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ZJXG Decoctions Enhance the Fracture Healing by Keeping the Serum Levels of GH, CT and PTH in Rats with Femur Fracture

Li Xiaodan, Ning Chi, Zhou Zhen, Wang Guanxi, Yang Guangwen and Guo Yunliang
Institute of Integrative Medicine Affiliated, Hospital of Qingdao University Medical College,
Qingdao, 266003, China

Abstract: The aim is to investigate the effects of Zhuang Jin Xu Gu (ZJXG) decoction on the serum levels of Growth Hormone (GH), Calcitonin (CT) and Parathyroid Hormone (PTH) and the femoral fracture healing in rats. Femur fractures were generated in 72 male adult Wistar rats by cutting femur transversely at the middle point. The ZJXG decoction was administered orally as drinking water after surgery for 28 days. The healing process was analyzed by X-ray, gross anatomy and Hematoxylin Eosin (HE) staining. The serum levels of GH, CT and PTH were assessed by enzyme linked immunosorbent assay (ELISA). X-ray, gross anatomy and HE staining indicated that the fracture line of the femoral fracture-end was clear at 7th day, fibrous callus tissue formed at 14th day, Calcium salt deposited at 21th day and osseous callus tissues formed at 28th day. During fracture (7-21 day), no difference of the callus tissue structure existed between model and treatment groups, but in treatment group it was better than that in model group at 28th day. ELISA results showed that the serum levels of GH, CT and PTH were higher than those in the control group at 7-28 days ($p < 0.05$). There were no significant difference between model and treatment groups at 7-21 days, but in treatment group it was higher than that in model group at 28th day ($p < 0.05$). So, ZJXG decoction could promote the fracture healing by reducing the decomposition of GH, CT and PTH and keeping their high levels in rats with femur fracture.

Key words: ZJXG decoction, fracture, calcitonin, parathyroid hormone, rats

INTRODUCTION

It is known to all that the hormones, growth factors and nerve system were composed the neuro-endocrine-local growth factor regulating system to participate in the whole process of bone formation (Andreassen and Oxlund, 2003). Growth Hormone (GH) is a kind of polypeptide secreted by pituitary eosinophils and a major regulator for the collagen growth (Raschke *et al.*, 2007). The growth hormone-insulin-like growth factor-I (GH-IGF-I) of the hypothalamus is an important axis of human endocrine metabolism which not only played an important role in the human growth and development but also closely related to the adult human tissue repair and reconstruction (Oreffo, 2004). Calcitonin (CT), Parathyroid Hormone (PTH) and active vitamin D₃ (VitD₃) are three main regulating hormones to maintain the balance of calcium phosphorus metabolism (Giardino *et al.*, 1997). The CT is a polypeptide composed of 32 amino acids which acted on the osteoblast to promote trabecular reconstruction and accelerate fracture healing (Garcia-Delgado *et al.*, 1997). The PTH is composed of 84 amino acid residues and its amino terminal consisted of 31-38 amino acid residues, stimulated human and animal bone growth (Yang *et al.*,

2006). Zhuangjin Xugu (ZJXG) decoction cited from the ancient book of "Shang Ke Da Cheng" written by Dr. Zhao Lian in Chinese Qing dynasty. Both animal experiments (Wang *et al.*, 2013) and clinical applications (Huang, 2012) proved that ZGXX decoction can promote fracture healing, but its mechanism is still not very clear, so this experiment tries to observe how ZJXG decoction influences the serum levels of CT and PTH after femoral fracture and to explore the effect and mechanism of promoting fracture healing.

MATERIALS AND METHODS

Animal grouping: Total of 72 healthy adult male Wistar rats, weighted 230-250 g. The SPF grade, provided by Qingdao Drug Inspection Animal Center ((SCXK(LU) 20120010). All animals are adaptive bred 7 days and then randomly divided into control group, fracture group and treatment groups consisting of 24 rats, respectively.

Model preparation: Experimental animals were anesthetized by injecting 10% chloral hydrate (300 mg kg⁻¹) intraperitoneally, fixed with prone position and aseptic operation. The operation was subjected with

lateral femoral incision and separated the femur through the thigh anterolateral intermuscular septum and cut the femur with low-speed dental micro-drilling (JBX-NE22, NSK Co. Ltd. Japan) in the middle of the femur (under the large rotor 1cm), then fixed retrogradely with a Kirschner needle (Diameter 1 mm, Shanghai Medical Needle Factory) intramedullary. At last skin was, sealed and bandaged aseptically. The surgical success rates are 100% (Wang *et al.*, 2013). In the control group, the rats were subjected to the same surgical procedures without cutting the femur.

Treatment methods: The ZJXG decoction was produced by our hospital pharmacy room based on Chinese Medical Institutions for Drug Management Practices" (National TCM [2009]3). Each medicine (Guo *et al.*, 2012) was fully decocted twice the final liquid was 224 mL containing crude drug 112 g, concentration 0.5 g mL⁻¹. According to the previous study results, the ideal dose was 1.25 g kg⁻¹ (2.5 mL kg⁻¹) (Wang *et al.*, 2013) for 28 days. The rats in fracture group and the control group were given an equal amount of normal saline at the same time.

Evaluation indexes: Six rats were selected from each group to be observed at 7, 14, 21, 28 days after treatment:

- **X-ray:** X-ray films (GE Revolution RE/d type, USA) were used to observe the processes of fracture healing
- **ELISA:** The rats were anaesthetized by 10% chloral hydrate (300 mg kg⁻¹) and 4 mL was collected from the abdominal aorta to centrifuge at 4000 rpm for 10 min to separate the serum. ELISA kits (Blue Gene Company) were used to determine the serum levels of GH, CT, PTH and VitD₃. Firstly, the serum samples were dissolved at room temperature and centrifuged again, then the absorbance value of supernatant sample 100 µL was measured by a microplate reader (Bio-Rad 550, USA) at 450 nm. The concentration of GH, CT and PTH in the corresponding coordinate (ng L⁻¹) was calculated according to the absorbance value of the sample
- **Path-anatomy:** After collecting the blood, whole femur was removed to be used observing fracture healing. Then the femur was fixed with 4% paraformaldehyde for 24 h, soaked in distilled water for 4 h and decalcified with a 20% solution of Ethylenediamine Tetraacetic Acid (EDTA) for 15 days. The tissue of fracture site was cut and dehydrated with ethanol, transparent by xylene and embedded in paraffin. The sections of thickness 5 µm

were cut along the longitudinal axis of the femur by microtome (Leica2015, Shanghai) and adhered to glass slides. The slides were stained by Hematoxylin Eosin (HE) to observe the bone callus tissue structures under the microscope

Statistical analysis: All data was presented by $\bar{x} \pm SD$ and analysed using SPSS11.0 statistical software. The $p < 0.05$ was considered significant.

RESULTS

X-ray: X-ray films showed the femoral cortical bone was integrity and continuous in control group rats (Fig. 1a), while the fracture line was clear and cortical bone discontinuity in model group rats (Fig. 1f). The fracture-ends was filled with fibrous tissue after fracture 7 days (Fig. 1b), but the fracture line still clear. At fracture 14 days (Fig. 1c), the fibrous callus tissue began to form, the calcium salt deposited at fracture 21 days (Fig. 1d) and the osseous callus tissues formed irregular trabecular bone at fracture 28 days (Fig. 1e). After treatment 7-21 days (Fig. 1g, h and i), no significant difference of bone callus structure between the model group and treatment group rats in the corresponding time, but the bone callus structure in treatment group (Fig. 1j) was better than that in the model group (Fig. 1f) after treatment 28 days, its cortical bone and marrow could be discriminated and imaged clearly.

Anatomy: Cortical bone was completely in control group rats (Fig. 2a) while the fracture fragments separated in fracture group rats (Fig. 2f). The fracture-ends were filled with granulation tissue at fractures 7 days (Fig. 2b), formed fibrous callus tissue at fracture 14 days (Fig. 2c), fibrous and cartilaginous callus increased at fracture 21 days (Fig. 2d) and cartilage and bone callus formed at fracture 28 days (Fig. 2e). After treatment 7-21 days (Fig. 2g, h and i), there was no significant difference of bone callus structure between treatment group and model group rats at the corresponding time, but at fracture 28 days the bone callus hardness in the treatment group (Fig. 2j) was significantly better than that in fracture group rats (Fig. 2e).

Histopathology: Bone structure was normally in control group rats (Fig. 3a) and the fracture-ends were filled with hematoma tissue in fracture group rats (Fig. 3f). The granulation tissue formed after fracture 7 days (Fig. 3b), fibrous-cartilaginous callus at fracture 14 days (Fig. 3c), scattered trabecular bone at fracture 21 days (Fig. 3d) and bone trabecular visibaly at fracture 28 days (Fig. 3e). After

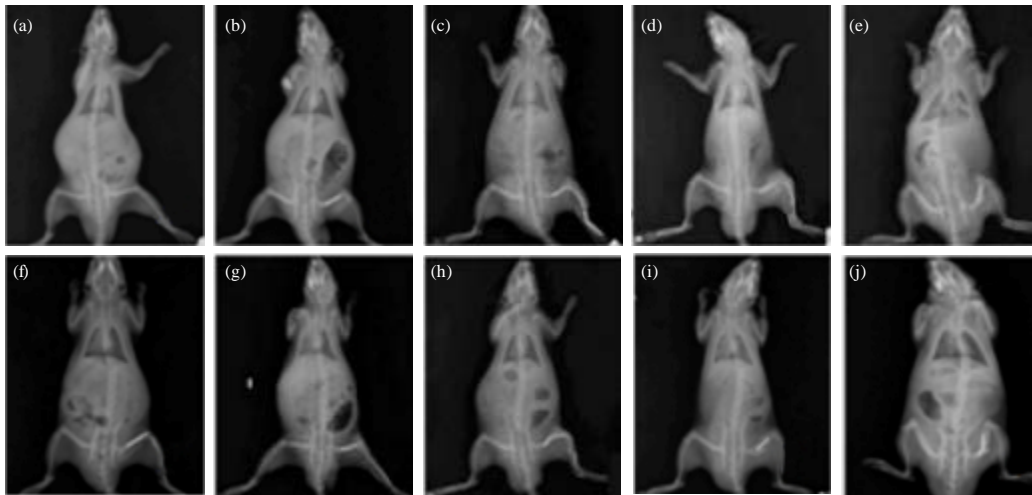


Fig. 1(a-j): X-ray films of femur fracture healing, (a) Control group, (b) Fracture 7 days, (c) Fracture 14 days, (d) Fracture 21 days, (e) Fracture 28 days, (f) Model group, (g) Treatment 7 days, (h) Treatment 14 days, (i) Treatment 21 days and (j) Treatment 28 days

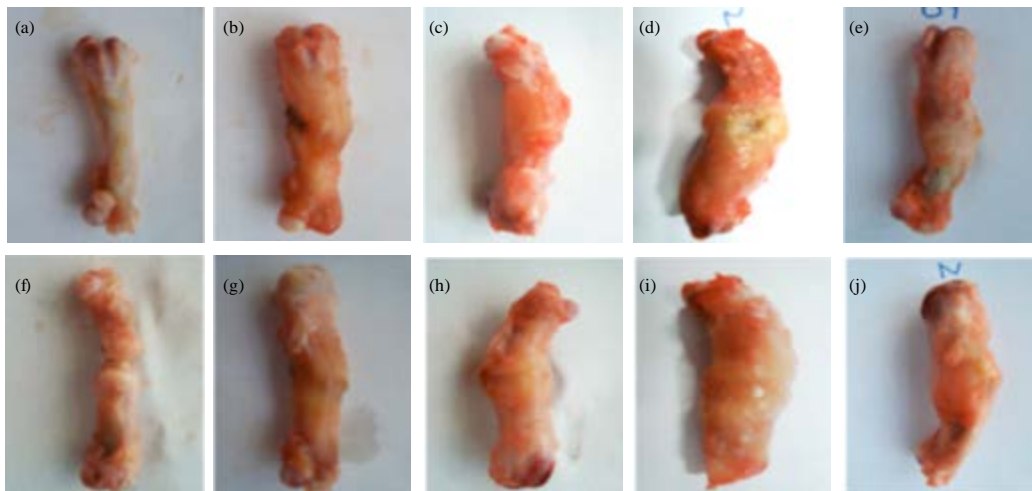


Fig. 2(a-j): Gross anatomy of femur fracture healing, (a) Control group, (b) Fracture 7 days, (c) Fracture 14 days, (d) Fracture 21 days, (e) Fracture 28 days, (f) Model group, (g) Treatment 7 days, (h) Treatment 14 days, (i) Treatment 21 days and (j) Treatment 28 days

treatment 7-21 days (Fig. 3g, h and i), bone callus structure existed no significant difference between the treatment and model groups in the corresponding time, but bone callus structure in the treatment group (Fig. 3j) was significantly better than that in fracture group rats (Fig. 3e) at fracture 28 days.

Serum level of GH: Compared from the aspect of treatment times, there was no significant difference in

serum levels of GH in the control group rats during fracture 7-28 days ($t = 0.10-0.58, p > 0.05$). And also no significant changes of serum levels of GH existed both in the fracture group and treated group during fracture 7-21 days ($t = 0.37-1.93, p > 0.05$), while it decreased significantly at fracture 28 days ($t = 2.64-10.46, p < 0.05$). Compared between the groups: The serum levels of GH in fracture group and treated group rats during fracture 7-28 days were significantly higher than those in control

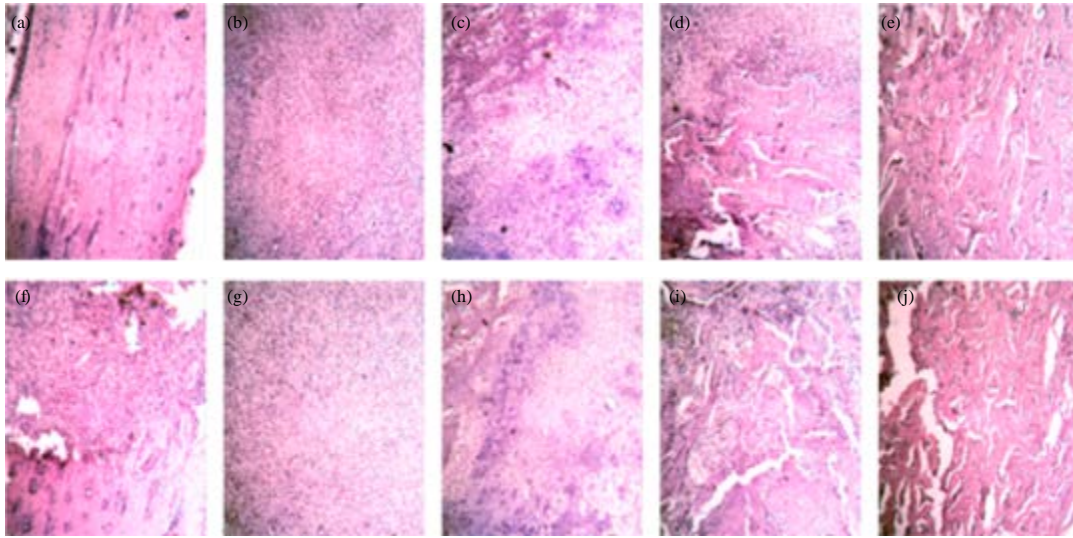


Fig. 3(a-j): Histopathology of femur fracture healing, HE×200, (a) Control group, (b) Fracture 7 days, (c) Fracture 14 days, (d) Fracture 21 days, (e) Fracture 28 days, (f) Model group, (g) Treatment 7 days, (h) Treatment 14 days, (i) Treatment 21 days and (j) Treatment 28 days

Table 1: Serum levels of GH after treatment ($\bar{x}\pm SD$, ng L⁻¹)

Groups	Time (days)			
	7	14	21	28
Control	14.05±0.61	14.08±0.67	14.05±0.61	14.21±0.63
Fracture	17.63±0.75 ^a	17.37±0.64 ^a	17.75±0.70 ^a	14.78±0.56 ^{bc}
Treatment	17.22±0.73 ^a	16.99±0.55 ^a	17.32±0.67 ^a	16.54±0.65 ^{abc}

^aCompared with the control group, $t = 2.13-12.60$, $p < 0.05$, ^bCompared with the fracture group, $t = 6.48$, $p < 0.05$, ^cCompared with the 21 days, $t = 2.64-10.46$, $p < 0.05$, $n = 6$

group in the corresponding time ($t = 2.13-12.60$, $p < 0.05$), after treatment 7-21 days, the serum levels of GH in treatment group rats showed no significant difference compared to the fracture group rats in the corresponding time ($t = 1.36-1.58$, $p > 0.05$), but at treatment 28 days were still significantly higher than those in the fracture group ($t = 6.48$, $p < 0.05$) and the control group ($t = 12.82$, $p < 0.05$) (Table 1).

Serum level of CT: According to treatment times, the serum levels of CT in the control group rats did not changed significantly after fracture 7-28 days ($t = 0.20-1.00$, $p > 0.05$) and also no significant changes during fracture 7-21 days both in the fracture group and treated group ($t = 0.03-1.56$, $p > 0.05$), but decreased significantly at fracture 28 days ($t = 5.99-13.36$, $p < 0.05$). Comparisons between the groups is the serum levels of CT in fracture group and the treatment group were significantly higher than those in the control group after fracture 7-28 days during the corresponding times ($t = 2.44-19.13$, $p < 0.05$). After 7-21 days, there was no

significant difference in the serum levels of CT between the treatment group and the fracture group in the corresponding time points ($t = 0.73-1.53$, $p > 0.05$), but at treatment 28 days it was still significantly higher than those in the fracture group ($t = 6.59$, $p < 0.05$) and the control group ($t = 11.75$, $p < 0.05$) (Table 2).

Serum PTH levels: Compared from the treatment time, no significant difference in serum levels of PTH existed in the control group rats after fracture 7-28 days ($t = 0.61-1.03$, $p > 0.05$). Moreover, the serum levels of PTH did not change significantly during 7-21 days both in the fracture group and the treatment group ($t = 0.36-1.74$, $p > 0.05$), but decreased significantly at fracture 28 days ($t = 5.28-13.00$, $p < 0.05$). Comparisons between the groups indicated that the serum levels of PTH in fracture group and the treated group after fracture 7-28 days were significantly higher than those in the control group in the corresponding times ($t = 2.42-14.10$, $p < 0.05$). In treatment group, the serum levels of PTH showed no significant difference after fracture 7-21 days compared to fracture group in the

Table 2: Serum levels of CT after treatment ($\bar{x}\pm SD$, ng L⁻¹)

Groups	Time (days)			
	7	14	21	28
Control	434.96±13.17	430.83±13.33	432.10±15.17	437.22±15.16
Fracture	541.79±14.60 ^a	541.61±12.56 ^a	543.77±15.38 ^a	453.57±14.81 ^{ac}
Treatment	528.70±15.45 ^a	532.91±12.28 ^a	538.96±14.00 ^a	498.87±15.89 ^{abc}

^aCompared with the control group, t = 2.44-19.13, p<0.05, ^bCompared with the fracture group, t = 6.59, p<0.05; ^cCompared with the 21 days, t = 5.99-13.36, p<0.05, n = 6

Table 3: Serum levels of PTH after treatment ($\bar{x}\pm SD$, ng L⁻¹)

Groups	Time (days)			
	7	14	21	28
Control	19.63±0.59	19.60±0.53	19.69±0.64	19.86±0.60
Fracture	25.14±1.17 ^a	25.31±0.93 ^a	26.05±1.04 ^a	20.63±0.81 ^{ac}
Treatment	24.80±1.05 ^a	24.53±0.97 ^a	25.32±1.06 ^a	23.17±0.73 ^{abc}

^aCompared with the control group, t = 2.42-14.10, p<0.05, ^bCompared with the fracture group, t = 7.37, p<0.05, ^cCompared with the 21 days, t = 5.28-13.00, p<0.05, n = 6

corresponding times (t = 0.68-1.84, p>0.05), but it was still significantly higher than that in the fracture group (t = 7.37, p<0.05) and control group (t = 11.08, p<0.05) after treatment 28 days (Table 3).

DISCUSSION

Bone remodeling or rebuilding included the re-sorption and the formation of osteoblasts, i.e., the bone absorbed calcium continuously from plasma for bone growth, at the same time, the bone calcium was absorbed and released into the blood. After fractures some bone growth factor promoted new bone formation and shortened fracture healing time (Turhani *et al.*, 2007). Animal experiments (Wang *et al.*, 2001) indicated that exogenous GH could promote fracture healing, but it is limited in clinical application because it could not promote fracture healing continuously and effectively due to its short half-life and high clearance rate in plasma and low bioavailability with non-injection. When a fracture happened, the expression of GH in pituitary eosinophils increased and the levels of GH in plasma increased significantly within fracture 24 h to promote the synthesis of osteocalcin (Vestergaard and Mosekilde, 2004). By stimulating the synthesis of Insulin-like Growth Factor (IGF), GH promoted the mitosis of osteoblasts, inhibited the formation of osteoclasts and induced apoptosis of osteoclasts (Liu *et al.*, 1998) thereby promoted fracture healing. GH improved calcium absorption from the intestine, excited the activity of hydroxylase in kidney and promote bone salt formation (Chen *et al.*, 1995). Also, GH could promote the synthesis of chondroitin sulphate and collagen, accelerate the cartilage calcification (Huang *et al.*, 2000), increase the content of cortical bone effectively

and improve the overall mechanical properties of bone (Hedstrom *et al.*, 2004).

In the early phase of fracture healing, CT could inhibit the expression of collagen type III mRNA and prevent excessive inflammation (Zheng *et al.*, 2009), while in late phase of fracture healing it could promote the expression of type I collagen mRNA and inhibits type II collagen mRNA in osteoblasts, thereby, promoting cartilaginous callus conversing to bone callus and maintaining normal morphology of trabecular bone (Mehta *et al.*, 2003). Its mechanism included that CT reduced bone re-sorption (Munoz-Torres *et al.*, 2004) by making intracellular calcium into the mitochondria, inhibiting osteoclast activity and inhibiting large mononuclear cells conversing to osteoclasts. Animal experiments (Wallach *et al.*, 1999) showed that the CT could shorten fracture healing time by promoting the formation of bone matrix including collagen, bone matrix mineralization and cartilage formation (Li *et al.*, 2003) and lighten disused osteoporosis due to fracture fixation (Petersen *et al.*, 1998) to improve bone mass and increase bone strength.

PTH could accelerate bone remodeling. Long-term high content of PHT in plasma could lead to bone re-sorption more than bone formation, so bone loss increased and Bone Mineral Density (BMD) reduced to induce deformation easily and pathological fractures (Arabi *et al.*, 2003). Injecting intermittently small doses of PTH could increase bone density, improve bone micro-architecture and reduce fracture risks (Gafni *et al.*, 2012). The double way effects of PTH (1-34) related closely to its multiple signal pathway. It acted mainly through activating PTHR1 receptor (G protein coupled receptor) (Mognetti *et al.*, 2011) on the surface of osteoblast to thereby activating several signal

transduction pathways including cAMP/PKA, NonPLC/PKC and PLC/PKC and so on (Yang *et al.*, 2007).

ZJXG decoction confirmed to the therapeutic principles of traditional Chinese medicine: Yuqu, Xinsheng and Guhe (clearing stasis, regeneration and bone remodeling). In this prescription, Chinese Angelica (*Radix Angelicae Sinensis*) activated blood circulation and nutrient or enriched blood; Rhizoma Chuanxiong (*Rhizoma Chuanxiong*) activated blood circulation and removed stasis; Radix Rehmanniae Preparata (*Radix Rehmanniae Preparata*) nourished Yin (essence) and enriched blood, invigorated essence and replenished marrow; Milkvetch Root (*Radix Astragali*) reinforced Qi (air), raised yang solid surface, induced diuresis for removing edema; Sanchi (*Radix Notoginseng*) removed stasis and hemostasis, reduced swelling and relieved pain; Himalayan Teasel Root (*Radix Dipsaci Asperoidis*) nutrient liver and kidney, strengthened bones and muscles, reconciled blood; Eucommia Bark (*Eucommia ulmoides Oliv*) enriched liver and kidney, strengthened bones and muscles; Fortune's Drynaria Rhizome (*Rhizoma Drynariae*) enriched kidney and strengthened bones; Safflower (*Flos Carthami*) activated blood circulation and recovered channels, dispersed blood stasis and relieved pain; White Paeony Root (*Radix Paeoniae Alba*) replenished blood, nourished yin and liver, relieved urgency and pain. Modern medical research showed (Liu *et al.*, 2004) that in hematoma period, the Rhizoma Chuanxiong and Safflower improved regional blood circulation of fracture fragments, removed blood clots and metabolites and provided an ideal condition for callus formation. In bone callus formation period, Himalayan Teasel Root and Fortune's Drynaria Rhizome were rich in collagen, calcium and trace elements, involved in the metabolism of protein synthesis and benefit to bone repair (Zhou and Wang, 1999). During the bone callus reconstruction period, Fortune's Drynaria Rhizome promoted protein-polysaccharide synthesis and calcification to complete successfully the process of new bone substitution (Lin and Zhou, 1993). In this study, we observed that ZJXG decoction did not increase the serum levels of GH, CT and PTH after fracture 7-21 days, but just only maintained the serum levels of GH, CT and PTH continued at a high level after 21 days. It indicated that ZJXG decoction did not promote the secretion of these hormones, but could only slow their degradation rates and prolonged their half-life in plasma to enhance the effect of hormonal activity, thereby promote fracture healing.

CONCLUSION

It is concluded that ZJXG decoction can promote the fracture healing by reducing the decomposition of GH, CT and PTH and keeping their high levels in femur fracture rats.

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