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Expression of FGF−2 and IGF−1 in diabetic rats with fracture

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ABSTRACT

Objective: To observe the change of fibroblast growth factor−2 (FGF−2), insulin−like growth factor−1 (IGF−1) in serum and bone callus after fracture in diabetic rats, and to explore molecular biological mechanism of healing of diabetic fracture. **Methods:** Thirty male SD rats were designed into normal (n=15) and control (n=15) groups randomly. Venous blood was extracted on the lst, 2nd, 4th, 6th, 8th week after surgery. It was certificated and the serum was obtained. Left lower extremity was observed by X− ray. Bone callus at broken ends was observed under light microscope. Expressions of FGF−2 and IGF−1 in tissue were detected by immunohistochemistry method, and ELISA was used to detect expression of FGF−2 and IGF−1 in serum. **Results:** The results showed a significant increase in the density and area of newly formed bone in the distraction gaps of normal rats compared to control rats. Increased cell proliferation was also found in the distraction gaps of normal rats versus control rats. There was significant difference in serum levels of FGF−2 and IGF−1 between two groups. **Conclusions:** The decrease of FGF−2 and IGF−1 both in the serum and in the fracture region is one of the reasons for bad bone healing or delayed union in rats' fracture with diabetes. There are some synergistic effects possibly between FGF−2 and IGF−1.

1. Introduction

Diabetes mellitus (DM) is a metabolic syndrome. Patients are often accompanied with fracture healing disorders and wound healing disorder. Fracture healing is a complex process with a variety of factors involved. With the recent development of molecular biology and the studies on related factors which can affect bone metabolism, we can explore the mechanism for slow fracture healing or non−healing of DM patients. In this study, we established the DM rat fracture model, observed the fibroblast Growth Factor−basic (FGF−2), insulin−like growth factor −1 (IGF−1) levels in serum after the fracture and analyzed their relevance, and then explored the mechanisms of DM fracture healing.

2. Materials and methods

2.1. Animals and grouping

A total of 30 male 6−week−old SD rats were selected, weighting 180−200 g, purchased from XX University Experimental Animal Division Department. They were randomly divided into two groups, the experimental group and control group (n=15), re−modeling rats were used as a supplement for the halfway death or abandonment. All rats were raised in a laboratory with constant humidity and temperature, animals received autoclave water for free drink. Rats in the experimental group were fed on high fat and high sugar diets, rats in the control group were fed with the routine diets. After the successful modeling of the diabetic model, the rats in the experimental group were used to establish tibia fracture model, while the rats in the control group only to establish fracture of tibia models.

2.2. Experimental instrument and reagents

Streptozotocin (STZ) was purchased from sigma company.Rat FGF–2, IGF–1 monoclonal antibody, SABC immunohistochemistry kit were purchased from Beijing Zhongshan Biotechnology Company. Rat FGF–2, IGF–1 ELISA kit were purchased from Wuhan Boster Reagent Company. Germany LeicaQwinV3 image analyzer, electronic scales, micro pipette, etc. were provided by the laboratory.

2.3. Diabetic model establishment

1% STZ, citric acid and citrate sodium buffer solution were injected into abdominal cavities of SD rats after 8 weeks of high fat and sugar diet. STZ injection volume is 50 mg/kg. Tail was cut after 1 d to collect blood, and weight and blood glucose were reexamined after one week. Animals with weight loss were assigned as diabetic rats, random non–fasting plasma glucose was ≥ 13.8 mmol/L.

2.4. Establishment of tibia fracture model

The rats of two groups were anesthetized with 10% chloral hydrate (300 mg/kg) by intraperitoneal injection. The upper and lower segment of the tibial were fixed after anesthesia, and the tibia were broken with external force. X–ray fluoroscopy was used to determine the middle segment non–displaced tibia fracture, then they underwent binging and fixation.

2.5. Serological examination

Three animals were sacrificed at 1, 2, 4, 6, 8 week, animal blood samples were obtained and FGF–2, IGF–1 levels were detected.

2.6. X–ray examination

Left tibia was detected at 1, 2, 4, 6, 8 weeks and then the growth condition of the callus was observed.

2.7. Histological examination

The left tibial bone ends were fixed in 10% formalin tissues. After dehydration, they were embedded, sectioned, and had routine HE stained. FGF–2, IGF–1 were detected by immunohistochemical assay. The immunofluorescent staining gray values of the expression were automatically detected by Leica–Qwin computer image analysis system.

2.8. Indicators observation

2.8.1. Pain behavior score
Standards were as follows: If the actions of the affected

limb and contralateral normal limb were the same, score was 0; If the affected limb was with claudication and need to raise during action, score was 1; If the affected limb was with obvious lameness and need to raise during action, score was 2; If the affected limb was not movable, score was 3.

2.8.2. Radiology score
All the results of the rats imaging were detected by X–ray Lane Sandhu scoring system. Standards were as follows: If the X–ray showed the fracture site did not heal, score was 0; If the X–ray showed the formation of the fracture fragments callus, score was 1;If the X–ray showed parts of fracture were healing, score was 2; If the X–ray showed no more fracture, score was 3; If the X–ray showed the fracture completely healed, score was 4.

2.8.3. Immunohistochemical score
All the results of the rats fracture site imaging were detected by Leica–Qwin scoring system after immunohistochemical. Gray value represents the transmittance in the per unit area. The greater gray value is, the less protein expression is. On the contrary, the protein expression is more.

2.9. Statistical analysis

The data was analyzed with SPSS 13.0 software. Data were expressed as mean±SD values. The differences between two groups were compared with *t* test. The relationship was analyzed by Spearman correlate analysis. *P*<0.05 was regarded as statistical significance.

3. Results

3.1. Pain behavior score

After the tibial fracture, all rats in two groups had movement disorder in their affected limb, and they were in a poor mental status and refusing food. The pain behavior score decreased with the healing of fractures, The pain behavior score were lower in the control group than the experimental group. In 2 weeks and 4 weeks, the pain behavior score of the control group were significantly lower than the experimental group (*P*<0.05). While in 1 week, 6 weeks and 8 weeks, there was no significantly different in the pain behavior score (*P*>0.05) (Table 1).

Table 1
Pain behavior score at different times after the fracture(mean± SD).

Groups	1 week	2 weeks	4 weeks	6 weeks	8 weeks
Experimental group	2.8±0.3	1.8±0.5*	0.7±0.4**	0.2±0.1	0
Control group	2.7±0.4	1.4±0.3	0.2±0.1	0	0

*: *P*<0.05, **: *P*<0.01 compared with control group.

2.2. Imaging results

After 1 week of the fracture, the broken end was very clear in the experimental group, and there was no significant callus. After two weeks of the fracture, there was a small amount of callus shadow in the experimental group, while the periosteal was thickening and the callus density was higher in the control group than the experimental group; After four weeks of the fracture, the callus shadow was much thicker than before between the bone ends in the experimental group, there was still transparent zone and the fracture lines were clear. While in the control group. the fracture lines were not clear and the transparence of the shadow was decline. After six weeks of the fracture, the fracture line was very clear in the experimental group, the callus could not fully wrapped fracture fragments; while in the control group, the fracture line disappeared, the callus could fully wrapped fracture fragments. After eight weeks of the fracture, the callus could wrapped fracture fragments completely in the experimental group, the fracture line was much clear than the control grop. While in the control group, the fracture line disappeared. After reexamination X-ray of rats in two groups, the result of the X-ray scores system showed that the scores of the control group were significantly higher than the experimental group ($P<0.05$). There was no significantly difference at 6 and 8 weeks ($P>0.05$) (Table 2).

Table 2
X-ray score at different times after the fracture (mean± SD).

Groups	1 week	2 weeks	4 weeks	6 weeks	8 weeks
Experimental group	0.4±0.3	1.3±0.5	2.7±0.4	3.6±0.4	3.9±0.1
Control group	0.7±0.3[*]	1.8±0.3[*]	3.7±0.3[**]	4.0±0.0	4.0±0.0

*: $P<0.05$, **: $P<0.01$ compared with control group.

3.3. Comparison of the histological results

After 1 week of the fracture, the cartilage in the experimental group were with a immature development and a small volume and arranged in a loosely way, While in the control group, the chondrocyte were with a mature development and arranged in clusters; After 2 weeks of the fracture, there were trabeculae formation in the experimental group, but with low density, while in the control group, mature cartilage began to degenerate, osteoblasts arranged into a cord-like trabecular bone; After 4 weeks of the fracture, the osteoblast among the bone matrix of the experimental group were with a big volume and irregular arrangement, while in the control group the osteoblast were with a regular arrangement. After 6 weeks of the fracture, there were formation of the braided callus in the experimental group, while in the control group, there were formation of the lamellar callus, and the fracture fragments were completely wrapped; After 8 weeks of the fracture, there were formation of the lamellar bone in the experimental group, while there were also formation of the lamellar bone in the control group. In the experimental group, there were weak expressions of the IGF-1, FGF-2 in the a small amount of mesenchymal cells and the granulation tissue, the expressions gradually increased until reached to the peak after 3 weeks of the fracture and then decreased gradually, and decreased gradually after 4 weeks; while in the control group, the IGF-1 and FGF-2 expression were higher than the experimental group, and the expression at the 1 week, 2 weeks, 4 weeks were significantly higher than the experimental group ($P<0.05$).There was no difference at 6 weeks and 8 weeks ($P>0.05$) (Table 3).

3.4. ELISA detection of FGF-2, IGF-1 concentrations

IGF-1 concentration at the 1 week and 2 weeks were significantly higher than the experimental group ($P<0.05$),

Table 3
Immunohistochemistry gray value of IGF-1 and FGF-2 after the fracture (mean± SD).

Groups	1 week		2 weeks		4 weeks		6 weeks		8 weeks	
	IGF-1	FGF-2	IGF-1	FGF-2	IGF-1	FGF-2	IGF-1	FGF-2	IGF-1	FGF-2
Experimental group	145.5±28.6[*]	95.7±16.7[*]	153.6±29.4[*]	102.3±17.2[*]	156.2±32.3[**]	122.7±22.5[**]	152.3±30.6	115.2±20.4	133.5±28.3	113.6±18.8
Control group	168.2±30.8	124.5±20.7	176.7±30.5	132.8±23.2	163.4±28.0	130.5±26.3	144.7±29.5	128.4±25.7	138.4±29.6	118.7±24.8

*: $P<0.05$, **: $P<0.01$ compared with control group.

Table 4
IGF-1 and FGF-2 concentrations after fracture (ng/mL, mean± SD).

Groups	1 week		2 weeks		4 weeks		6 weeks		8 weeks	
	IGF-1	FGF-2	IGF-1	FGF-2	IGF-1	FGF-2	IGF-1	FGF-2	IGF-1	FGF-2
Experimental group	3.8±0.1	2.0±0.2	4.0±0.1	2.5±0.2	4.1±0.1	2.8±0.2	4.0±0.1	2.5±0.2	3.9±0.1	1.9±0.2
Control group	4.0±0.1[*]	3.1±0.2[*]	4.3±0.1[*]	4.2±0.2[*]	4.0±0.1	3.8±0.2[*]	4.0±0.1	2.8±0.2	3.8±0.1	2.3±0.2

*: $P<0.05$ compared with control group.

while the difference at 4 weeks, 6 weeks and 8 weeks were not significant ($P>0.05$). The difference in FGF–2 at 6 weeks and 8 weeks was not significant ($P>0.05$) (Table 4).

3.5. Correlation analysis of IGF–1 and FGF–2 expression

In diabetic rats,the correlation analysis of IGF–1 and FGF–2 showed there was a positive correlation between them ($r = 0.736$, $P = 0.008$).

4. Discussion

A fracture is a medical condition in which there is a break in the continuity of the bone. Fracture healing is a complex and continuous process. According to the histological and cytological changes, it includes three phases: (1) the stage of organization of hematoma and inflammation, which refers to the necrosis of the soft tissue and bone tissue after fracture, and then cause the inflammatory response, thus cause the organization of hematoma and the formation of granulation tissue; (2) The stage of primary porosis. Bridge callus formated, which represents the formation of callus; (3) The moulding period of bone plates[1–4]. DM is a metabolic clinical syndrome. Patients with diabetes are more likely to suffer fracture than the general population and slow healing, with nonunion or delayed healing rate and pseudarthrosis rate[5–8].

With the development of molecular biology,the research about the fracture healing of diabetic patients are more deeper and extensive. Liu et al[9] confirmed that inflammatory cytokines (IL–6, IL–8 and TNF– α) have a synergistic effect in the process of fracture healing in diabetic rats, which can provide a reference to determine the progress of diabetes fracture healing. Wang et al[10] observed IGF–1 gene transfected mesenchymal stem cell transplantation on the impact of fracture healing in diabetic rats, they think IGF–1 gene transfected BMSCs transplantation can promote fracture healing of diabetic rats. J iang et al[11] showed during the stage of early fracture, the TGF– β 2, BFGF, PDGF levels decreased, and the IL–6, IL–8 and TNF– α were increased to participate in the process of fracture healing in diabetic rats, which is one of the reasons for the delayed healing and nonunion. Wang et al[12] observed the effect of bone morphogenetic protein–2 (BMP–2) gene transfection BMSCs transplantation on diabetic fracture healing, and detected BMP–2 by immunohistochemistry and ELISA assay, the result showed that BMP–2 gene transfected BMSCs transplantation can promote fracture healing of diabetic rats. Therefore, the first thing of our study is to establish an animal model of diabetic rats, and then we observed the FGF–2, IGF–1 expression in diabetic rats after fracture and the change of morphology and function during the fracture healing. We compared the difference of the tissues and cells between normal fracture healing and the diabetic fracture healing and provided new ideas for the prevention and treatment of diabetes fractures. Our observation showed that after the tibial fracture, rats in two groups were both with a movement disorder in their affected limb, and they were both in a poor mental status and refusing food. The pain behavior score decreased with the healing of fractures. However, at 2 weeks and 4 weeks the behavior score of experimental rats was significantly higher than the control group. The X–ray examination showed after the 1, 2, 4, 8 weeks of the fracture, the callus formation of the experimental group was worse than the control group, the difference was statistical significant. After 1, 2, 4, 6, 8 weeks of the fracture, the cartilage development and the trabecular bone formation showed that the healing of diabetic rats were slow and delayed than normal rats. All these showed a successful modeling.

The expression of FGF–2, IGF–1 in the experimental group and the control group were detected by immunohistochemistry, the result showed the expressions were located in a small amount of the mesenchymal cells and granulation tissue in the experimental group. The IGF–1, FGF–2 expression reached the peak at 2 weeks in the control group, then was gradually reduced. That happened one week later in the experimental group than the control group, the expression reached the peak at 3 weeks and then decreased gradually, and it decreased gradually after 4 weeks, both of the expressions were higher in the control group than in the experimental group, which is also consistent with Wang et al[13–17]. The expression of IGF–1 and FGF–2 of diabetic rats in the early stages were lower than the control group. Hyperglycemia may have inhibitory effect on IGF–1, FGF–2 expressions and lead to the delayed fracture healing. After 1 week and 2 weeks of the fracture, the peripheral blood IGF–1 concentrations in the control group were significantly higher than the experimental group, while after 1 week, 2 weeks and 4 weeks, the FGF–2 concentrations of the experimental group were significantly lower than the control group. After 6 weeks and 8 weeks of the fracture, there was no significantly differences of the IGF–1 and FGF–2 of the rats in each groups. Long–term hyperglycemia can decrease the IGF–1 binding and lead to easily discharge, so there was a low expression of IGF–1 in early fracture.FGF–2′s ability to accelerate fracture healing have been reported[18–23]. The Spearman rank analysis showed that there is a positive correlation between IGF–1 and FGF–2 in the process of fracture healing of the diabetic rat.

Fracture healing is systemic and localized mutual

adjustment process[24,25]. The diabetes fracture healing requires the participation of a variety of cytokines, and the interactions of the cytokine can influence the fracture healing. Studies have confirmed that it is correlated between IGF–1 and FGF–2 expression of the diabetic fracture, the specific mechanism needs further study.

Conflict of interest statement

We declare that we have no conflict of interest.

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