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# Effects of insulin-like growth factor I on alveolar bone remodeling in diabetic rats

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*Background and Objective:* Diabetes is a chronic hyperglycemic disorder and results in a tendency to develop osteoporosis. Furthermore, the delayed healing of tooth-extraction wounds, the activation of alveolar resorption and the suppressed formation of bone around implants are difficult for dentists to resolve. In diabetes, insulin-like growth factor I (IGF-I) appears to enhance the differentiation of osteoblasts and to activate the mineralization of bone. Hence, the aim of this study was to investigate the effects of insulin-like growth factor I on the remodeling of alveolar bone in diabetic rats.

*Material and Methods:* Diabetes was induced in 40 male Sprague-Dawley rats by intravenous administration of alloxan. The teeth of the rats were extracted to investigate remodeling of alveolar bone. Insulin-like growth factor I was administered, via intraperitoneal injection, to diabetic rats following tooth extraction. The remodeling of alveolar bone was determined using radiographic data, histological analyses and tetracycline fluorescence labeling.

*Results:* Compared with the control group, diabetes decreased alveolar bone formation. The height of alveolar bone and the bone-formation rate was significantly lower in the untreated diabetic group than in the control group or in the treated rats. Treatment with insulin-like growth factor I not only regulated abnormal blood glucose levels but also increased the height of the alveolar bone and increased the bone-formation rate relative to the results in diabetic animals. Furthermore, the expression of glucose transporter-1, the main transporter of glucose, was changed by hyperglycemia.

*Conclusion:* The results suggest that insulin-like growth factor I treatment increases the volume of newly formed bone following tooth extraction and normalizes the expression of glucose transporter-1 in diabetic rats, which may play an important role in bone formation and mineralization.

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Glucose is a major source of energy for most mammalian cells and is necessary for cells to maintain normal glucose transport and metabolism. However, abnormally high levels of glucose, as seen in diabetes, lead to the development of a number of chronic complications, including diabetic retinopathy, diabetic skin complications, diabetic nephropathy, as well as osteopenia and infection (1). Osteopenia and infection are involved in a number of diabetes-associated dental conditions, including delayed wound healing following tooth extraction, the activation of

alveolar bone resorption and impaired bone formation around dental implants (2–4).

Glucose transporter-1 (GLUT1) is the main cellular transporter that mediates the uptake of glucose. A number of studies have demonstrated that high glucose levels change the expression of GLUT1 in retinal endothelial cells, skin keratinocytes and mesangial cells (5–7). It is believed that changes in GLUT1 expression contribute to the pathogenesis of diabetic retinopathy, diabetic skin complications and diabetic nephropathy (1,8). Several studies have also reported that GLUT1 is expressed in developing and mature articular cartilage and may be required for the maintenance and homeostasis of this tissue (9). In addition, it has been reported that GLUT1 and GLUT3 mediate glucose uptake in UMR-106 osteoblasts (10) and that insulin can regulate the expression of *GLUT1* mRNA in these cells. In fact, insulin-like growth factor I (IGF-I) shares sequence homology with human proinsulin and exhibits insulin-like effects (11,12). However, the influence of high glucose levels on osteoblasts remains controversial, and the role of IGF-I in regulating glucose levels through GLUT1 expression during osteoblast development remains to be elucidated. Therefore, using an *in vivo* model of hyperglycemia, we investigated the influence of hyperglycemia and IGF-I on the remodeling of alveolar bone in diabetic rats and explored the mechanism of IGF-I therapy for the treatment of diabetes-associated osteoporosis and osteopenia.

## Material and methods

### Animal model

Forty, 21-d-old, male Sprague-Dawley rats (50–60 g in weight) were used in this study. The rats were raised and handled in agreement with guidelines set forth by the Legal and Ethical Committee of China. Diabetes was induced in these rats by intravenous administration of alloxan, as previously described (13–15). The rats were maintained in a diabetic condition for 1 wk before being randomly divided into four groups (normal control group, diabetic group, diabetic + insulin group and diabetic + IGF-I group) of 10 animals per group and exposed to experimental conditions. Briefly, 45 mg/kg of alloxan dissolved in 0.5 mL of physiologic saline was injected intravenously into 12-h fasted rats. One week after alloxan injection, diabetes was confirmed by the presence of blood glucose concentrations of > 11.1 mM or blood-fasting sugar concentrations of > 7 mM. The stability of the diabetes mellitus model was confirmed by the maintenance of diabetes for 1 wk in the rats. The left first mandibular molar was extracted from each animal. The blood glucose levels of each group were monitored using a glucometer (MediSense Products, Bedford, MA, USA), and the blood glucose levels of the treatment groups were maintained at 7–9 mM by the administration of insulin (5–6 units/d; Sigma, St Louis, MO, USA) or IGF-I (30 μg/kg/d; Sigma) via intraperitoneal injection, depending on the experimental group, until the rats were killed. Animals in the control and untreated diabetes mellitus experimental groups were injected with an equivalent volume of saline.

### Radiography and histology

The rats were killed 2 or 4 wk after tooth extraction by a lethal dose of sodium pentobarbital and the heart was perfused with 40 g/L of freshly depolymerized paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The left mandible was removed and immediately fixed in 4% neutral-buffered formalin. A digitized image of the left mandible was obtained for each rat, and gray-scale resolution of the alveolar bone density was determined using an image-analysis system (Leica Q500MC; Leica Instruments, Bensheim, Germany) and the height of alveolar bone was measured also using an image-analysis system (CDR; Schick, Long Island City, NY, USA). The mandibles were then decalcified in 0.5 M EDTA for 5–6 wk, frozen and sectioned serially at 6 μm along the coronal plane on a cryostat (Leica M1900; Leica Instruments). The alveolar bone of the first mandibular molar was stained with hematoxylin and eosin, and a histological examination was performed to observe pathological changes in bone tissue. For histomorphometric assessment of new alveolar bone, the number of cube and polygonal osteoblasts in every section ($n = 5$) from each animal ($n = 4$) of each group was counted. Only osteoblasts attached to the bone surface were counted. The number of osteoblasts per millimetre of alveolar wall in the socket was calculated.

### Tetracycline fluorescence labeling

To examine changes in bone morphology, a tetracycline fluorescence labeling study was performed. Briefly, four animals in each group were given two intramuscular injections of 1% tetracycline hydrochloride (50 mg/kg; Chemical Reagent Factory, Shang Hai, China) 3 or 9 d before they were killed. Four weeks after tooth extraction, the rats were killed with a lethal dose of sodium pentobarbital and the heart was perfused with 40 g/L of freshly depolymerized paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The left mandible was removed and immediately fixed in 80% ethanol. After embedding in methyl methacrylate, each mandible was serially sectioned at 30 μm along the coronal plane on a cryostat. Unstained sections were mounted in a nonfluorescent mountant (Fluormount, Gallard-Schlesinger INC., NY, USA) and examined in transmitted ultraviolet light. For histomorphometric assessment of the rate of alveolar bone formation, the distance between the two tetracycline-labeled lines was measured in the sections from each animal ($n = 5$) of each group. Results are given as mm/d.

### Expression of GLUT1 protein in newly formed bone

Immunohistochemistry was performed using a streptavidin-biotinylated enzyme complex system (Strept ABC Kit; Boster Biological Technology Co., Ltd. Wuhan, China). Rabbit polyclonal GLUT1 Ig (Chemicon International, Inc., Temecula, CA, USA) was used at a

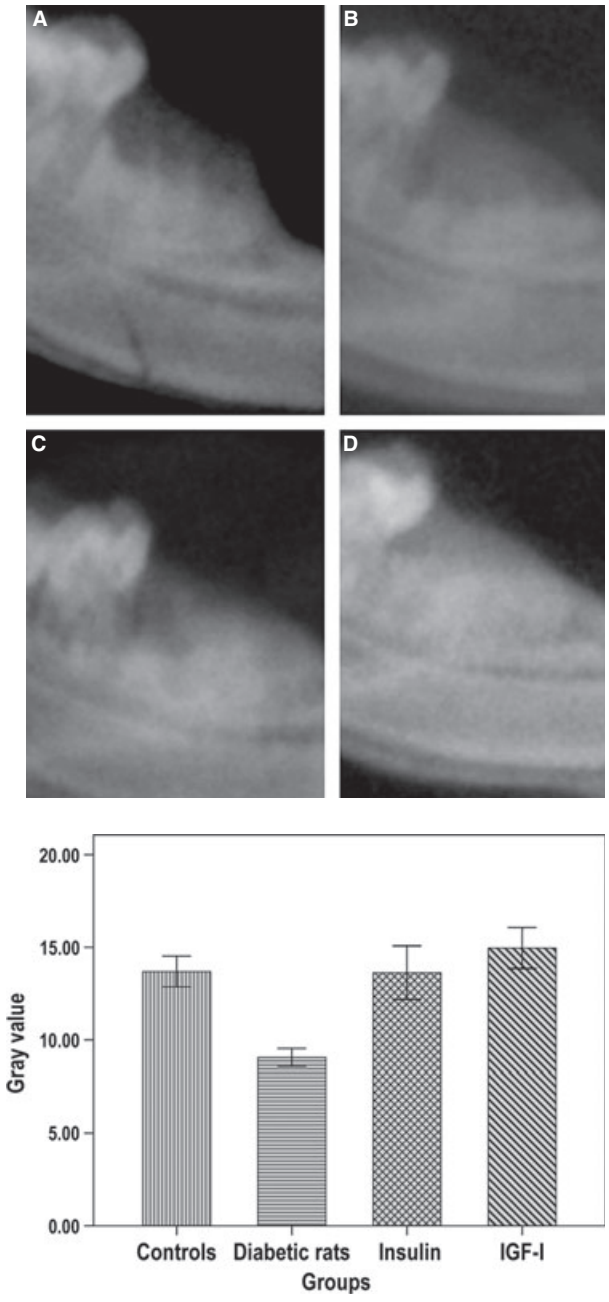


*Fig. 1.* X-ray images 4 wk after tooth extraction; the gray-scale value was analyzed using software. (A) Control group. (B) Untreated diabetic group. (C) Insulin-treated diabetic group. (D) Insulin-like growth factor I (IGF-I)-treated diabetic group.

dilution of 1 : 200. The number and gray-scale resolution of immunopositive regions in mandibular sections was determined using an image-analysis system (Leica Q500MC; Leica Instruments).

### Statistical analysis

In all experiments, one-way analysis of variance was performed to determine the effects of the different treatments at different time points. When the analysis of variance indicated a significant difference among the groups, statistical differences between individual groups were evaluated using the Student Newman-Keuls test. All experiments were repeated at least three times. All data are expressed as mean ± standard deviation. Statistical analyses were performed using the SPSS statistics base 17 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

## Results

### Radiographic analysis of alveolar bone

Diabetes was induced in Sprague-Dawley rats by intravenous administration of alloxan. Two weeks after tooth extraction, the boundary of the tooth socket was analyzed in each cohort. Bone density was evaluated from the digital X-ray photographs using a computer software program (LeicaQ500MC; Leica Instruments). The same exposure parameters were used each time, and the procedure was carried out by the same well-trained technician. When new bone formation was determined by a well-trained radiologist, the diagnosis was almost the same as that obtained using the software program. In the control and treated diabetic groups, new bone was observed and the tooth socket depth was decreased. In contrast, rats in the untreated diabetic cohort presented disordered and rarefied alveolar bone structures. By 4 wk after tooth extraction, the socket was filled with new bone and the bone trabecula had reached the alveolar ridge crest in each group. The height of the alveolar ridge was calculated to be 1.3811 ± 0.17 cm for the controls, 0.8433 ± 0.13 cm for the diabetic rats, 1.1678 ± 0.27 cm for the insulin-treated diabetic rats, and 1.2433 ± 0.18 cm for the IGF-I-treated diabetic rats. Statistical analysis demonstrated a significant difference in the alveolar ridge height between control and diabetic rats ($p < 0.01$), and between diabetic and IGF-I-treated and insulin-treated diabetic rats ($p < 0.01$). No significant difference was observed between control and treated diabetic rats or between IGF-I-treated and insulin-treated diabetic rats ($p > 0.05$). Analysis of gray-scale values, however, showed that the alveolar bone density in the treated groups was significantly greater than that in the diabetic groups, and diabetic rats showed a 40% decrease in bone density compared with that of the

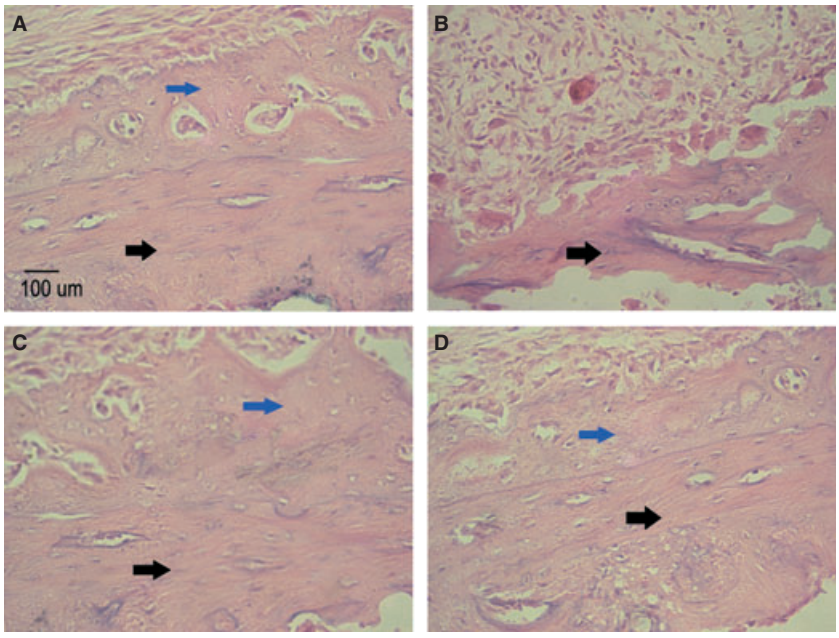


*Fig. 2.* Images of hematoxylin and eosin-stained mandibular sections 4 wk after tooth extraction (magnification 200×). (A) Control group. (B) Untreated diabetic group. (C) Insulin-treated diabetic group. (D) Insulin-like growth factor I (IGF-I)-treated diabetic group. The blue arrow indicates newly formed bone, and the black arrow indicates old bone.

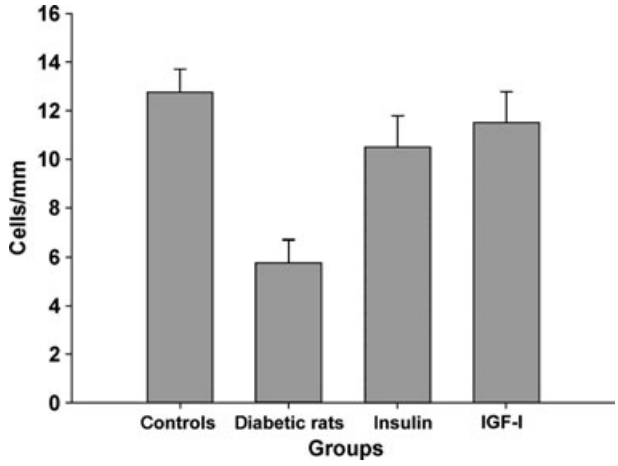


*Fig. 3.* Number of osteoblasts (expressed per mm of alveolar wall) in the socket 4 wk after tooth extraction. IGF-I, insulin-like growth factor I.

control group. Interestingly, IGF-I treatment was able to restore alveolar bone density almost completely (to 85% of the alveolar bone density observed in control rats) (Fig. 1).

### Histological analysis of alveolar bone

We analyzed, histologically, the regrown alveolar bone in rats from each of our four treatment cohorts. Two weeks after tooth extraction, we investigated granulation, tissue necrosis, fibroblast infiltration and new bone formation in the socket. In the untreated diabetic cohort, an increased number of inflammatory cells were present, and fibroblasts were in a state of disorder. Analysis of the control and diabetic cohorts 4 wk after tooth extraction demonstrated increased levels of new bone formation as well as the presence of numerous cube and polygonal osteoblasts. In contrast, the untreated diabetic group exhibited increased bone reabsorption in the alveolar tissue and minimal new bone formation 4 wk postextraction (Fig. 2). In both insulin and IGF-I treatment groups, the number of cube and polygonal osteoblasts was nearly equivalent to those observed in the control. The lowest number of cube and polygonal osteoblasts was found in the diabetic group compared with the control and treated diabetic groups. Statistical analysis demonstrated a significant difference in the number of osteoblasts between control and diabetic rats ($p < 0.01$), and between diabetic and IGF-I-treated and insulin-treated diabetic rats ($p < 0.01$). No significant difference was observed between control and treated diabetic rats or between IGF-I-treated and insulin-treated diabetic rats ($p > 0.05$) (Fig. 3).

### Formation rate of alveolar bone

The tetracycline fluorescence labeling technique clearly demonstrates alveolar bone formation. Using this methodology, two clear tetracycline-labeled lines corresponding to each injection time were identified on the labial and lingual regions of the alveolar wall, as well as on the alveolar ridge crest, in the control rats. These two labeled lines represent continuous bone formation by osteoblasts. Comparison of the control group with the diabetic group showed that diabetic rats exhibited a significant reduction in bone formation, as demonstrated by a distinct reduction in the width of the labeling lines. The alveolar ridge crest of the diabetic rats showed little or no tetracycline labeling, whereas the labial and lingual surfaces of the alveolar bone were labeled. Although two labels were found on the labial and lingual surfaces of the alveolar bone in the diabetic rats, the space between the two lines was markedly narrower than in the controls. In the sections from the IGF-I-treated and insulin-treated rats, this space appeared to be identical to that in the control sections. Quantification of bone volume formed on the alveolar

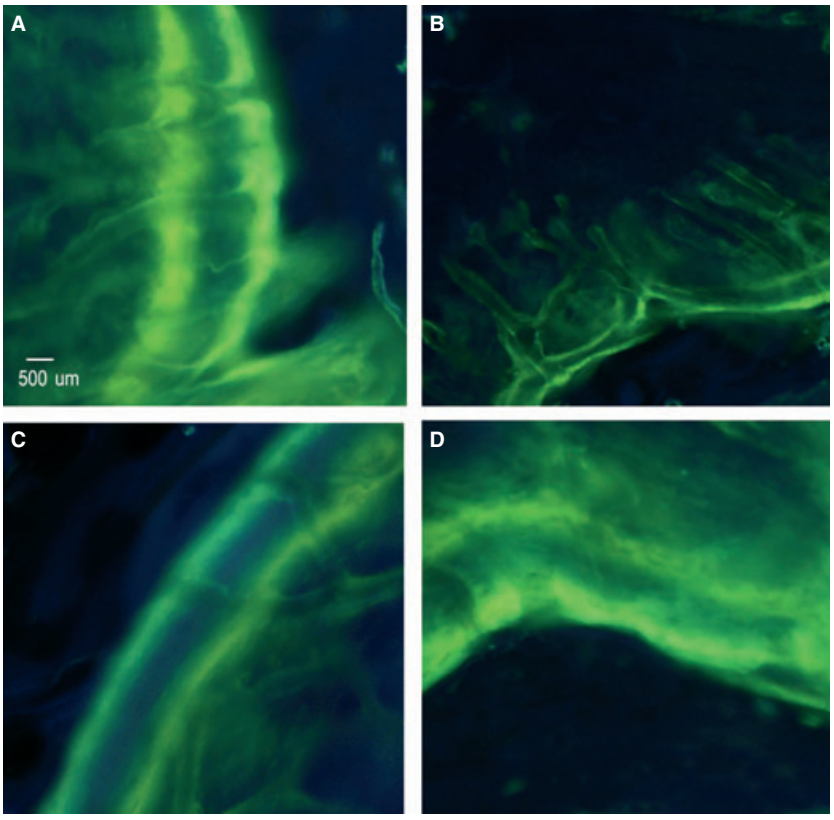


*Fig. 4.* The tetracycline fluorescence labeling technique clearly demonstrates bone formation in the alveolar bone after tooth extraction (magnification 20×). (A) Control group. (B) Untreated diabetic group. (C) Insulin-treated diabetic group. (D) Insulin-like growth factor I (IGF-I)-treated diabetic group.

wall over a 6-d period was calculated to be 0.993 ± 0.04 mm for the controls, 0.511 ± 0.01 mm for the diabetic rats, 0.845 ± 0.04 mm for the insulin-treated diabetic rats and 0.881 ± 0.02 mm for the IGF-I-treated diabetic rats (Fig. 4). In addition, the volume of bone formed was decreased by 48% in diabetic rats compared with control rats, and treatment of diabetic rats with IGF-I was able to restore alveolar bone growth to 88% of that observed in the control rats. In all groups, the rate of bone formation, as determined by tetracycline fluorescence labeling, was similar to the volume of bone formed. A statistically significant difference in the volume of new bone formed was observed between the control and the diabetic rats as well as between the untreated and treated diabetic rats ($p < 0.05$). No significant difference in bone volume was observed between the control and the treated diabetic rats ($p > 0.05$).

### Expression of GLUT1 protein in alveolar bone

We next investigated whether IGF-I is able to change GLUT1 expression during alveolar bone regrowth. Four weeks after tooth extraction, a weakly immunopositive GLUT1 signal was detected in both the control and treated diabetic groups and there was no significant difference in the amounts of protein expressed among these cohorts. However, in the untreated diabetic group, the levels of GLUT1 protein became increasingly higher compared with those in the control or treated diabetic groups. GLUT1 was expressed at similar levels in both the IGF-I-treated group and the insulin-treated group ($p > 0.05$) (Fig. 5). These data suggest that IGF-I treatment is able to normalize the increased expression of GLUT1 in the alveolar osteoblasts of diabetic rats to a level comparable to that obtained by treatment with insulin.

## Discussion

In this study, we utilized *in vivo* models of diabetes to investigate the influences of hyperglycemia and IGF-I on alveolar bone formation in rats. Radiographic data, histological analysis and tetracycline fluorescence labeling collectively demonstrated that alveolar bone formation *in vivo* is repressed in diabetic rats following tooth extraction, as seen by the pathological changes in the bone structure of the socket and decreased alveolar bone height. IGF-I was not only able to regulate abnormal blood-glucose levels but also to increase the height of alveolar bone and the bone-formation rate relative to those parameters in diabetic animals.

These data were explained by the results of our previous cell culture studies (16). During development, pre-osteoblasts undergo a series of differentiation stages, including proliferation, matrix development and matrix mineralization. Calcium accumulation begins in the matrix-development phase and peaks during the matrix-mineralization stage. There is a reciprocal correlation between osteoblast proliferation and osteoblast differentiation (2,17). Previous studies indicated that enhanced periodontal regeneration was observed in sites receiving growth and differentiation factors such as IGF-I, basic fibroblast growth factor and growth differentiation factor-5, compared with control sites. Transcriptional regulation of tissue-specific factors represses proliferation while permitting differentiation (18). Likewise, during cellular proliferation, differentiation factors are inhibited. Hyperglycemia increased cellular proliferation while concurrently inhibiting osteoblast mineralization. Furthermore, treatment with IGF-I reversed these effects and restored the balance between cellular proliferation and differentiation. Based on the results of our *in vivo* studies, IGF-I increases calcium deposition in the matrix and bone formation, contributing to wound healing and new bone formation in diabetic rats.

Although IGF-I appears to enhance bone mineralization in diabetes, very little is known about the molecular

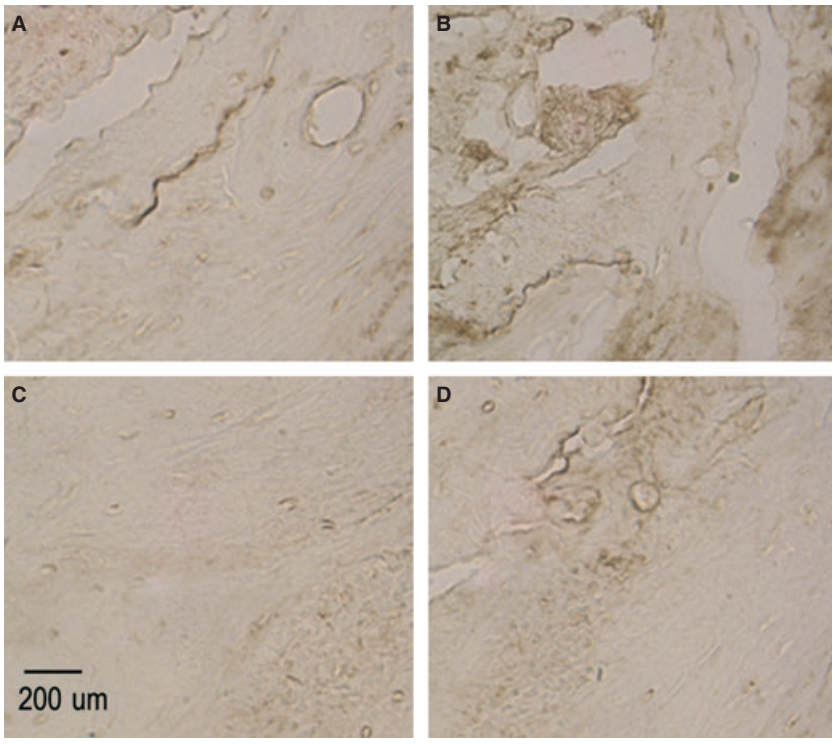


*Fig. 5.* Expression of glucose transporter-1 (GLUT1) protein in regenerated alveolar bone. Immunohistochemical analyses comparing GLUT1 protein expression in bone from control (A), untreated diabetic (B), insulin-treated (C) and IGF-I-treated (D) rats, 4 wk after tooth extraction (magnification 100×).

mechanism by which this occurs (3,10,16). As glucose is the main energy source for cellular activity and GLUT1 is the main transporter of glucose, altered expression of GLUT1 may directly affect the development of osteoblasts and the formation of new bone. Therefore, we hypothesized that IGF-I may be involved in the regulation of GLUT1 in response to high glucose levels in diabetic rats. Consistent with this hypothesis, we observed that in the untreated diabetic group, the levels of GLUT1 protein became increasingly higher compared with those in the control group, or in the IGF-I-treated or insulin-treated diabetic groups. These studies demonstrated that changes in GLUT1 expression may be the molecular mechanism responsible for decreased alveolar bone formation under hyperglycemic conditions. In addition, accompanied by the increased expression of GLUT1, the mineralization of osteoblasts decreased. However, when GLUT1 expression was down-regulated by IGF-I, this effect was reversed. These data suggest that IGF-I may be effective for the treatment of diabetes-associated bone disease. So, what might be the most appropriate route of administration of IGF-I, especially in humans? In this animal research, intraperitoneal injection, as a systemic application of IGF-I, was used; however, adverse effects may occur with the systemic application of IGF-I. There are various reports on the effect of the route of administration of IGF-I. Victory *et al.* (19) reported that the administration of IGF-I preserves or restores salivary gland function following radiation therapy for head and neck cancer. Systemically applied IGF-I could improve whole-body glucose uptake and glucose tolerance (20), but many reports show that IGF-I crucially influences malignant development (21–23). A higher level of serum IGF-I in older men is associated with an increased risk of death from cancer (22). Regarding those potential side effects, we consider that local application of IGF-I is the route of administration most suitable for humans, especially for older patients with diabetes who receive tooth extraction.

Although our previous *in vitro* cell study showed that calcium deposition in the matrix of IGF-I-treated osteoblasts was greater than that in cells in the insulin-treatment group (16), no significant difference in bone volume was observed between IGF-I-treated and insulin-treated diabetic rats. In fact, a previous study showed that GLUT1 might play a role in cementogenesis and could serve as a biomarker to differentiate between cells of cementoblastic and osteoblastic lineages (24). It seems that our results were different from those of other researchers, and we think that this may be a result of the different cells selected for use in each study. In our next study we will explore whether there is any direct evidence linking GLUT1 with the regulation of bone formation in osteoblasts.

In summary, the results suggest that IGF-I treatment increases the volume of newly formed bone following tooth extraction and normalizes the expression of GLUT1 protein in diabetic rats, which may play an important role in bone formation and mineralization. Although a number of questions remain, including the most appropriate mode of delivery of IGF-I, these results suggest that IGF-I may be an effective drug for the treatment of diabetes-associated bone diseases, specifically diabetes-related dental disease.

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