bone balance, prevents bone loss,

and maintains bone stability (Lee and Herzog, 2009; Teixeira et al., 2009). NPY participats in fracture remodeling not only from the central nervous system, but also from the tissue surrounding fracture (Liu et al., 2009). 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, it increases the risk of infection and the rates of delayed union, and nonunion (Zhao et al., 2011). Traditional Chinese Medicine Zhuang Jin Xu Gu Decoction (ZJXG Decoction) has been clinically used for promoting fracture healing for many years (Li et al., 2009). The exact therapeutic mechanism by which ZJXG Decoction enhances healing in rodent model, however, still remains unclear. Here we aimed to elucidate if the effects of ZJXG Decoction in fracture repair was related to the expression of NPY.

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MATERIALS AND METHODS

Animal model and grouping

Fifty male adult *Wistar* rats (Experiment Animal Center of Qingdao Drug Inspection Institute, SCXK (LU) 20090010) weighting 190-210g were used in this study. All experimental procedures were approved by the Ethics Committee of Qingdao University Medical College (No. QUMC 2011-09). The rats were anesthetized with intraperitoneal injection of 100g/L chloral hydrate (300mg/kg) and then restrained in a supine position for operation. The animal's hind limb was shaved and then a medial parapatellar incision was created. The femoral fracture model was established by cutting the femur transversely at middle section (about 1.0 cm below great trochanter) (Wang et al., 2005). 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 subjected to the same procedure except without cutting femur. Animals were 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 5g/kg respectively while the vehicle was given at the same volume to sham and control group rats. At the time points of 7d or 14d, rats were subjected to X-ray image taking after chloral hydrate anesthesia and then euthanized for blood and tissue collection.

Preparation of Zhuang Jin Xu Gu 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: Chinese Angelica (*Radix Angelicae Sinensis*) 12g, Rhizoma Chuanxiong (*Rhizoma Chuanxiong t*) 12g, Radix Rehmanniae Preparata (*Radix Rehmanniae Preparata*) 10g, Milkvetch Root (*Radix Astragali*) 12g, Eucommia Bark (*Eucommia ulmoides Oliv*) 12g, Himalayan Teasel Root (*Radix Dipsaci Asperoidis*) 12g, Fortune's Drynaria Rhizome (*Rhizoma Drynariae*) 12g, Sanchi (*Radix Notoginseng*) 10g, White Paeony Root (*Radix Paeoniae Alba*) 10g, Safflower (*Flos Carthami*) 10g, Total 112g. 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^{0}C with relative humidity ≤85%, and then cooked to the boil, kept

on simmer for 10-15 min to concentrate the extracts, protecting and maintaining all essential ingredients. The same procedure was repeated for 2 times. The yeild obtained from the two extractions was 224ml liquid medicinal decoction containing 112g of dry weight (concentration of 0.5g/ml) which was packed with sterilized plastic bags and stored at -20^{0}C until use.

Radiological evaluation

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).

Histological analysis

The anesthetized rats were then sacrificed and the femurs were taken out and incubated in 40g/L formaldehyde solution for 4 h and rinsed in distilled water for 4h, and then decalcificated for 10 days in 20% ethylenediamine tetraacetic acid (EDTA). The samples were then dehydrated using graded ethanol, immersed in dimethylbenzene for 2h, embedded by paraffin The 7μm thickness slices were made by mirotome (Leica RM2015, Shanghai Leica Instruments, China) and attached to poly-L-lysine processed slides. Paraffin sections were deparaffinaged in dimethylbenzene, hydrated in gradient ethanol and rinsed with distilled water. The sections were stained with hematoxylin-eosin (HE).

Immunohistochemical staining

For immunostaing, antigen retrieval was made using a microwave oven. The sections were incubated with rabbit anti-rat NPY polyclonal antibodies at 4℃ overnight. Negative control used PBS instead of primary antibodies. Immunohistochemical procedures were performed strictly according to the SABC kit manual. Four serial sections from each experimental rat were observed under a light microscope (manufacture). LEICA Qwin microgramme analytical system was used to analyze the expression of immunosignals, illustrated by Absorbance values (A).

Enzyme linked immunobsorbent assay (ELISA)

About 4 ml blood was aseptically collected from abdominal aorta of each rat and centrifugalized for 10 minutes at 4000 r/min at 4 ℃ to separate the serum which was then kept at -20 ℃ until required for analysis.
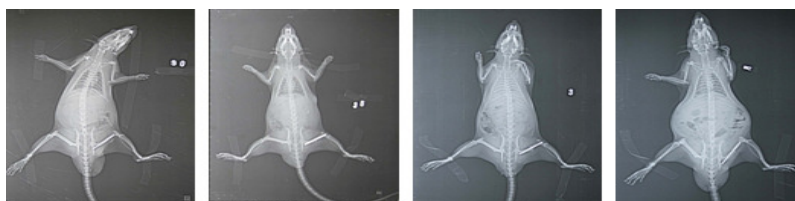
Figure 1. The X-ray films of femur fracture healing on day 7 in control group (A), day 7 in low-dose treated group (B), day 14 in control group (C) and day 14 in low-dose treated group (B)
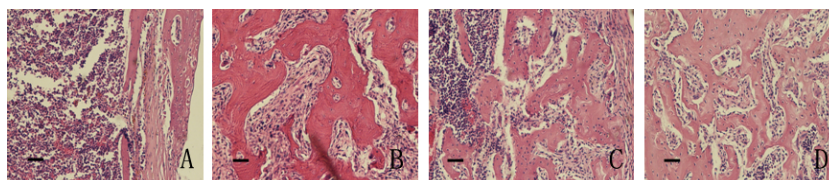

Figure 2. Hematoxylin and eosin staining on tissues collected on day 7 in control group (A) and treatment group (B); on day 14 in control group (C) and treatment group (D). Scale bar =50µm.

The serum level of NPY were measured using commercially available ELISA kits (Blue Gene Co. Ltd). The procedure was performed following manufacturer's instruction. The ODs were calculated with BioRad 550 microplate reader (USA) set to 450nm to reflect the level of NPY.

Statistical analysis

The data was expressed by mean ± standard deviation ($\bar{x}$±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-ray examination

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 (Figure 1). There was no statistical significance between groups of low-dose, medium-dose and high-dose groups.

HE staining

On day 7, in control group, the inflammatory cell infiltration, formation of granulation tissues occurred between fracture fragments. The proliferation of fibroblast and osteoblasts under periosteum was localized in the fracture gap. On day 14 of control rats, the number of fibroblasts and osteoblasts increased and fibrous callus had formed with a smallcartilaginous callus. In the treated groups, the inflammatory cells decreased and the fibroblasts and osteoblasts increased in the fractured bone end 7 days after treatment compared to control, while on day 14 a lot of fibrous, cartilaginous and osseous callus tissues had developed and newly formed bone trabeculae appeared (Figure 2).

Immunohistochemistry

Minimal expression of NPY was detected in the sham group ($P >0.05$). NPY 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 (F=14.12, q=2.39-7.69, $P<0.05$). In paired comparisons of groups, the grade of values of absorbance (A) of NPY was significantly higher in the treatment groups compared to control group (F=14.12, q=2.39-7.69, $P<0.05$). It was not significantly different among the high-dose, medium-dose and low-dose treated groups ($P >0.05$) (Table 1 and Figure 3).

Table 1. The expression values of absorbance and serum concentration of NPY (X±S, n=5)

Groups	Dose	Absorbance (A)		Concentration (ng/L)	
		7 d	14 d	7 d	14 d
Sham group	NS	0.27±0.05	0.25±0.07	121.27±13.55	135.20±203.20
Control group	NS	0.38±0.06[a]	0.46±0.11[a c]	283.36±20.06[a]	363.61±28.05[a c]
Low-dose group	1.25g/kg	0.56±0.10[b]	0.70±0.10[b c]	562.35±46.78[b]	706.00±65.10[b c]
Medium-dose group	2.5g/kg	0.57±0.12[b]	0.71±0.13[b c]	573.32±50.32[b]	710.00±70.36[b c]
High-dose group	5.0g/kg	0.57±0.16[b]	0.68±0.13[b c]	575.55±53.16[b]	688.64±63.73[b c]

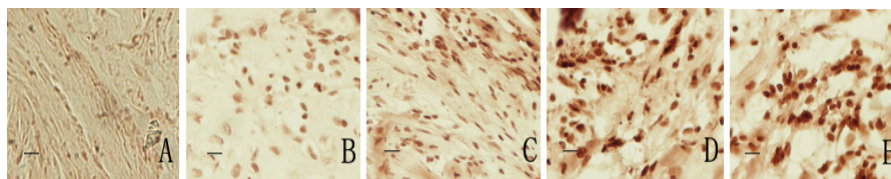a $P<0.05$ *vs* sham group, b $P<0.05$ *vs* control group, c $P<0.05$ *vs* treatment group 7 d

Figure 3. Immunohistochemistry of NPY-7, DAB × 200, Scale Bar = 25μm. A: Sham group, B 7 days in control group; C: 7 days in low-dose group; D: 14 days in control group, E: 14 days in low-dose group

The serum levels of NPY

There was no significant difference of serum levels of NPY between day 7 and day 14 in sham operation group ($P>0.05$). It is significantly different between day 7 and day 14 within other each group, with higher levels on day 14 ($P<0.05$). At same time points, the serum levels of NPY in the control group were 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 1).

DISCUSSION

Previous clinical studies have demonstrated that applications of ZJXG Decoction enhanced healing of fractured humerus and femur (Zhang et al., 2006; Liang et al., 2005; Kuang et al., 2003; Li and Zhang, 2010). 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 (Westerhuis, et al., 2005). In the present study, we hypothesized that the efficacy of ZJXG Decoction be mediated via the up-regulation of local and systematic NPY. Immunohistochemical experiments revealed that neuropeptides including NPY widely distributed in bones

(Elefteriou, 2005). Several lines of evidence suggest that NPY mediate the bone reconstruction. Nunes et al. (2010) demonstrated that NPY could promote the synthesis of osteoblasts, cartilage cells and bone cells and. increase the bone mass by increasing ceramide contents during embryonic and adult periods. The bone fracture remodeling was enhanced not only by the NPY from the central nervous system, but also by the peripheral NPY surrounding the fractures (Long et al., 2010). NPY also influenced the osteoblasts activity by inhibiting synthesis of cAMP in osteoblasts (Lindblad et al., 1994). In this experiment, higher level of NPY was detected in the fibroblasts and osteoblasts in fracture callus following treatment with ZJXG Decoction and the concentration of NPY also exhibited an increase in blood serum of rats with ZJXG Decoction by ELISA. At the same time points, X-ray showed the fracture line became dim and the fibrous and cartilaginous callus formed at fracture-site by HE staining after treatment with ZJXG decoction. In combination, our findings suggest that accelerated fracture healing induced by ZJXG decoction may also be mediated by an increase in NPY expression.

CONCLUSIONS

The current data demonstrated that ZJXG Decoction significantly promotes the fracture healing in a fracture rat model. Moreover, the fracture healing effects by ZJXG decoction might partially be due to its influence on the expression of growth factors of NPY. This study

provided information useful for elucidating the mechanistic details underlying the therapeutic effects of ZJXG decoction on bone. Future studies will be needed to investigate which signaling pathways are affected by ZJXG decoction.

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