

ORIGINAL ARTICLE

Effects of insulin therapy on fracture healing and expression of VEGF in diabetic rats

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Summary

This study was designed to investigate effects of insulin on fracture healing and expression of vascular endothelial growth factor (VEGF) in diabetic rats. Wister rats were randomly divided into diabetic control (n=66), diabetic insulin (=66) and non-diabetic control group (n=66). Diabetes was established by peritoneal injection of alloxan. Tibia fracture was surgically created and was allowed to heal. Radiological and biomechanical examinations were performed on the healing tibia. Immunohistochemistry was used to assess VEGF expression in the healing fracture tissues. Cortical reconstruction of the fracture sites in non-diabetic control and diabetic insulin groups was more rapid than in diabetic control group within 6 weeks of the fracture. Mechanical strength of the affected tibia in the diabetic insulin and non-diabetic control group was superior to diabetic control group. Histological examination of the fracture sites revealed a delay in chondrocyte maturation and hypertrophy in diabetic control group. VEGF expression was widely distributed in fracture sites within the first 4 weeks in control and diabetic insulin treatment group. However VEGF expression in the callus and periosteum in diabetic control group was much less than in diabetic insulin or non-diabetic control group. In conclusion, diabetes delays fracture healing and adversely affects callus formation with a reduced VEGF expression at the fracture sites. Insulin therapy improves fracture healing in diabetes rats, possibly through enhancing VEGF expression in the fractured bones.

Key words: insulin; fracture; VEGF; diabetes; rats

INTRODUCTION

Diabetes is a common chronic disease that is associated with significant morbidity and mortality. Patients with diabetes who sustain a fracture are at increased risk for complications including higher

rates of in hospital mortality, in-hospital postoperative complications, delayed bone healing and nonunion (Gaston and Simpson 2007, Wukich et al. 2011). In diabetic animals, the recovery of structural and material strength in healed fractures is delayed by at least 1 week compared with that in controls (Funk et al. 2000). Even few weeks after healing, fracture callus from the diabetic animals had a 29% decrease in tensile strength and a 50% decrease in stiffness compared with the controls (Macey et al. 1989). The mechanisms of delayed fracture healing in diabetic patients or animal models are unclear. Experimental studies suggested that impaired osteoclast function may contribute to decreased cartilage resorption and delayed endochondral ossification in diabetes

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(Kasahara et al. 2010). Diabetes was also found to cause an upregulation of proapoptotic genes during the transition from cartilage to bone in fracture healing, increasing chondrocyte apoptosis (Kayal et al. 2010). In addition, inadequate expression of genes that regulate osteoblast differentiation (Lu et al. 2003), suppression of cellular proliferation of chondroprogenitor cells and inhibition of chondrocytes in soft calluses during early stages of healing (Ogasawara et al. 2008), may also be responsible for the delayed fracture healing in diabetic animals.

Insulin seems to alleviate the delays in fracture healing in diabetic animals. In diabetic animals treated with insulin, the tensile strength and stiffness of the callus at 2 weeks was restored to the same level as the controls (Macey et al. 1989). Direct delivery of insulin to the fracture site in diabetic rats returned fracture healing to normal (Gandhi et al. 2005). When diabetic mice was treated with slow release insulin to maintain normal serum glucose levels, the loss of cartilage and enhanced osteoclastogenesis seen in the control group was normalized by treatment with insulin (Kayal et al. 2009). Higher level of chondrocyte apoptosis in diabetic mice was also blocked by insulin (Kayal et al. 2009). The mechanisms by which insulin alleviates loss of cartilage and facilitates endochondral bone formation during fracture repair in diabetic animals are unclear. As vascular endothelial growth factor (VEGF) played a role in the healing of fractures in animal models (Huh et al. 2009, Kidd et al. 2010) or in humans (Sarahrudi et al. 2009), we hypothesized that VEGF may be involved in the insulin-stimulated fracture healing in diabetic rats.

MATERIALS AND METHODS

Animal model

This study was approved by the institutional review board of Taishan Medical University, and principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed. Male Wistar rats (n=198, weight 280–320 g) were randomly divided into diabetic control (n=66), diabetic insulin (n=66) and non-diabetic control group (n=66). Diabetes was induced in diabetic control and diabetic insulin group by intraperitoneal injection of 5% solution alloxan (200 mg/kg body weight, Sigma, USA). Venous blood glucose was tested 48 hours after the injection. Animals whose blood glucose was greater than 16.7 mmol/l were chosen as diabetic animals (Islam and Loots 2009, Lee et al. 2010). In the diabetic insulin group, insulin was administered subcutaneously (Novolin 30R, Novo Nordisk, Denmark) at

a daily dose of 8–10U/kg/d for 8 weeks. Fasting blood glucose was measured daily and the dose of insulin was adjusted to maintain the blood glucose between 4 to 9 mmol/l. In the non-diabetic control group, no alloxan was administered, but normal saline was subcutaneously injected daily throughout the experiment. Under sterile conditions and general anesthesia with sodium pentobarbital, a closed tibia fracture of the right hind leg was surgically created in all animals.

Radiological and biomechanical examination

At 1, 2, 4, 6, 8 weeks following tibia fracture, 14 rats from each group were euthanized and the right hind leg was imaged by X-ray. X-ray films were examined by a radiologist to assess healing of the fracture sites and the growth of callus. A self-designed scoring system was used by the radiologist to semi-quantitatively assess the fracture healing and callus growth, in order to maintain consistencies between animals. In the second cohort of 14 rats from each group, the fractured tibias at week 4, 6 and 8 of the experiment were retrieved and soft tissues removed. In each group, 7 rats were used for bending strength test for the tibia, and the other 7 were used for torsional strength testing.

Histology and immunohistochemistry

At day 3, week 1, 2, 4, 6 and 8 following a tibia fracture, 4 animals from each group were randomly selected and euthanized. The fracture site of the tibia was sliced into 0.5 cm long specimens. After washing with normal saline, the specimens were placed in 10% neutral formalin and fixed for 24 hours. After embedding in paraffin, 20% EDTA was added to the specimens for decalcification. Bone tissues from the fracture sites were cut into 5µm thick sections, which were stained by hematoxylin and eosin stain. VEGF in the bone tissue was measured with ready-to-use SABC immunohistochemistry kit (Boster Biological Engineering, Wuhan, China). Tissues were stained with rat anti-VEGF antibody. Sections of samples were deparaffinized in xylene, hydrated through a graded series of ethanol, and then immersed in 3% hydrogen peroxide in 100% methanol for 30 min. The sections were boiled in 10 mM citrate buffer, pH 6.0 for 30 min. After being rinsed in phosphate-buffered saline (PBS), the sections were incubated with normal rat serum for 10 min. They were subsequently incubated overnight at 4 °C in humid chambers with rat anti-VEGF antibody. After washing with PBS, the slides were treated with peroxidase-conjugated streptavidin for 20 min, followed by treatment with 0.01% H₂O₂ and 0.05% diaminobenzidine tetrahydrochloride for 3 min.

Positive VEGF expression rate was measured as following: under light microscope (x40), cells with positive VEGF expression and total number of cells were counted in each visual field. VEGF expression was classified as: no staining (-); weak staining (\pm); moderate staining; and strong staining (++).

Statistical analysis

Statistical analysis was performed with SPSS 12.0. Data were expressed as mean \pm standard deviation (SD). One way ANOVA was used at the significance level $2\alpha=0.05$.

RESULTS

Diabetes was established after injection of alloxan in all animals of the diabetic control and diabetic insulin group. A tibia fracture was successfully established and all animals completed the 8 week study.

Radiological and biochemical examination results

The radiological examination results of the fractures are shown in Fig. 1. One week after fracture, a fracture line and a periosteal thickening was present. The periosteal thickening in diabetic insulin (Fig. 1A) and non-diabetic control group (Fig. 1C) group was greater than in diabetic control group (Fig. 1B). Two weeks after the fracture, trabecular bones were connecting at the fracture site. The bone formation and bone membrane in diabetic insulin group (Fig. 1D) and non-diabetic control group (Fig. 1F) was greater than in diabetic control group (Fig. 1E). Four weeks after the fracture, there was a clear trabecular connectivity in diabetic insulin (Fig. 1G) and non-diabetic control group (Fig. 1I) with a greater bone mass, but the fracture lines were still clear in diabetic control group (Fig. 1H) with less bone mass. Six weeks after the fracture, there was a further increase in bone mass at the fracture site, the fracture line was blurred and fully wrapped by callus in diabetic insulin (Fig. 1J) and non-diabetic control group (Fig. 1L). The fracture line was still clearly seen in the diabetic control group (Fig. 1K). At the end of week 8, bone mass in diabetic insulin (Fig. 1M) and non-diabetic control group (Fig. 1O) was further increased with little trace of fracture lines. In diabetic control group (Fig. 1N), fracture was completely wrapped by the callus, but the fracture line was still present.

At 4, 6 and 8 week following tibia fracture, three-point bending strength (Fig. 2) and torsional stiffness (Fig. 3) in diabetic insulin or non-diabetic control group was greater than in diabetic control group (statistically significant).

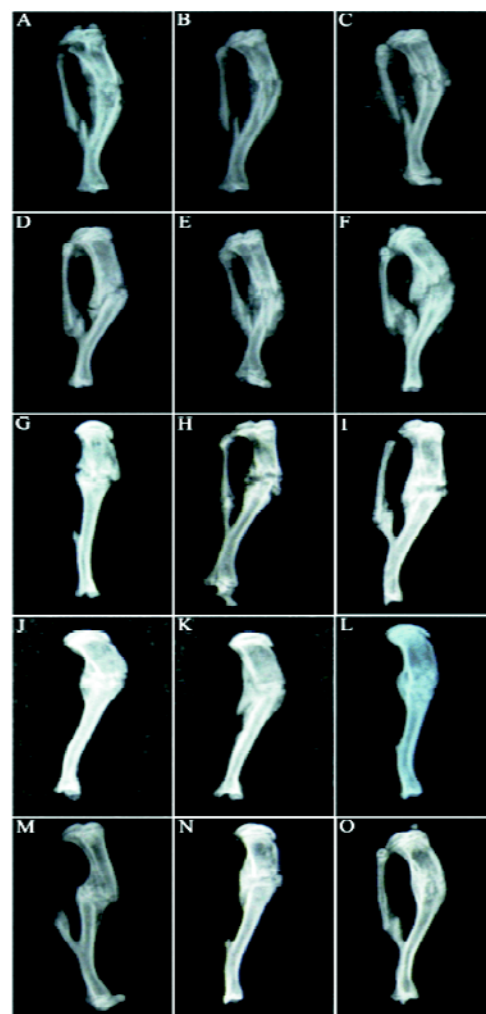


Fig. 1. Radiological examination of fractured tibia.

A, B, C, diabetic insulin, diabetic control and non-diabetic control group 1 week after fracture; D, E, F, insulin, diabetic control and non-diabetic control group 2 weeks after fracture; G, H, I, insulin, diabetic control and non-diabetic control group 4 weeks after fracture; J, K, L, insulin, diabetic control and non-diabetic control group 6 weeks after fracture; M, N, O, insulin, diabetic control and non-diabetic control group 8 weeks after fracture.

Histological examination results

The histological changes during fracture healing are shown in Fig. 4. Three days after fracture, sections of callus from diabetic insulin group (Fig. 4A) showed large amount of red blood cells, but few inflammatory cells and fibroblasts. A large number of red blood cells, inflammatory cells and fibroblasts were also observed in diabetic control group (Fig. 4B), with a greater number of inflammatory cells in each high

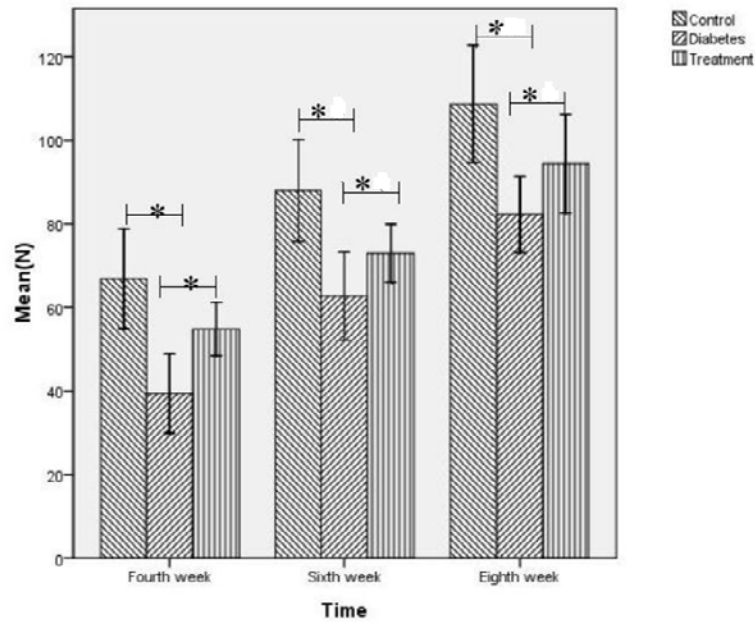


Fig. 2. Mean bending rigidity tests of 7 healing tibias in non-diabetic control, diabetic control and diabetic insulin group.
* statistically significant

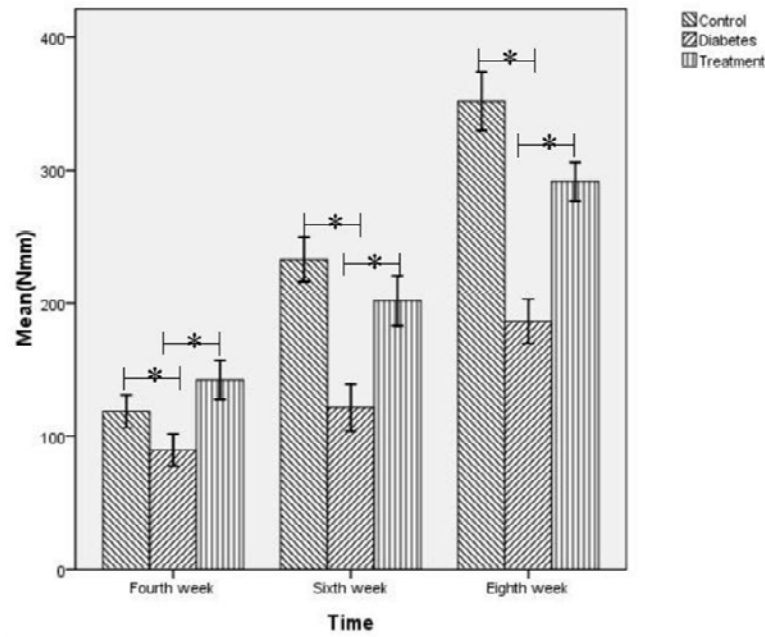


Fig. 3. Means of torsional strength tests of 7 tibias from control, diabetes and insulin group. Symbols as in Fig. 2.

power field than in diabetic insulin group. In non-diabetic control group (Fig. 4C), granulation tissues formed at fracture site, showing fibroblasts, and less number of inflammatory cells than in the diabetic control group.

One week after fracture, the boundaries between reserve cartilage zone and proliferative zone was marked in diabetic insulin (Fig. 4D) and non-diabetic control group (Fig. 4F), with mature chondrocytes arranged in clusters. In diabetic control group

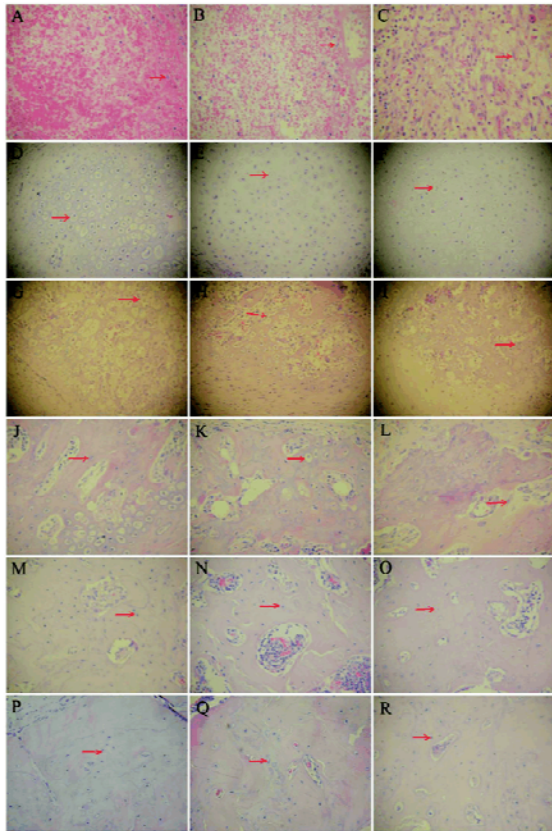


Fig. 4. Histological examination results of fracture sites in the diabetic insulin, diabetic control and non-diabetic control group. A, B, C, diabetic insulin, diabetic control and non-diabetic control group 3 days after fracture; D, E, F, diabetic insulin, diabetic control and non-diabetic control group 1 weeks after fracture; G, H, I, diabetic insulin, diabetic control and non-diabetic control group 2 weeks after fracture; J, K, L, diabetic insulin, diabetic control and non-diabetic control group 4 weeks after fracture; M, N, O, diabetic insulin, diabetic control and non-diabetic control group 6 weeks after fracture; P, Q, R, diabetic insulin, diabetic control and non-diabetic control group 8 weeks after fracture.

(Fig. 4E), chondrocytes were small, immature and loosely arranged.

Two weeks after the fracture, in diabetic insulin (Fig. 4G) and non-diabetic control group (Fig. 4I), mature chondrocytes degraded and osteoblasts arranged in monolayer on the surface of bone matrix to form cord-like trabecular bone. Trabecular bones seen in the diabetic insulin and non-diabetic control group were sparse in diabetic control group (Fig. 4H).

After 4 weeks, woven bones were formed at the fracture site, with small and scattered bone marrow cavity in the diabetic insulin (Fig. 4J) and non-diabetic control group (Fig. 4L). In diabetic

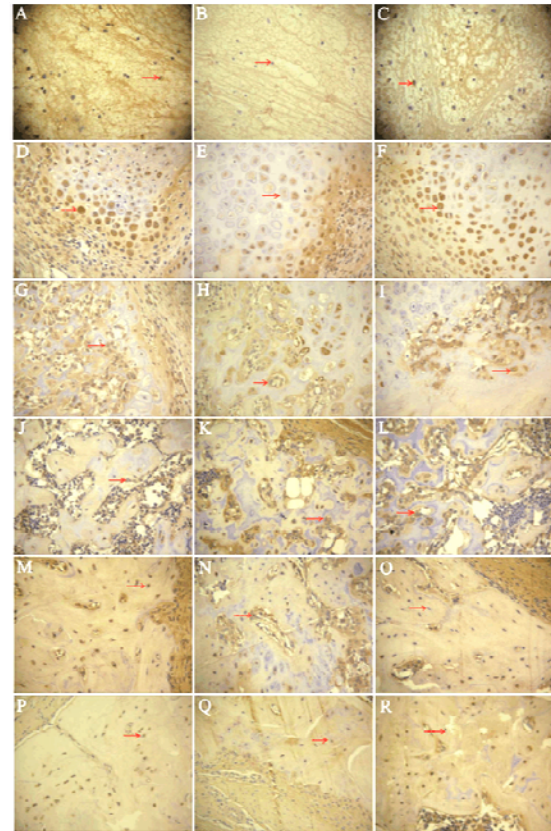


Fig. 5. VEGF expression in healing fractures. A, B, C, diabetic insulin, diabetic control and non-diabetic control group 3 days after fracture; D, E, F, diabetic insulin, diabetic control and non-diabetic control group 1 weeks after fracture; G, H, I, diabetic insulin, diabetic control and non-diabetic control group 2 weeks after fracture; J, K, L, diabetic insulin, diabetic control and non-diabetic control group 4 weeks after fracture; M, N, O, diabetic insulin, diabetic control and non-diabetic control group 6 weeks after fracture; P, Q, R, diabetic insulin, diabetic control and non-diabetic control group 8 weeks after fracture (DAB \times 200).

control group (Fig. 4K), callus matrix was mainly basophilic staining cartilage matrix, and osteoblasts in bone matrix were bulky, and irregularly arranged. After 6 weeks, woven bone calluses were combined to form lamellar bone in the diabetic insulin (Fig. 4M) and non-diabetic control group (Fig. 4O), marrow cavity gradually formed a large medullary cavity. In diabetic control group (Fig. 4N), woven bone calluses were more mature than before, bone mass were increased. At the end of week 8, calluses formed lamellar bone in three groups (Figs 4P, 4Q and 4R), but in the diabetic control group, osteocytes in bone matrix were bulky and irregularly arranged.

Table 1. Immunolocalization of VEGF in healing fractures.

		Weeks of healing								
		Week 1			Week 2			Week 4		
		Cont	DM	Ins	Cont	DM	Ins	Cont	DM	Ins
Intramembranous ossification	Osteogenic cells	+	+	+	+	+	+	+	±	±
				+			+			
	Osteoblasts in the subperiosteal bone	+	+	+	+	+	+	+	+	+
				+			+			
Endochondral ossification	Fibroblast-like cells	+	+	+	+	±	+	–	–	–
				+			+			
	Proliferating chondrocytes	+	+	+	+	+	+	–	–	–
				+			+			
	Mature chondrocytes	+	+	+	+	±	+	–	–	–
				+			+			
	Hypertrophic chondrocytes	–	–	–	–	–	–	–	–	–
	Osteoblasts in the trabecular bone near to endochondral ossification front	+	+	+	+	+	+	+	±	+
				+			+			

Cont, control; DM, diabetes group; Ins, insulin group; – no staining; ± weak staining; + moderate staining; + + strong staining.

Immunohistochemistry results

The expression of VEGF in the healing fractures is shown in Table 1. The intensities of expression of VEGF during fracture healing are shown in Fig. 5. The brown-yellow staining represents positive expression of VEGF. There was almost no expression of VEGF in normal bone tissues.

Three days after fracture, slices of callus showed that there were mainly hematomas at fracture site in three groups with visible inflammatory cells. VEGF staining was positive in extracellular matrix, stronger staining in diabetic insulin (Fig. 5A) and non-diabetic control group (Fig. 5C) than in the diabetic control group (Fig. 5B).

At the end of first week, diabetic insulin and non-diabetic control group (Figs 5D and F) showed stronger expression of VEGF than in diabetic control group (Fig. 5E) in the cytoplasm of subperiosteal

mesenchymal cells, chondroblast and chondrocytes at the fracture sites. The difference in VEGF expression between diabetic insulin or non-diabetic control group and the diabetic control group continued until week 4. At the end of week 8, there was still positive VEGF expression in osteocytes in the diabetic insulin group (Fig. 5P). However, only a small amount of VEGF expression can be seen in the diabetic control group (Fig. 5Q), whereas in the non-diabetic control group there was no trace of VEGF expression in the cytoplasm of the osteocytes (Fig. 5R).

DISCUSSION

Alloxan is a β -cell toxic agent, rendering irreversible damages to islet β -cells in the pancreas. It has been

used to induce diabetes in animals such as rats (Islam and Loots 2009, Lee et al. 2010). Dysfunction of insulin secretion occurs soon after alloxan injection, with a sustained high level of blood glucose after 24 hours. Alloxan-induced diabetes in rats is similar to human type 1 diabetes (Lee et al. 2010), but some investigators believe alloxan-induced diabetes also share many features of type 2 diabetes (Islam and Loots 2009). In the present study, a single injection of alloxan produced diabetes in all animals in which surgical fracture of the tibia was created to study the impact of diabetes on fracture healing.

Fracture repair involves several distinguishable processes, such as immediate response to injury, intramembranous bone formation, chondrogenesis, endochondral bone, and bone remodelling (Barnes et al. 1999). These healing processes are largely regulated by a number of growth factors, such as fibroblast growth factor, platelet-derived growth factor, transforming growth factor-beta, or bone morphogenetic protein (Barnes et al. 1999). In diabetes, there is a decreased expression of platelet-derived growth factor, transforming growth factor-beta and other growth factors such as insulin-like growth factor (Gaston and Simpson 2007). The reduction of these factors may reduce cell proliferation in the early phase of fracture healing (Gaston and Simpson 2007). There was also evidence that the level of VEGF was reduced in diabetes (Gaston and Simpson 2007), but the role of this reduction in the fracture healing process was not clear.

In the present study, histological examination of fracture sites showed that in untreated diabetic rats, periosteal reactions were weaker than in control or insulin treated animals. Maturity of chondrocytes in the diabetes group was also much delayed than in the control or insulin group. Biomechanical assessments revealed that bending strength and torsional stiffness in the diabetes group were inferior than in control or insulin group 4 weeks after the fracture. These results were consistent with previous reports that diabetes delays fracture healing and decreases biomechanical performance of the fractured bones (Macey et al. 1989, Funk et al. 2000, Lu et al. 2003, Kasahara et al. 2010, Kayal et al. 2010).

In the present study, VEGF expression was found in different stages of fracture healing. It was expressed in the fibroblasts and inflammatory cells in the hematoma shortly after fracture. One week after fracture, strong expression of VEGF was seen in the mesenchymal cells and in chondroblasts. Thereafter, the expression gradually increased in the osteoblasts, but began to weaken from week 6. We also found that VEGF expression in diabetes group was weaker than

in non-diabetic control group within the first 6 weeks of fracture. Insulin treatment improved VEGF expression within the first 4 weeks. These results suggest that insulin treatment following fracture is associated with an increased VEGF biosynthesis in the fractured tissues, which may be responsible, at least in part, for the improved bone healing observed in this group of animals.

In summary, this study showed that in diabetic rats, healing of fracture was delayed and biomechanical strength of the callus was decreased. The expression of VEGF in the fractured bone tissues of the diabetes rat was also reduced, indicating that VEGF reduction may play a role in the compromised fracture healing processes of the diabetes rats. Insulin therapy and maintenance of relatively normal blood glucose restored the level of VEGF expression in the fractured bones and promoted fracture healing in diabetic animals. These results indicate that adequate control of blood glucose, and therapies that enhance VEGF expression may facilitate fracture healing in patients with diabetes mellitus.

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