

Effect of Fluoride on Insulin Level of Rats and Insulin Receptor Expression in the MC3T3-E1 Cells

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Abstract Studies on the role of insulin and insulin receptor (InsR) in the process of skeletal fluorosis, especially in osteogenic function, are rare. We evaluated the effect of increasing F^- doses on the marker of bone formation, serum insulin level and pancreatic secretion changes in vivo and mRNA expression of InsR and osteocalcin (OCN) in vitro. Wistar rats ($n=50$) were divided into two groups, i.e. a control group and fluoride group. The fluoride groups were treated with fluoride by drinking tap water containing 100 mg F^-/L . The fluoride ion-selective electrode measured the fluoride concentrations of femurs. The alkaline phosphatase (ALP), OCN, insulin and glucagon of serum were tested to observe the effect of fluoride action on them. Meantime, the pancreas pathological morphometry analysis via β cells stained by aldehyde fuchsin showed the

action of fluoride on pancreas secretion. MC3T3-E1 cells (derived from newborn mouse calvaria) were exposed to varying concentrations and periods of fluoride. The mRNA expression of InsR and OCN was quantified with real-time PCR. Results showed that 1-year fluoride treatment obviously stimulated ALP activity and OCN level along with increase of bone fluoride concentration of rats, which indicated that fluoride obviously stimulated osteogenic action of rats. In vitro study, the dual effect of fluoride on osteoblast function is shown. On the other hand, there was a significant increase of serum insulin level and a general decrease of glucagon level, and the histomorphometry analysis indicated an elevated insulin-positive area and increase in islet size in rats treated with fluoride for 1 year. In addition, fluoride obviously facilitated the mRNA expression of InsR in vitro. To sum up, there existed a close relationship between insulin secretion and fluoride treatment. The insulin signal pathway might be involved in the underlying occurrence or development of skeletal fluorosis.

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Introduction

Skeletal fluorosis is endemic in some parts of the world due to life-long ingestion of high amounts of fluoride in drinking water. Fluoride (F^-) is a trace element that is incorporated into bone mineral during bone formation. The action of fluoride on bone has been extensively studied, and this ion has been shown to have an effect on bone mineral, bone cells and bone architecture [1]. Previous studies have demonstrated that high bone turnover and

active osteoblast function is the key process to the occurrence and development of skeletal fluorosis [2]. Bone turnover is a complex process, and vitamin D, parathyroid hormone and reproductive hormones are traditionally thought to be involved in such remodeling [3]. The most recent discoveries in bone biology have uncovered the role of insulin as an additional factor involved in the skeletal remodeling process and the recognition of a new bone–pancreas endocrine loop [4]. Fulzele et al. [5] previously attempted to demonstrate a role for insulin which has been complicated by the presence of insulin-like growth factor 1 receptor in osteoblasts. Sequentially, they found that mice lacking insulin receptor (InsR) in osteoblasts dramatically impaired postnatal trabecular bone acquisition and showed a decrease in bone volume and the number of osteoblasts [6]. So far, it was demonstrated that InsR is expressed by osteoblasts, and exposure of primary osteoblasts or osteoblast-like cell lines to physiological levels of insulin increases bone anabolic markers including collagen synthesis, alkaline phosphatase (ALP) production and glucose uptake, and localized insulin delivery accelerates fracture healing by enhancing osteogenesis in a mouse model [7]. These results provide crucial evidence linking insulin action in osteoblast control of postnatal bone development and bone remodeling. This research assessed the effects of increasing F^- doses on the marker of bone formation, serum insulin level and pancreatic secretion changes in vivo, and mRNA expression of InsR and osteocalcin (OCN) in vitro. The present study indicated the elevated insulin level and InsR expression along with improvement in bone formation and osteoblast function and revealed the underlying mechanism of skeletal fluorosis.

Materials and Methods

Animals

Fifty Wistar rats, 25 males and 25 females, age 2 months, were purchased and acclimated for 1 week. During the experimental period, the animals were kept in a room under standard temperature condition on a 12-h light–dark cycle, with a stand diet and water available ad libitum. Routine hygiene procedures included cleaning of plastic cages and daily changes of tap water throughout the experiment period. Fifty rats were divided into two groups: control and fluoride treatment. Thirty rats were treated with drinking tap water containing 100 mg F^-/L (per 221 mg sodium fluoride into 1 L tap water). Twenty rats were raised as

control group. After 1 year of fluoride dosing, all rats were euthanized. Eleven fluoride-treated rats and ten control rats were used for further analyses.

Measurement of Fluoride Concentration of Bone

One femur was collected for measurement of fluoride level. The connective tissue was completely removed, and the bone marrow was rapidly flushed for removal. Bone samples were dry ashed in a muffle furnace, at between 450 and 500 °C for up to 2 h, and then the ash was dissolved in hydrochloric acid, pH was adjusted to 4–5, and a constant volume of 10 ml plus 1 ml TISAB. The fluoride ion-selective electrode (Model 96-09, Orion Research, USA) was used to measure the fluoride concentrations of samples.

Measurement of Serum ALP and OCN Level

Serum collected from rats was separated, and the activity of ALP was detected immediately by automatic biochemistry analysis (Sanyo Inc., Japan). Serum OCN level was analysed by enzyme-linked immunosorbent assay (ELISA).

Measurement of Insulin and Glucagon Level

The level of insulin was tested with Insulin Radioimmunoassay Kit (provided by Institute of Diabetes Research, West China Medical Center of Sichuan University). The level of glucagon was tested with glucagon Radioimmunoassay Kit (provided by Beijing North Institute of Biological Technology). Both procedures were operated according to their instructions.

Pancreas Pathological Morphometry Analysis

The paraffin sections of Bouin-fixed rat pancreas were sliced into 5 μ m and dewaxed. A pre-oxidation, e.g. with an acid solution of potassium permanganate reacted for 5 min. The pancreas was stained by aldehyde fuchsin (that is a more sensitive stain for β cells), and transparent by dehydration, then cemented by neutral resin. The pancreatic β cell particles in the cytoplasm were purple to deep purple. Multi-parameter analysers via the histochemical methods with an image quantitative analysis were operated. Test conditions were eyepiece 10 \times and objective \times 10. For each slice, the islet relatively concentrated area was selected and determined under the five visions. The ratio of insulin-positive reaction product to the pancreatic area (Ins $^{+/-}$ of Panc. %) in each vision was tested. Five large cross sections of the islet, under the objective lens \times 20, with the cursor were delineated along the islet

border. For each islet insulin reaction-positive product area (the Ins⁺ area), the islet area (Isl. area) was measured, and the ratio of insulin-positive products to islet area (the Ins⁺ /Isl %) was calculated. Each islet of the shape factor, i.e. circular degrees (shape factor), was analysed.

Cell Culture and Treatment

The MC3T3-E1 (subclone4) cell line, derived from newborn mouse calvaria, was maintained in α -MEM modified Eagle's medium (α -MEM) (Hyclone), supplemented with 10 % fetal bovine serum and penicillin/streptomycin mixture (100 U/mL penicillin G, 100 g/mL streptomycin sulfate) and cultured in a humidified atmosphere of 5 % CO₂ and 95 % air. The experiment included three fluoride-treated groups ($n=6$) and one control α -MEM group ($n=6$). Cells were seeded (2×10^6 cells per well) into six-well plates and treated with α -MEM (2 % calf serum) containing 2.0, 8.0 and 20.0 mg/L of fluoride for 2, 4 and 10 days, respectively. Cells in the control group were cultured with α -MEM (2 % calf serum, fluoride-free) for the same period. At the end of each treatment period, cells were washed twice with ice-cold 0.9 % NaCl and lysed into debris after being treated with TRIzol reagent (Invitrogen, Groningen, Netherlands).

Reverse Transcription and Real-Time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen Inc., USA) and quantified by a scanning spectrophotometer. First-strand cDNA was synthesized from 1 μ g of total RNA with the use of Oligo (dT)₁₈ primer and reverse transcriptase (Takara, Japan). Quantitative PCR (qPCR) was performed in a total reaction volume of 20 μ l with an SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) per well. An ABI 7500 thermocycler (Applied Biosystems, Foster City, CA) was used for PCR. The reaction conditions were as follows: 95 °C preheating for 1 min, followed by 40 cycles of 95 °C for 15 s (denaturation), 60 °C for 20 s (annealing) and 72 °C for 32 s (elongation). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference. The qPCR primer pairs are as follows: InsR (NM_010568.2, 121 bp): forward, ATGG GCTTC GGGAGAGGAT, reverse, GGATGTCCATAC CAGGGCAC; OCN (NM_031368.4, 121 bp): forward, CCAAGCAGGAGGGCAATA, reverse, TCGTCA CAAGCAGGGTCA; GAPDH (XM_003085777.1, 122 bp): forward, GGCTGCCAGAACATCAT, reverse, CGGACACATTGGGGGTAG.

Statistical Analysis

Data were expressed as mean \pm SD. Differences between two groups were analysed by the LSD and Duncan's test. Independent samples *t* test or one-way analysis of variance was used in comparison between groups. The Pearson's correlation coefficient was used to measure the strength of the association between bone fluoride concentration and insulin level, ALP activity or OCN level. All statistical analyses were performed using SPSS for Windows ver. 18.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Fluoride Concentration of Bone

The femurs were collected from rats with 1-year fluoride treatment. The fluoride ion selective electrode tested fluoride concentration of the femur. The concentrations of fluoride ions in 30 samples of fluoride-treated group and 20 samples of the control group were analysed. The concentration of fluoride in treatment groups was significantly higher than that in the control, which showed a large number of fluorine accumulation in the bone (Fig. 1).

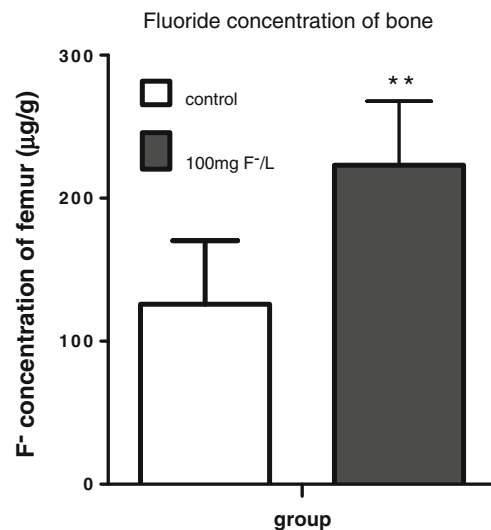


Fig. 1 The fluoride level of femur in rats treated with overdose of fluoride. Femurs were collected from rats drinking tap water containing 100 mg fluoride per liter (100 mg F⁻/L) for 1 year. The fluoride ion-selective electrode detected fluoride concentration of femur. Average fluoride content is represented as mean \pm SD (fluoride treatment group, $n=11$; control group, $n=0$). ** $P < 0.01$, significantly different from the control

ALP and OCN Level of Serum

A number of bone-related factors, such as OCN and ALP, both of which are commonly used as markers of osteogenic differentiation, were detected in the researches about the metabolic bone disease. In the current study, the serum ALP activity markedly increased in the experimental group in comparison with the control (Fig. 2a). A significant correlation was observed between bone fluoride concentration and ALP activity ($r=0.428$, $P<0.05$; Fig. 2b). The OCN level of serum in the experimental group was generally higher than that in the control (Fig. 3a); however, Pearson's correlation coefficient analysis showed an obvious correlation between bone fluoride concentration and osteocalcin level ($r=0.426$, $P<0.05$; Fig. 3b). These data indicated that 1-year fluoride treatment obviously stimulated the active osteogenic status of rats.

Fig. 2 The level of ALP in rats treated with overdose of fluoride. Blood was collected from rats drinking tap water containing 100 mg fluoride/L for 1 year. **a** Serum alkaline phosphatase (ALP) activity was detected by automatic biochemistry analysis. Average level of ALP is represented as mean \pm SD (fluoride treatment group, $n=11$; control group, $n=10$). $*P<0.05$, significantly different from the control. **b** Pearson's correlation coefficient (r) was used to investigate the relationship between bone fluoride concentration and ALP activity. A marked correlation ($r=0.428$, $P<0.05$) was found between ALP activity and bone fluoride concentration

Insulin and Glucagon Level of Serum

The body blood glucose was maintained in a very narrow range. Insulin and glucagon are the hormones that make this happen. Insulin and glucagon are hormones secreted by islet cells within the pancreas. They are both secreted in response to blood sugar levels. In this study, insulin level in rats treated by fluoride significantly increased more than ten times than that in the control. On the contrary, glucagon level of serum generally decreased in the fluoride-treated group, but the difference did not show statistical significance (Fig. 4a). These changes might be caused by the direct action of fluoride on the insulin secretion instead of the regulation of blood glucose. On the other hand, it showed a significant positive correlation between insulin level and bone fluoride concentration ($r=0.663$, $P<0.01$) through Pearson's correlation coefficient analysis (Fig. 4b).

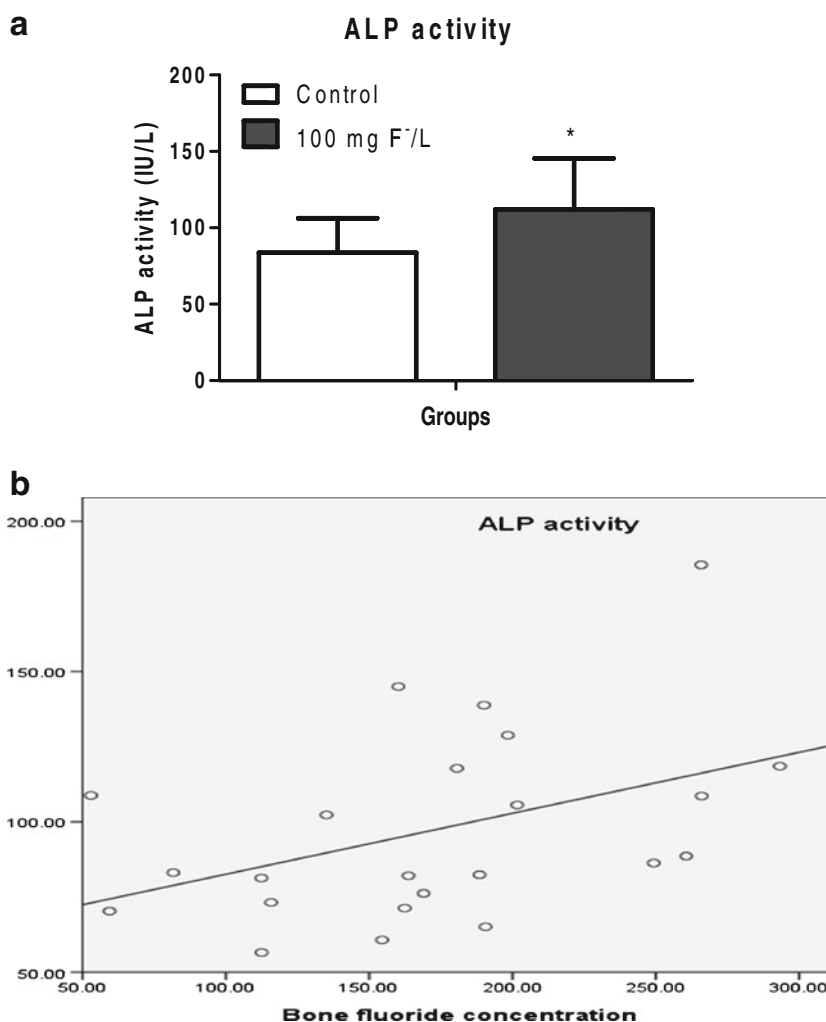
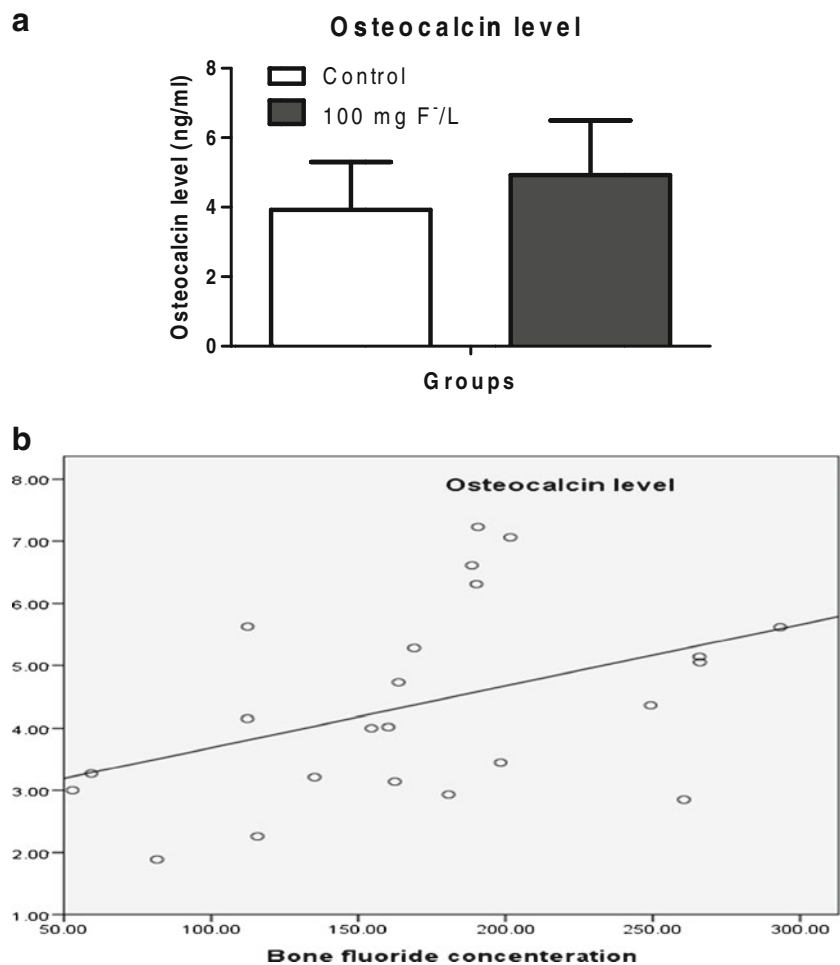


Fig. 3 The level of osteocalcin in rats treated with overdose of fluoride. Blood was collected from rats drinking tap water containing 100 mg fluoride/L for 1 year. **a** Serum osteocalcin content was analysed by ELISA. Average level of osteocalcin is represented as mean \pm SD (fluoride treatment group, $n=11$; control group, $n=10$). **b** Pearson's correlation coefficient (r) was used to investigate the relationship between bone fluoride concentration and osteocalcin level. A significant correlation ($r=0.426$, $P<0.05$) was found between osteocalcin level and bone fluoride concentration in rats exposed to fluoride



Pancreas Pathological Morphometry Analysis

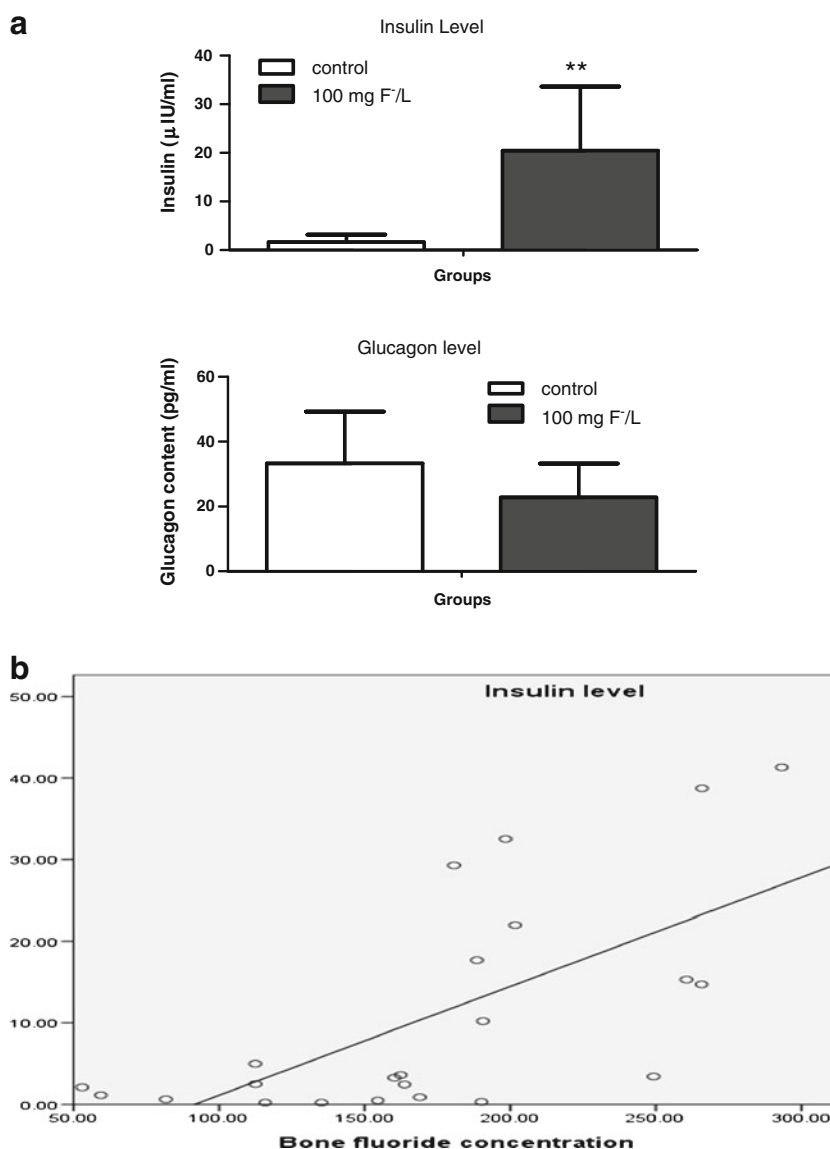
Pathological changes of pancreas were analysed via the histochemical methods with a quantitative image analysis software. It showed that the insulin-positive area in the rats treated by fluoride increased in comparison to that in the control; accordingly, the ratio of insulin-positive area to pancreas or islet area declined in rats treated by fluoride (Fig. 5). The islet size and shape analysis indicated that the islet size in the rats treated by fluoride obviously increased, but its shape factor obviously decreased in comparison to that in the control. These results showed that fluoride treatment could improve the insulin secretion and islet function (Fig. 6).

The mRNA Expression of InsR and OCN

MC3T3-E1 cells were exposed to varied fluoride concentration and periods. At the end of the experiment, real-time

PCR was used to analyse quantitatively the expression of InsR and OCN. It indicated that the expression of InsR in cells exposed to 20 mg F⁻/L for 2 days significantly increased in comparison to the respective control. After the cells were exposed to fluoride for 4 days, the InsR expression was markedly higher in cells of 2 and 20 mg F⁻/L groups. After cells were exposed to fluoride for 10 days, the InsR expression increased significantly in three fluoride-exposed groups. It showed that fluoride exposure obviously stimulated the InsR gene expression. The mRNA expression of OCN significantly increased in cells exposed to 2 and 8 mg F⁻/L for 4 days. On the contrary, the intracellular OCN expression all decreased in the 20 mg F⁻/L group in the three experimental periods, and its decrease was significant in the 20 mg F⁻/L group of 2- and 10-day periods. These data indicated the dual effect of fluoride on osteoblast function, i.e. stimulatory at low concentration, inhibitory at high concentration in vitro (Fig. 7).

Fig. 4 The level of insulin and glucagon in rats treated with overdose of fluoride. Blood was collected from rats drinking tap water containing 100 mg fluoride/L for 1 year. **a** Serum insulin and glucagon levels were detected by radioimmunoassay. Average concentration of insulin and glucagon is represented as mean \pm SD (fluoride treatment group, $n=11$; control group, $n=10$). $**P<0.01$, significantly different from the control. **b** Pearson's correlation coefficient (r) was used to investigate the relationship between bone fluoride concentration and insulin level. A highly significant correlation ($r=0.663$, $P<0.01$) was found between bone fluoride concentration and insulin level



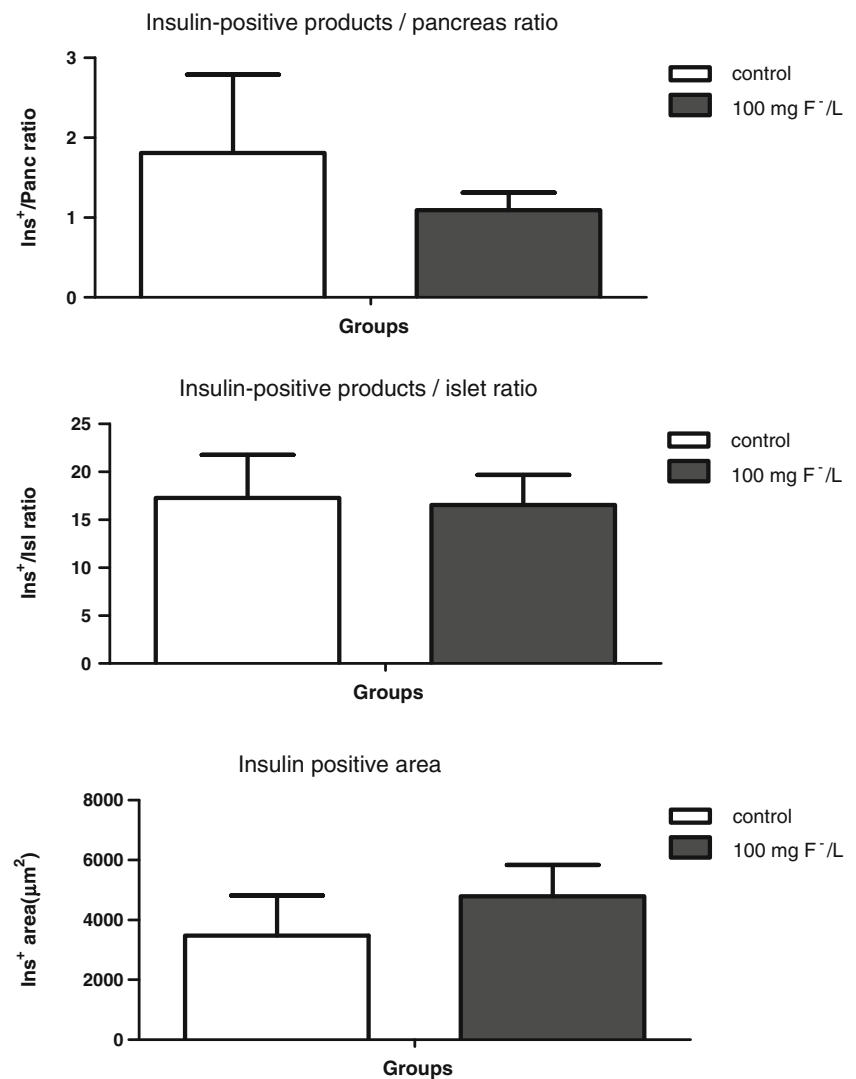
Discussion

In a previous study [8, 9], we showed that increasing doses of fluoride had different effects on intracellular endoplasmic reticulum stress, and low calcium nutrition aggravated the fluoride toxicity. There is mounting interest in the role of bone turnover as a key process in pathogenesis of skeletal fluorosis. Bone turnover means the sequential and coordinated actions of the osteoclasts to remove bone and the osteoblasts to replace it. The balance between bone formation and resorption is tightly controlled by osteoblastic and osteoclastic activities of main bone cells, the osteoblasts and osteoclasts, respectively [10]. In this study, ALP activity increased and OCN level was elevated along with the increase of fluoride ion in the bone, which indicated that 1-year fluoride treatment obviously stimulated the active osteogenic status of rats along with the

increase of fluoride concentration of the femur. As it is known, osteoblasts are indispensable cells for bone growth, development and maintenance, and these cells help to maintain normal homeostatic balance of the bone. In vitro study showed the dual effect of fluoride on osteoblast function, i.e. stimulatory at low concentration and inhibitory at high concentration. Others also observed the dose-dependent effects on mechanical properties of fluorosis [11, 12]. The data demonstrated that fluoride treatment induced active osteoblast function and high osteogenic status.

The osteogenic effect of fluoride had been illuminated in previous literatures [13–15], but the underlying mechanisms are still not fully understood. Actually, the mechanism of skeletal fluorosis involved oxidative stress, endoplasmic reticulum stress, hormones, etc. [9, 13, 16, 17]. Insulin signaling is one of the most well-dissected pathways in both

Fig. 5 Quantitative analysis of insulin-positive product in rats treated with overdose of fluoride. The pancreas was collected from rats drinking tap water containing 100 mg fluoride/L for 1 year. The β cells of the pancreas were stained by aldehyde fuchsin. Multi-parameters of the insulin-positive product were measured by a quantitative image analysis. The ratio of insulin-positive reaction product to the pancreatic area ratio ($\text{Ins}^+/\text{Panc. \%}$), ratio of insulin-positive products to islet area (the $\text{Ins}^+/\text{Isl}\%$), and each islet insulin reaction-positive product area (Ins^+ area) were analysed. Average value of each parameter is represented as mean \pm SD (fluoride treatment group, $n=11$; control group, $n=10$)



mammalian and non-mammalian systems. Gene analysis suggests that insulin signaling facilitates bone formation [18]. Fulzele et al. [6] found the insulin receptors (InsR) present in the osteoblasts and the finding that insulin through interacting with the cell surface receptors facilitates bone formation by suppressing Twist2 (Runx2 inhibitor). However, there are few literatures about the relationship between skeletal fluorosis and insulin; most of them are about the indirect effect of fluoride on glucose level. Studies on rats have suggested that fluoride toxicity may produce glucose intolerance and abnormalities in insulin secretion and pancreatic structure [19, 20]. Trivedi et al. [21] observed that chronic fluoride toxicity in humans could result in significant abnormalities in glucose tolerance, which are reversible upon removal of the excess fluoride. There is a decrease in the insulin concentration and increase in the C-peptide level in blood serum of 72 workers of cryolytes industries detected by radio-immunological method. These changes were caused by fluoride intoxication of workers [22]. Conversely, the present

study showed a significant increase of serum insulin level and a general decrease of glucagon level in rats treated with NaF for 1 year. Moreover, there was a direct correlation between bone fluoride concentration and insulin level. The histomorphometry analysis indicated an elevated insulin-positive area and islet size, which suggested the improvement of pancreatic secretion caused by fluoride exposure. Following varying concentrations of fluoride treatment in MC3T3-E1 cells, mRNA levels of InsR markedly increased, which suggested that fluoride obviously stimulated the expression of InsR in osteoblasts. Linked with the above results, fluoride exposure facilitated the elevated insulin secretion along with active osteogenic status in vivo, and stimulated the expression of InsR and osteoblasts function in vitro. In short, there must be a close relationship between insulin secretion and fluoride treatment. In view of insulin and InsR incorporated in the process of bone remodeling or bone turnover as mentioned previously, the insulin signal pathway may be involved in the occurrence or development of skeletal fluorosis.

Fig. 6 Quantitative analysis of islet size and shape in rats treated with overdose of fluoride. The pancreas was collected from rats drinking tap water containing 100 mg fluoride/L for 1 year. The β cells of the pancreas were stained by aldehyde fuchsin. The islet size and shape were measured by a quantitative image analysis. The Isl. area and shape factor were analysed. Average value of each parameter is represented as mean \pm SD (fluoride treatment group, $n=11$; control group, $n=10$). * $P<0.05$, significantly different from the control

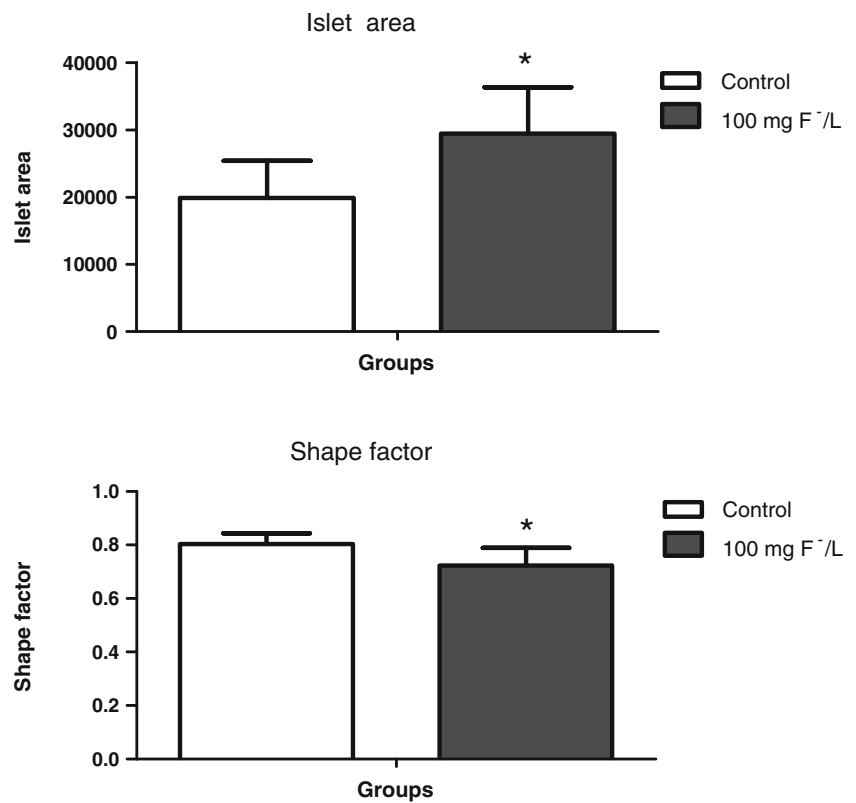
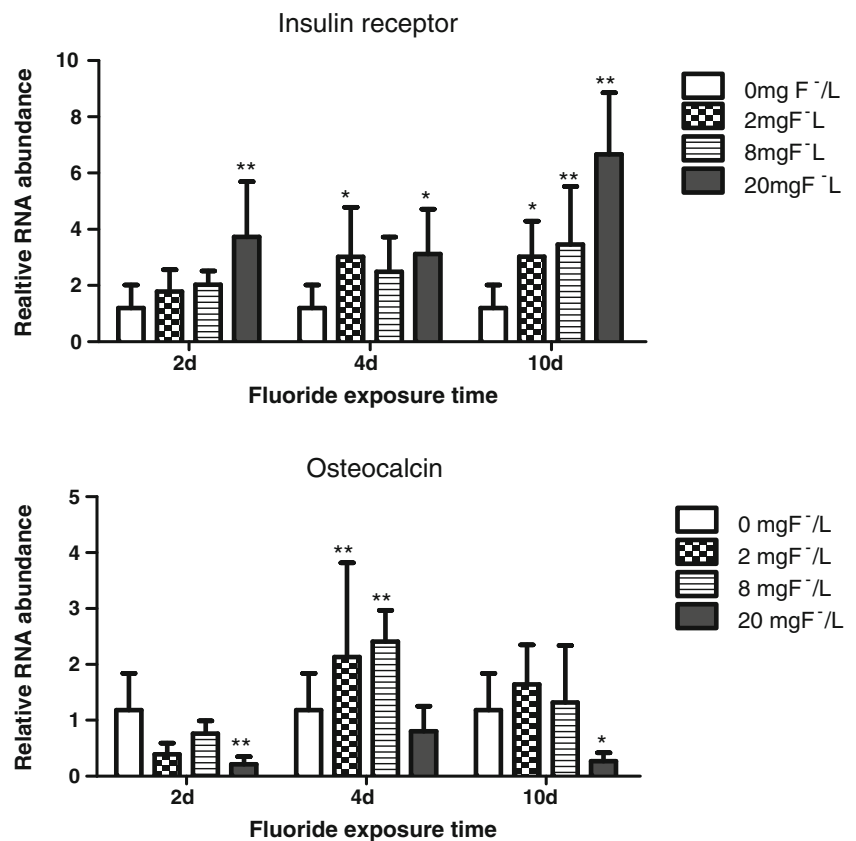


Fig. 7 The relative RNA abundance of insulin receptor and osteocalcin in MC3T3-E1 cells exposed to fluoride. The MC3T3-E1 (subclone4) cells derived from newborn mouse calvaria were exposed to varied concentrations of fluoride. Cells treated with α -MEM (2 % calf serum) containing 2.0, 8.0 and 20.0 mg/L of fluoride for 2, 4 and 10 days, respectively. Cells in the control group were cultured with α -MEM (2 % calf serum, fluoride-free) for the same period. Quantitative PCR was performed in a total reaction volume of 20 μ l with an SYBR Green PCR Master Mix. GAPDH was used as reference. Average relative RNA abundance of insulin receptor (InsR) and osteocalcin (OCN) is represented as mean \pm SD ($n=6$). * $P<0.05$, significantly different from the control; ** $P<0.01$, significantly different from the control



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