ISSN 2347-6893

The Effect of ZJXG Decoction on The Serum Levels of GH, CT, PTH and VitD3 in Femur Fracture Rats

Hua Zhang, Hanzhong Zhu, Jidong Zhang, Guangzhen Zhou, Zhen Zhou*
Institute of Integrative Medicine, Qingdao University Medical College, Qingdao 266021, China
E-mail: w4zh@163.com, zhuhanzhong1974@163.com,
zjd8919319@163.com, guangzheng1983@163.com,
Corresponding to Zhen Zhou, Tel.: +86-0532-82991711,
E-mail: lnlyfly@163.com

ABSTRACT

In this paper, we aim to investigate the effects of Zhuang Jin Xu Gu(ZJXG) decoction on the serum levels of growth hormone (GH), calcitonin (CT), parathyroid hormone (PTH) and active vitamin D3 (VitD3) and the femoral fracture healing in rats. Femur fractures were generated in 72 male adult *Wistar* rats by cutting femur transversely at middle point. ZJXG decoction was administered orally after surgery for 7~28. The healing process was analyzed by gross anatomy and hematoxtlin Eosin (HE) staining in rats. The serum levels of GH and CT were assessed by enzyme linked immunobsorbent assay (ELISA). Gross anatomy indicated that the fibrous callus tissue at the femoral fracture-end increased at 7-28 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. ELISA results showed that the GH and CT levels in serum increased significantly 7-14 d following treatment with ZJXG decoction. It is concluded that ZJXG decoction could enhance the fracture healing by reducing the decomposition of GH and CT in femur fracture rats.

Keywords:

ZJXG decoction; Fracture; Growth hormone, Calcitonin; Parathyroid hormone; Vitamin D3;

Academic Discipline And Sub-Disciplines

Experimental Studies

SUBJECT CLASSIFICATION

Integrative Medicine

TYPE (METHOD/APPROACH)

Quasi-Experimental

Council for Innovative Research

Peer Review Research Publishing System

Journal of Advances in Biology

Vol. 7, No. 2

www.cirjab.com

editorsjab@gmail.com , editor@cirjab.com

ISSN 2347-6893

INTRODUCTION

In bone tissue formation process, hormone regulation and growth factor regulation, the nervous system regulate together form the nerve-endocrine-local growth factor regulating network, the entire process involved in the formation of bone tissue[1]. Growth hormone (GH) is a peptide released by the pituitary gland, and is a major regulator of collagen growth[2]. Growth hormone- insulin-like growth factor-I (GH-IGF-I) in the hypothalamus is an important human endocrine metabolic axis, it not only plays an important role in regulation to the person's growth and development, but also close regulates adult human tissue's repair and reconstruction[3]. Calcitonin (CT), parathyroid hormone (PTH) and the active Vitamin D3 (VitD3) comprise of the three major regulatory hormone to maintain human body calcium and phosphorus metabolism balance [4]. CT is a polypeptide composed of 32 amino acids, acting on the bone cells, promote the reconstruction of trabecular bone, accelerate fracture healing[5]. PTH consists of 84 condensed amino acid residues, its amino terminal - amino acid residues 31-38 fragments stimulated the growth of human and animal bone[6]. 1,25(OH)2D3 is the only active form of vitamin D in vivo, Calcium can be used by bone only under the action of active VitD3,and the active VitD3 can improve the activity of osteoblasts, inhibit osteoclast activity, enable the trabecular bone form normally, cartilage callus normally ossify[7]. Clinical application proved that ZJXG decoction can promote fracture healing, but the mechanism is not very clear[8]. This experiment aimed to elucidate if the effect of ZJXG decoction in fracture repair was related to the serum levels of CT, PTH and VitD3, and to investigate its mechanism of enhancing fracture healing.

MATERIALS AND METHODS

1.1 Animal models

Total of 72 male adult *Wistar* rats purchased from Experiment Animal Center of Qingdao Drug Inspection Institute, SCXK (LU) 20120010), weighted 230~250g. All rats were given time to adapt to the laboratory environment for 7 days, then divided randomly into control group (n=24), model group (n=24) and treated group (n=24).

1.2.Model preparation

The rats were anesthetized with intraperitoneal injection of 100g/L chloral hydrate (300 mg/kg) and then restrained in a prone position for operation. The femoral fracture model was established by cutting the femur transversely with low speed dental drill (JBX-NE22 , NSK Co. Ltd. Japan) at the middle section (about 1.0cm below the great trochanter) from medial parapatellar incision.The fractured femur was fixed with intramedullary Kirschner wires (diameter 1.0mm, 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% [8].

1.3 Treatment methods

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 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 two extractions yielded an amount of 224ml liquid medicinal decoction containing 112g of dry weight (concentration of 0.5g/ml). Rats in treatment group were administered orally with ZJXG decoction of 1.25g/kg according to the previous research results [8], one time a day for 28 days, while the saline solution was given at the same volume to sham and control group rats.

1.4 Evaluation index

Six rats in each group were selected to observe after treated 7, 14, 21, 28 days respectively.

1.4.1 X-ray examination

The rats were anesthetized by injecting intraperitoneally 10% chloral hydrate (300mg/kg) for X-ray evaluation (GE Revolution RE/d, USA).

1.4.2 Enzyme linked immunosorbent assay (ELISA)

About 4 ml blood were centrifugalized for 10 minutes at 4000 r/min at 4°C to separate the serum (2ml). The serum level of GH, CT, PTH and VitD3 measured using commercially available ELISA kits (Blue Gene Co. Ltd). The procedure was performed following manufacturer's instruction. The OD was calculated with Bio-Rad 550 microplate reader (USA) set to 450 nm to reflect the level of GH, CT, PTH and VitD3 (ng/L).

1.4.3 Histological staining

The femur were incubated in 4% formaldehyde solution for 24 h and decalcificated for 15 days in 20% ethylenediamine tetraacetic acid (EDTA). The interception of the fracture (including hematoma, callus) was dehydrated using graded ethanol, immersed in dimethylbenzene, embedded by paraffin. The 5μm thickness slices were made by mirotome (Leica RM2015, Shanghai Leica Instruments, China), the sections were stained with Hematoxylin Eosin (HE).

1.5 Statistical analysis

The data was expressed by mean ± standard deviation ($\bar{x} \pm s$) and analyzed with SPSS 16.5 statistical software. $P<0.05$ was considered significantly.

ISSN 2347-6893

RESULTS

2.1 Xray films

X-ray films revealed the femoral cortical bone was integrity and continuous in control group rats. The fracture-end was filled with fibrous tissue after fracture 7 days and became fibrous callus at fracture 14 days, and bony callus formatted at fracture 28 days. There were no statistical difference of callus structure existed between treated group and model group during fracture 7-21 days, but the bone callus structure in treated group was better than that in model group after treatment 28 days.

2.2 Anatomical observation

Cortical bone was complete in control group rats while the fracture fragments separated in model group rats. 7 days after fracture, the fracture-end was surrounded by fibrous granulation tissue. fibrous callus formed and turned hard 14 days after fracture. 21 days after fracture, fibrous callus and cartilage callus increased and became more hard, and fibrous callus was gradually replaced by cartilage and bone callus 28 days after fracture. There was no statistical difference between treated groups and model group during fracture 7-21 days, but callus in treated group became more hard than that in model group 28 days after fracture.

2.3 Histopathology

The bone structure of rats was normal in control group. The breaking-ends of rats in model group rats were filled with hematoma organization. On fracture 7 days, inflammatory cells infiltrated and granulation tissues occurred between fracture gaps. On day 14, fibroblast and osteoblasts proliferated of under periosteum in fracture fragments. Osteoclasts and osteoblasts were activated and trabecular bone formed at fracture 21 days, and trabecular bone was clearly visible until fracture 28 days. In treatment group, callus structure had no significant difference compared with model group at the same time during fracture 7-21 days, but by 28 days it was significantly better than that in model group.

2.4 Enzyme linked immunosorbent assay (ELISA)

2.4.1 The serum level of GH: Compared from the aspect of treatment times, there was no significant difference of serum levels of GH in control group during fracture 7-28 days (t=0.10-0.58, P>0.05). And also no significant difference of serum level of GH both in model group and treatment group during fracture 7-21days (t=0.37-1.93, P>0.05), while it significantly decreased at fracture 28 days (t=2.64-10.46, P<0.05). In paired comparisons of groups, the serum levels of GH in the treatment group and model group were significantly higher than those in control group during 7-28 days (t=2.13-12.60, P<0.05). There was no significant difference of serum levels of GH in model group and treatment group in the corresponding time during fracture 7-21 days (t=1.36-1.58, P>0.05), but still significantly higher than those in the model group (t=6.48, P<0.05) and control group (t=12.82, P<0.05) at treatment 28 days.(Table 1).

Table 1 Serum levels of GH ($\bar{x}$±SD, ng/L)

Groups	n	7d	14d	21d	28d
Control group	6	14.05±0.61	14.08±0.67	14.05±0.61	14.21±0.63
Sham group	6	17.63±0.75 [a]	17.37±0.64 [a]	17.75±0.70 [a]	14.78±0.56 [a c]
Treated group	6	17.22±0.73 [a]	16.99±0.55 [a]	17.32±0.67 [a]	16.54±0.65 [a b c]

[a] Compared with control group, t=2.13-12.60, P<0.05; [b] Compared with sham control group, t=6.48 , P<0.05;

[c] Compared with the 21 day, t=2.64-10.46, P<0.05

2.4.2 The serum level of CT: In control group, no significant difference of serum level of CT existed between 7 days to 28 days (t=0.20-1.00, P>0.05). Also there was no significant difference of serum level of CT both in model group and treatment group during fracture 7-21 days (t=0.03-1.56, P>0.05), while it decreased significantly at fracture 28 days (t=5.99-13.36, P<0.05). Compared in paired groups, the serum level of CT in treatment group and model group was significantly higher that in control group during fracture 7-28 days (t=2.44-19.13, P<0.05). There was no significant difference of serum level of CT between 7-21 days in model group and treatment group (t=0.73-1.53, P>0.05), but at fracture 28 day, they were still higher than those in the model group (t=6.59, P<0.05) and control group (t=11.75, P<0.05) (Table 2).

Table 2 Serum levels of CT ($\bar{x}$ SD, ng/L)

Groups	n	7d	14d	21d	28d
Control group	6	434.96±13.17	430.83±13.33	432.10±15.17	437.22±15.16
Sham group	6	541.79±14.60 [a]	541.61±12.56 [a]	543.77±15.38 [a]	453.57±14.81 [a c]
Treated group	6	528.70±15.45 [a]	532.91±12.28 [a]	538.96±14.00 [a]	498.87±15.89 [a b c]

[a] Compared with control group, t=2.44-19.13, P<0.05; [b] Compared with sham control group , t=6.59, P<0.05;

[c] Compared with the 21 day, t=5.99-13.36, P<0.05

ISSN 2347-6893

2.4.3 The serum level of PTH: There was no significant difference of serum level of PTH in control group among fracture 7-28 days (t=0.61-1.03, P>0.05). And also no significant difference of serum level of PTH both in model group and treatment group during fracture 7-21 days (t=0.36-1.74, P>0.05), while decreased significantly at fracture 28 days (t=5.28~13.00, P<0.05). In paired comparisons of groups, the serum level of PTH in treatment group and model group was significantly higher than that in control group among 7-28 days (t=2.42-14.10, P<0.05). There was no significant difference of serum level of PTH both in model group and treatment group among 7-21 days (t=0.68-1.84, P>0.05), but in treatment group it was still significantly higher than that in model group (t=7.37, P<0.05) and control group (t=11.80, P<0.05) at fracture 28 day.(Table 3).

Table 3 Serum levels of PTH ($\bar{x}$±SD, ng/L)

Groups	n	7d	14d	21d	28d
Control group	6	19.63±0.59	19.60±0.53	19.69±0.64	19.86±0.60
Sham group	6	25.14±1.17 [a]	25.31±0.93 [a]	26.05±1.04 [a]	20.63±0.81 [a c]
Treated group	6	24.80±1.05 [a]	24.53±0.97 [a]	25.32±1.06 [a]	23.17±0.73 [a b c]

[a] Compared with control group, t=2.42-14.10, P<0.05; [b] Compared with sham control group, t=7.37, P<0.05;

[c] Compared with the 21 day, t=5.28-13.00, P<0.05

2.4.4 The serum level of VitD3: There was no significant difference of serum level of VitD3 between day 7 and day 28 in control group (t=0.55-1.21, P>0.05). Also no significant difference of serum level of VitD3 between day 7 and day 21 in model group and treatment group (t=0.36-1.90, P>0.05), while decreased at fracture 28 days (t=5.90-14.00, P<0.05). In paired comparisons of groups, the serum level of VitD3 both in treatment group and model group was significantly higher that in control group between day 7 and day 28 (t=7.66-23.11, P<0.05). There was no significant difference of serum level of VitD3 between day 7 and day 21 in model group and treatment group (t=0.23-1.86, P>0.05), but on day 28, the levels was still significantly higher than that in model group (t=9.51, P<0.05) and control group (t=17.00, P<0.05) (Table 4).

Table 4 Serum levels of VitD3 ($\bar{x}$±SD, ng/L)

Groups	n	7d	14d	21d	28d
Control group	6	47.70±1.07	48.02±1.06	47.43±1.12	47.87±1.03
Sham group	6	57.85±1.14 [a]	58.34±1.15 [a]	58.16±1.09 [a]	51.45±1.06 [a c]
Treated group	6	58.43±1.05 [a]	58.23±1.03 [a]	59.15±1.16 [a]	56.13±1.14 [a b c]

[a] Compared with control group, t=7.66-23.11, P<0.05; [b] Compared with sham control group, t=9.51, P<0.05; [c] Compared with the 21 day, t=5.90-14.00, P<0.05

DISCUSSION

Some bone growth factor could promote the formation of new bone after fracture, shorten the time of fracture healing[9]. Pituitary eosinophilic cells enhances expression of GH, the level of serum GH is significantly increased in the 24h, promotes osteocalcin synthesis[10]. By stimulating the synthesis of IGF, promoting bone cell mitosis, inhibiting the formation of osteoclast, inducing osteoclastic apoptosis[11], GH thereby promote fracture healing.By improving intestinal calcium absorption, exciting kidney hydroxylase activity, GH promotes the formation of bone salt[12]. In the early stage of fracture healing, CT inhibits COL-III mRNA expression, to prevent excessive inflammatory reaction[13]. In the late phase promotes the expression of osteoblasts COL-I mRNA, inhibits COL-IImRNA expression, so as to promote the conversion of cartilage callus to bony callus, maintain the normal morphology of the trabecular bone[14]. Animal experiments [15] show that, CT can promote bone matrix formation,bone mineralization and cartilage formation, shorten the healing time[16], reduce the disuse osteoporosis following fracture fixation[17], improve bone mass, increase bone strength.PTH can accelerate the process of bone remodeling, long-term elevated PHT can lead to bone resorption exceeds bone formation, Induced bone loss, bone mineral density (BMD) decreased, easy deformation and pathological fracture[18]. Intermittent small dose injection of PTH can increase the bone density, improve bone microstructure, reduce the risk of fractures[19]. Shibatab reported[20], As a prodrug of active VitD3, alfacalcidol in mouse model of osteoporosis, pharma-cological dose to maintain normal blood calcium can inhibit bone resorption. Duque found that [21], the active VitD3 can inhibit the apoptosis induced by serum deprivation removal, increase osteoblast survival time. The active VitD3 plays an important role in maintaining the normal calcification, calcium balance and intestinal calcium absorption[22]. Supplement with calcium and the active VitD3 can increase bone mass, improve the bone biomechanical properties.

ZJXG decoction accord with "promoting blood circulation to remove blood stasis, tissue hyperplasia, fracture healing" principles of treatment. Research shows that[23], during the organization of hematoma, radix salviae miltiorrhizae, Rhizoma Chuanxiong, safflower etc. in ZJXG decoction improve the local blood circulation, remove blood clots and metabolic product, provide the conditions for callus formation. In the formation of callus, teasel root, Drynaria contain rich

collagen, calcium salts and trace elements, involved in protein synthetase metabolism, is beneficial to bone repair. In callus transformation period, pilose antler, ginseng, Rhizoma Drynariae, promote protein synthesis of polysaccharides and calcification, in order to complete the new bone creeping substitution process smoothly. This experiment indicated that in the first 3 weeks after fracture, ZJXG decoction did not increase the serum levels of GH,CT,PTH and VitD3, but could only maintained those bone growth factors in high level continuously after fracture 21 days. This showed that ZJXG decoction could not promote the secretion of endogenous bone growth cytokines, perhaps, only reduced down the decomposition rates of endogenous GH,CT,PTH and VitD3, prolonged their half life to enhance their activities, and thus promoted fracture healing.

REFERENCES

[1] Andreasen TT, Oxlund H. Local anabolic effects of growth hormone on intact bone and healing fractures in rats. Calcif Tissue Int, 2003, 73(3):258-264.

[2] Raschke M, Rasmussen MH, Govender S, et a1. Effects of growth hormone in patients with tibial fracture : a randomized, double-blind, placebo-controlled clinical trial. Eur J Endocrinol, 2007, 156(3):341-351.

[3] Oreffo RO. Growth factors for skeletal reconstruction and fracture repair[J]. Curr Opin Investig Drugs, 2004, 5(4):419~423.

[4] Giardino R, Fini M, Nicdi Aldini N, et a1. Effects of synthetic salmon calcitonin and alendronate on bone quaiity in ovariectomized rats. Minerva Med, 1997, 88(3):469-477.

[5] Garcia Delgado I, Preto S,Gil Fraguas L. Calcitonin , etidronate , and calcidiol treatment in bone loss after cardiac transplantation. Calcif Tissue Int, 1997, 60(2):155-159.

[6] Yang D, Guo J, Divieti P, et al. Parathyroid hormone activates PKC-delta and regulates osteoblastic differentiation via a PLC-independent pathway. Bone, 2006,38(4):485-96.

[7] Wu HW. Research on prevention and treatment of secondary osteoporosis[J]. China Journal of Modern Medicine, 2004, 14(7):75-78.

[8] Wang XJ, Pan YX, Du ZX, et al. Effects of Zhuangjin Xugu Decoction on the expressions of GH and NPY of fracture callus in rats. China Journal of Traditional Chinese Medicine and Pharmacy, 2013, 28(8):2420-2422.

[9] Turhani D, Weissenbock M, Stein E, et al. Exogenous recombinant human BMP-2 has little initial effects on human osteoblastic cells cultured on collagen type Icoated/non coated hydroxyapatite ceramic granules. J Maxillofac Surg, 2007, 65(3):485-493.

[10] Vestergaard P. Mosekilde. Fracture risk is decreased in acromegaly-a potential beneficial effect of growth hormone. Osteoporos Int, 2004, 15(2):155-159.

[11] Liu HF, Chen JJ, Yang PS, et al. Research of Sanhuajiegu Powder promote growth hormone and fracture healing . Chinese Journal of Traditional Medical Traumatology & Orthopedics, 1998, 6 (2): 7-11.

[12] Chen JJ, Yang PS, Pan SHCH, et al. Experimental report of the Sanhuajiegu Powder for treating bone fracture. Beijing Medical Journal, 1995, 1(1):58-60.

[13] Zheng PF, Dong Zh. Wang JG, et al. Calcitonin regulate expression of Cbfα1 mRNA in osteoblast by ERK-MAPK signaling pathway. Journal of Clinical Medicine in Practice, 2009, 13 (4): 35~38.

[14] Mehta NM, Malootian A, Gilligan JP. Calcitonin for osteoporosis and bone pain. Curr Pharm Des, 2003, 9(2):2659~2676.

[15] Wallach S, Rousseau G, Martin L. Effects of calcitonin on animal and in vitro modds of skeletal metabolism. Bone, 1999, 25(4):509-516.

[16] Li XL, Luo XL, Yu NSH, et al. Effects of Salmon calcitonin on the fracture healing of ovariectomized rats. Chinese Osteoporosis Magazine, 2003, 9 (2): 111-113.

[17] Petersen MM, Lauritzen JB, Schwarz P. Effect of nasal calcitonin on post-traumatic osteopenia following ankle fracture, a randomized double-blind placebo-controlled study in 24 patients. Acta Orthop Scand, 1998,69(3):347-350.

[18] Arabi A, Garnero P, Porcher R. et al. Changes in body composition during post-menopausal hormone therapy: a 2 year prospective study. Hum Reprod, 2003, 18(10):1747-1752.

[19] Gafni RI, Brahim JS, Andreopoulou P, et al. Daily parathyroid hormone 1~34 replacement therapy for hypoparathyroidism induces marked changes in bone turnover and structure. J Bone Miner Res, 2012,27(8):1811-1820.

[20] Shibata T, Shira-ishi A, Sato T, et al. Vitamin D hormone inhibits osteoclastogenesis in vivo by decreasing the pool of osteoclast precursors in bone marrow. J Bone Miner Res, 2002, 17(4): 622-629.

[21] Duque G, El Abdaimi K, Henderson JE, et al. Vitamin D inhibits Fas ligand-induced apoptosis in human osteoblasts by regulating components of both the mitochondrial and Fas-related pathways. Bone, 2004, 35(1):57-64.

[22] Adams JS, Kantorvich V, Wu C, et al. Resolution of vitamin D insufficiency in osteopenic patients results in rapid recovery of bone mineral density. J Clin Endocrinol Metab, 1999, 84(7):729-2732.

[23] Liu ZH, Yang SR, Hui C. Promote healing of experimental Chinese medicine Research Survey. Journal of Changchun University of Traditional Chinese Medicine, 2004, 20(2):63-65.