

Analysis of the Role of Insulin Signaling in Bone Turnover Induced by Fluoride

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Received: 11 August 2015 / Accepted: 22 October 2015 / Published online: 31 October 2015
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Abstract The role of insulin signaling on the mechanism underlying fluoride induced osteopathology was studied. We analyzed the expression of genes related with bone turnover and insulin signaling in rats treated by varying dose of fluoride with or without streptozotocin (STZ) *in vivo*. Furthermore, insulin receptor (InR) expression in MC3T3-E1 cells (pre-osteoblast cell line) was interfered with small interfering RNA (siRNA), and genes related with osteoblastic and osteoclastic differentiation were investigated in cells exposed to fluoride *in vitro* for 2 days. The *in vivo* study indicated the possible role of insulin in bone lesion induced by excessive amount of fluoride. Fluoride activated the InR and Insulin-like growth factor 1 (IGF1) signaling, which were involved in the mechanism underlying fluoride induced bone turnover. The TGF β 1 and Wnt10/ β -catenin pathway took part in the mechanism of bone lesion induced by fluoride, and insulin probably modulated the TGF β 1 and β -catenin to exert action on bone turnover during the development of bone lesion. The *in vitro* study showed the concomitant decrease of OPG, osterix and OCN with inhibition of InR expression in osteoblast, and three genes still was low in cells co-treated with fluoride and InR siRNA, which suggested that fluoride

probably stimulated the expression of OPG, osterix and OCN through InR signaling. In conclusion, insulin played the important role in bone lesion induced by excessive amount of fluoride through mediating InR receptor signaling, and IGF1 signaling probably exerted action on bone turnover caused by overdose of fluoride.

Keywords Fluoride · Insulin · Insulin receptor · Bone turnover · Insulin-like growth factor 1

Introduction

Fluorides are naturally present in the environment. Excessive systemic uptake of fluoride frequently caused disturbances of bone homeostasis named as skeletal fluorosis. It was demonstrated that the severity of fluorosis is depended on fluoride dose and the duration of fluoride exposure. Skeletal fluorosis results in a change in the shape of the skeleton and appears as osteosclerosis, osteoporosis, osteopenia and ligament calcifications [1, 2]. The complicated features of skeletal fluorosis lead to difficulty to understand the mechanism underlying the effect of fluoride on bone. Recently, the feedback loop between pancreas and osteoblast has been discovered and suggests that bone is a key insulin-utilizing tissue [3]. Researchers recognized that insulin and its downstream signaling pathway are necessary for postnatal bone growth and turnover through influencing on both osteoblast and osteoclast development and insulin signaling regulates both bone formation and bone resorption [4]. Experiments involving animals exposed to excessive fluoride have observed changes of insulin secretion in body of fluorosis, and suggest the role of insulin on the process of skeletal fluorosis [5, 6]. The previous works indicated fluoride influenced insulin release, activity and sensitivity, and the insulin state *in vivo* interfered in the osteogenesis in turn,

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which implied the possible relation between insulin and skeletal fluorosis [7]. Bone remodeling or turnover involves the coupling of resorption of existing bone and the formation of new bone and assessment of bone turnover remains essential in understanding pathophysiology, and skeletal response to treatment. Therefore, this study mainly analyzed the gene level of insulin signaling and bone turnover related factors *in vivo* and *in vitro* to investigate the possible role of insulin signaling on bone lesion induced by excessive amount of fluoride.

Materials and Methods

Animals and Treatment

The male Wistar rats, weighing 150–180 g, were from the Experimental Animal Center of Bethune Medical College, Jilin University. All rats were kept in individual cage with standard food and water *ad libitum* in proper environmental conditions. Standard pellet diet (except for the fasting period) and tap water were supplied *ad libitum*. All experimental protocols related to using and treating animals were approved by the Ethics Committee on the Use and Care of Animals of Jilin University (Changchun, China). Rats were divided into three groups: the control, low fluoride and high fluoride groups. According to the fluoride doses used in the literature about experimental fluorosis rats, one third of rats were administered sodium fluoride (NaF) (Sigma-Aldrich, USA) by gavage at doses 10 mg fluoride/kg.day as low fluoride group, and one third of rats were administered 20 mg fluoride/kg.day as high fluoride group and the remaining were raised as control group. After one month's period, half of rats in every group were treated with Streptozotocin (STZ) for once. The rats were fasted overnight then intraperitoneally (*i.p.*) administered 52 mg/kg STZ that was dissolved in citrate buffer (pH 4.5) and kept at 4 °C. Sixty rats were divided into six groups ($n = 10$ per group): (1) normal control, (2) 10 mg fluoride/kg.day group, (3) 20 mg fluoride/kg.day group, (4) STZ control, (5) STZ + 10 mg fluoride/kg.day group, (6) STZ + 20 mg fluoride/kg.day group. The control rats were administered with water by gavage and normal saline by *i.p.* injection.

Isolation of RNA from Bone

One femur of rats was collected for RNA extraction and completely removed all connective tissue, then rapidly flushed to remove bone marrow. Femur was resected a subjacent 3-mm-wide band of the metaphyseal trabecular primary spongiosa with a bone cutter (dip in 1 mol/L NaOH and DEPC-treated water each for 5 s before use). Bone was placed in an RNase-free mortar and pestle, poured liquid nitrogen in and grinded into a powder while being immersed in liquid nitrogen. Bone powder was transferred into an eppendorf tube

with 2 ml TRIzol reagent (Invitrogen Inc., USA) and stored at -80 °C for use.

Cell Culture and Treatment

The MC3T3-E1 cell line (Cell Bank of the Chinese Academy of Sciences) has been isolated from C57BL/6 mouse calvaria and used widely as a cell model for osteoblast differentiation. In this experiments, MC3T3-E1 cells were cultured in α -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) at 37 °C with 5 % CO₂. The NaF was administered to investigate action of fluoride on osteoblastic cells. Experiment included three fluoride-treated groups.

Small Interfering RNA for InR Transfection

The small interfering RNA (siRNA) targeted against mouse InR mRNA were performed with ON-TARGETplus SMARTpool reagents (Thermo Scientific Dharmacon RNAi Technologies). MC3T3-E1 cells were plated in 6-well plate at a density of 1×10^5 cells per well. After 24 h incubation in medium without antibiotics, cells were transfected with 50 nM siRNA effectively decreased gene expression of InR, which was demonstrated by means of real-time PCR. Subsequently, this experiment was designed to include the control (cells were not treated with siRNA reagents), the negative control (cells were treated with non-specific scrambled siRNA reagents), the positive control (cells were treated with specific 50 nM InR siRNA reagents) and three fluoride-exposed groups (cells were treated with specific 50 nM InR siRNA reagents +1, 2 and 8 mg/L of fluoride, respectively). Cells were treated with α -MEM containing different reagents for 2 days. After 2 days of fluoride exposure and siRNA InR transfection, cells were lysed with TRIzol kit to detect gene level of InR. The real-time reverse transcription (RT) PCR was used to detect gene expression of InR downstream and factors related with bone turnover.

Reverse Transcription and Real-Time PCR

The total RNA was extracted from the femoral trabecular bone and MC3T3-E1 cells using TRIzol reagent according to the manufacturer's instruction. Total RNA was quantified by scanning spectrophotometer. The RNA was digested with DNase to eliminate any contaminating genomic DNA. The amount of RNA was quantified by spectrophotometry. The first-strand cDNA was synthesized from 1 μ g of total RNA with the use of Oligo(dT)₁₈ primer and reverse transcriptase (Applied Biosystems, USA). Then SYBR green detection method was used to examine the expression of genes in bone tissue and MC3T3-E1 cells with Power SYBR Green PCR

Reagents Kit (Applied Biosystems, USA). Glyceraldehyde-3-phosphatedehydrogenase (GAPDH) served as a control and the expression of a given gene was expressed as proportion relative to the average value of GAPDH. All experiments performed in triplicate. The relative ration of expression was calculated and used for statistics. The primers used in vivo and in vitro experiments were synthesized by Sangon Biotech (Shang Hai, China) and shown in Tables 1 and 2.

Statistics Analysis

Data are expressed as mean \pm SD. Differences between two groups were analyzed by the LSD and Duncan's test. One-way analysis of variance was used in multiple group

comparisons. All statistical analyses were performed using SPSS for Windows ver. 18.0 (SPSS Inc., Chicago, IL, USA). The $p < 0.05$ was considered statistically significant.

Results

Osteogenicity in Femur of Rats Exposed to Fluoride with or Without Streptozotocin

The osteogenic markers and transcription factors was investigated in this study. Gene expression of alkaline phosphatase (ALP) and osteocalcin (OCN) determines the response of osteogenic cells to fluoride and STZ. It showed that treatment

Table 1 The quantitative PCR primer sequences of rats

Gene name	Accession number	Sequences
ALP	NM_013059.1	Forward: 5'-AACGTGGCCAAGAACATCATCA -3' Reverse: 5'-TGTCCATCTCCAGCCGTGTC -3'
Runx2	NM_053470	Forward: 5'-CGCATTCTCATCCCAGTAT -3' Reverse: 5'- GCCTGGGGTCTGTAATCTGA-3'
OCN	NM_013414.1	Forward: 5'-GGTGCAGACCTAGCAGACACCA -3' Reverse: 5'-AGGTAGCGCCGGAGTCTATTCA -3'
TRACP	M76110.1	Forward: 5'-CAGCCCTTATTACCGTTTGC -3' Reverse: 5'-GAATTGCCACACAGCATCAC -3'
Cathpsin K	NM_031560.2	Forward: 5'- TGTCTGAGAACTATGGCTGTGG -3' Reverse: 5'-ATACGGGTAAGCGTCTTCAGAG -3'
MMP9	NM_031055.1	Forward: 5'-CGTGGCCTACGTGACCTATGA -3' Reverse: 5'-TGCACCGCTGAAGCAAAAG -3'
M-CSF	AF515736.1	Forward: 5'-CTTGGCTTGGGATGATTCTCA -3' Reverse: 5'-TCTCTGCCAAAAGCTGCATA -3'
OPG	U94330.1	Forward: 5'-GCCAACACTGATGGAGCAGAT -3' Reverse: 5'-TCTTCATTCCCACCAACTGATG -3'
RANKL	NM_057149.1	Forward: 5'-TCAGGAGTTCAGCTATGAT -3' Reverse: 5'-CCATCAGCTGAAGATAGTCC -3'
InR	NM_017071.2	Forward: 5'-TTCATTGAGGAAGACCTTCGA -3' Reverse: 5'-AGGCCAGAGATGACAAGTGAC -3'
IRS	NM_012969.1	Forward: 5'-GCCTGGAGTATTATGAGAACGAGAA -3' Reverse: 5'-GGGGATCGAGCGTTTGG -3'
IGF1	NM_178866	Forward: 5'-GCACTCTGCTTGCTCACCTTTA-3' Reverse: 5'-CTTGGTCCACACACGAACTGA -3'
IGFBP4	NM_001004274	Forward: 5'-GCATCCCAACAACAGCTTCAA-3' Reverse: 5'-CCCCACAACCTTATCTTGCT -3'
IGFBP6	NM_013104	Forward: 5'-AGGCCAGTCTCTGTTCAAGAT-3' Reverse: 5'-TGCTTGCGGTAGAAACCTCTG -3'
β -catenin	NM_053357.2	Forward: 5'-GCCAAGTGGGTGGCATAGA-3' Reverse: 5'-TCCCTGTCACCAGCACGAA -3'
Wnt10b	NM_001108111.1	Forward: 5'-CCTCAAGCGCGTTTCC -3' Reverse: 5'-CAGCAGCCAGCATGGAGAA -3'
GAPDH	NM_017008.4	Forward: 5'-AGTTCAACGGCACAGTCAG -3' Reverse: 5'-TACTCAGCACCAGCATCACC -3'

Table 2 The quantitative PCR primer sequences of mice

Gene name	Accession number	Sequences
InR	NM_010568	Forward: 5'- AGATGAGAGGTGCAGTGTGGCT-3' Reverse: 5'- GGTTCCTTTGGCTCTTGCCACA-3'
Esp	NM_007955	Forward: 5'- TAGAGGCTGAGCATCCTGCCAA-3' Reverse: 5'- CAGAATGAGGCTCTCCTGATAGC-3'
FoxO1	NM_019739	Forward: 5'- CTACGAGTGGATGGTGAAGAGC-3' Reverse: 5'- CCAGTTCCTTCATCTGCACTCG-3'
IRS1	NM_010570	Forward: 5'- TGTCACCCAGTGGTAGTTGCTC-3' Reverse: 5'- CTCTCAACAGGAGGTTTGGCATG-3'
Runx2	NM_001145920.1	Forward: 5'- CCGGTCTCCTTCCAGGAT -3' Reverse: 5'- GGGAAGTGTGTGGCTTC -3'
OCN	NM_031368.4	Forward: 5'- CCAAGCAGGAGGGCAATA-3' Reverse: 5'- TCGTCACAAGCAGGGTCA -3'
osterix	NM_130458.3	Forward: 5'- AGCGACCACTTGAGCAAACAT-3' Reverse: 5'- AGCGACCACTTGAGCAAACAT -3'
OPG	NM_008764.3	Forward: 5'-CGGAAACAGAGAAGCCACGCAA -3' Reverse: 5'-CTGTCCACCAAAAACACTCAGCC -3'
RANKL	NM_011613.3	Forward: 5'-GTGAAGACACTACTGACTCC -3' Reverse: 5'-GCCACATCCAACCATGAGCCTT -3'
GAPDH	NM_008084.2	Forward: 5'-GGCTGCCAGAACATCAT -3' Reverse: 5'-CGGACACATTGGGGGTAG -3'

with 10 mg/kg.day of fluoride significantly promoted ALP gene expression in femur of rats; however, the 20 mg/kg.day of fluoride inhibited its expression. The OCN expression increased after treatment with fluoride. On the other hand, administration with STZ markedly decreased the ALP and OCN expression, and co-treatment with fluoride and STZ yet reduced their gene expression in femur of rats compared to that in the control. Runx2 acted as a key transcription factor regulating osteoblast differentiation. The expression of Runx2 was significantly unregulated in 10 mg/kg.day of fluoride group, but slightly downregulated in co-treatment with fluoride and STZ groups by comparison with that in the control group (Fig. 1). These results showed the low dose of fluoride enhanced ALP and Runx2 expression, and high dose of fluoride slightly increased the expression of OCN and Runx2. In the contrast, co-administration of fluoride and STZ significantly decreased the expression of ALP, OCN and Runx2.

Osteoclastogenesis in Femur of Rats Exposed to Fluoride with or Without Streptozotocin

The receptor activator of NF- κ B ligand (RANKL), osteoprotegerin (OPG) and macrophage colony-stimulating factor (M-CSF) gene expression were produced by osteoblast and mediated osteoclast differentiation. The Fig. 2 showed that fluoride treatment significantly promoted the RANKL expression in femur of rats and obvious upregulation was observed in 10 mg/kg.day of fluoride group by comparison with the

control. The single STZ administration or co-treatment with fluoride and STZ further increased the RANKL expression. The OPG expression in co-treatment with fluoride and STZ groups markedly increased at gene level compared to that in the control, and it was significantly higher than that in the same dose of single fluoride treatment. Furthermore, the M-CSF indicated the obvious upregulation expression in both fluoride-treated groups, but single STZ or co-treatment with fluoride and STZ significantly inhibited its expression (Fig. 2). The gene expression of three essential transcription factors for osteoclast differentiation increased in varying degrees after fluoride treatment. The STZ mainly inhibited M-CSF expression, whereas expression of RANKL and OPG still was upregulated in co-treatment with fluoride and STZ.

Osteoclast Markers in Femur of Rats Exposed to Fluoride with or Without Streptozotocin

The cathepsin K, matrix metalloproteinase 9 (MMP9) and tartrate-resistant acid phosphatase (TRACP) are frequently called bone resorption markers. It showed that the 10 mg/kg.day of fluoride treatment obviously stimulated the expression of cathepsin K compared to that in the control and the co-treatment with same dose of fluoride and STZ groups. While the single STZ administration or co-treatment with fluoride and STZ slightly decreased cathepsin K expression. Similarly, the expression of TRACP was significantly upregulated in 10 mg/kg.day of fluoride group. Moreover, the

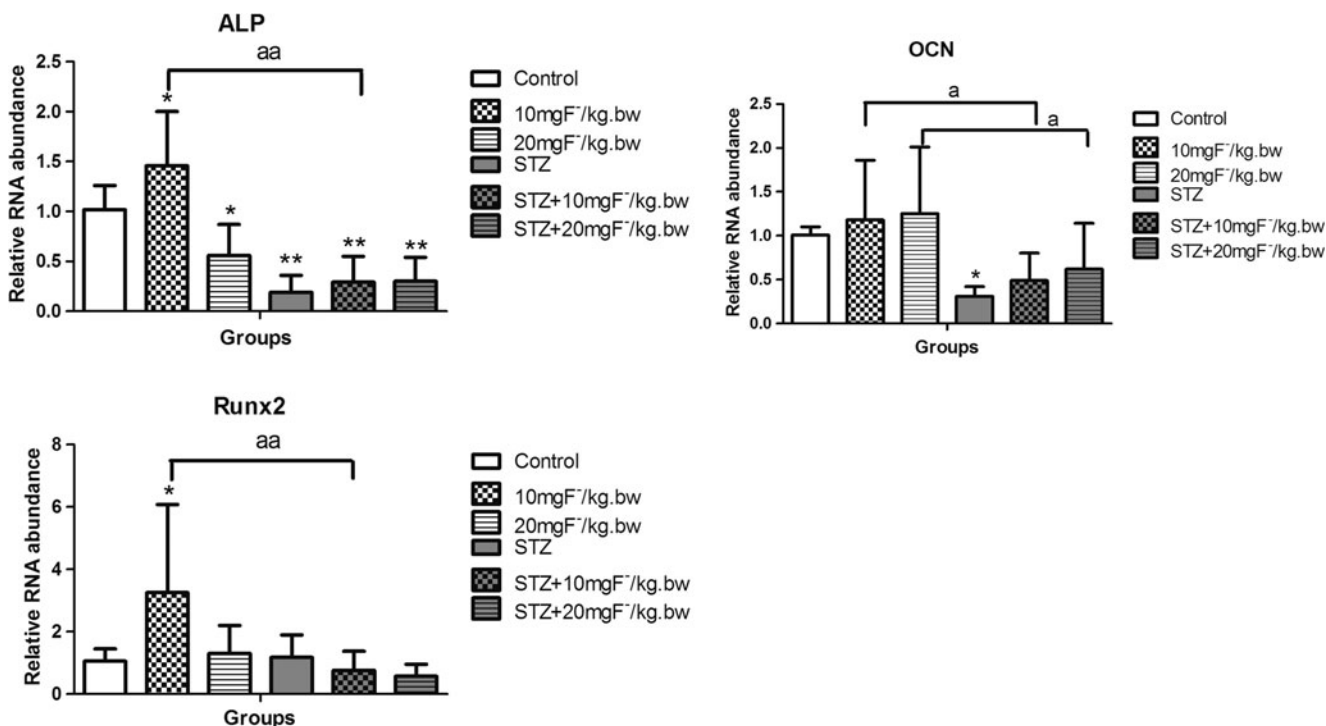


Fig. 1 Relative RNA abundance of osteogenesis associated genes in femur of fluoride-treated rats with or without Streptozotocin. The Wistar rats were treated with fluoride by gavage and Streptozotocin by intraperitoneal injection. Quantitative analysis of alkaline phosphatase (ALP), osteocalcin (OCN) and runt-related transcription factor (Runx2)

expression levels was performed via real-time RT PCR. Results are expressed as mean ± SD ($n = 3$). It indicated the significant changes (*, $P < 0.05$; **, $P < 0.01$) compared to the control group, and the significant changes (a, $P < 0.05$; aa, $P < 0.01$) between two groups

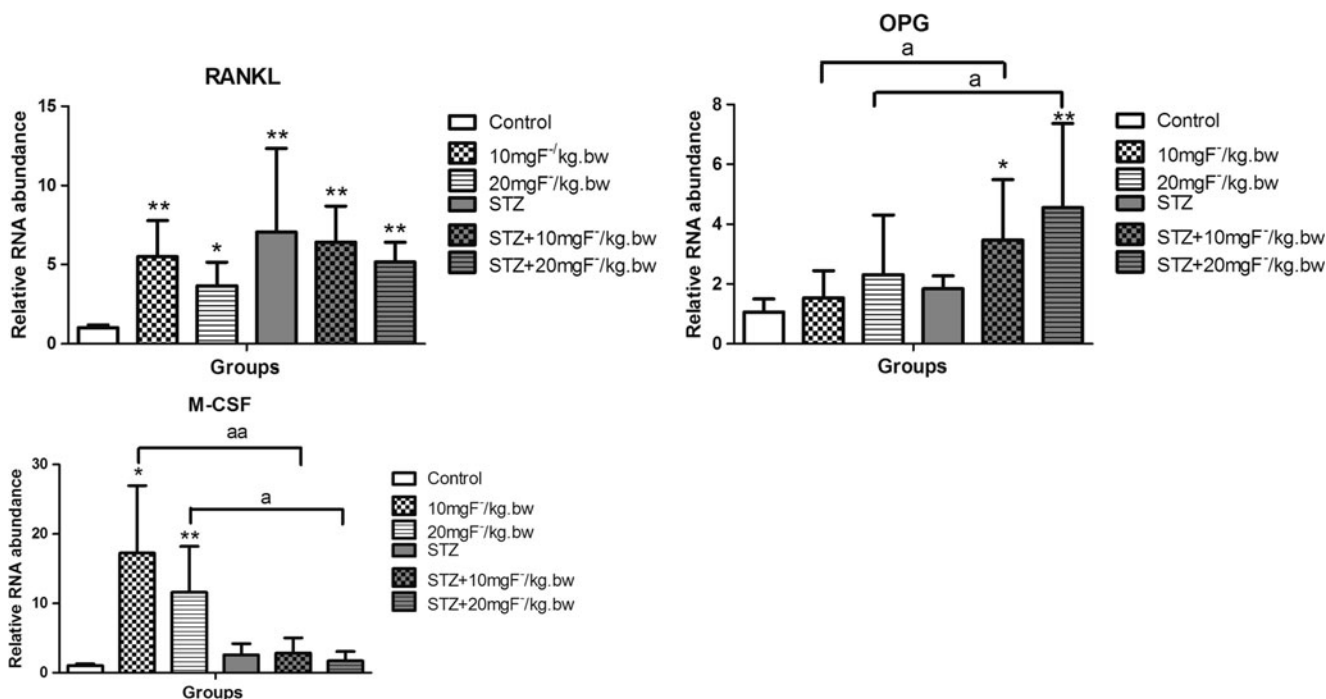


Fig. 2 Relative RNA abundance of osteoclastogenesis associated genes in femur of fluoride-treated rats with or without Streptozotocin. The Wistar rats were treated with fluoride by gavage and Streptozotocin by intraperitoneal injection. Quantitative analysis of receptor activator of NF-κB ligand (RANKL), osteoprotegerin (OPG) and macrophage

colony-stimulating factor (M-CSF) expression levels was performed via real-time RT PCR. Results are expressed as mean ± SD ($n = 3$). It indicated the significant changes (*, $P < 0.05$; **, $P < 0.01$) compared to the control group, and the significant changes (a, $P < 0.05$; aa, $P < 0.01$) between two groups

expression of MMP9 was significantly down regulation in STZ administration groups compared to that in the control. The co-treatment of fluoride and STZ markedly inhibited MMP9 expression by comparison with that expression in only same dose of fluoride treatment (Fig. 3). These results showed that low dose of fluoride stimulated bone resorption but single STZ or linked with fluoride administration inhibited bone resorption.

Insulin Signaling in Femur of Rats Exposed to Fluoride with or Without Streptozotocin

The intracellular insulin signaling molecules including insulin receptor (InR), insulin-like growth factor 1 (IGF-1), insulin-like growth factor binding protein 4 (IGFBP4) and insulin-like growth factor binding protein 6 (IGFBP6) were analyzed. Fluoride treatment stimulated InR expression, and this action was significant in the 10 mg/kg.day of fluoride treatment compared to that in the control. The co-treatment of fluoride and STZ markedly decreased the expression of InR by comparison with only same dose of fluoride treatment. Moreover, fluoride treatment markedly enhanced the expression of IGF1 and this action had not been inhibited in STZ administration groups, while its expression still lower in co-treatment with fluoride and STZ by comparison with that in single same dose of fluoride treatment. The upregulation of IGFBP4 and IGFBP6 expression occurred in both fluoride treatment

groups, and co-treatment with fluoride and STZ slightly inhibited IGFBP4 expression compared to that in the control. However, the STZ administration still significantly stimulated the expression of IGFBP6, though the stimulating action was significantly lower compared to that action induced by single same dose of fluoride treatment (Fig. 4). These results indicated the fluoride obviously stimulated insulin and IGF1 signaling in bone tissue, and STZ administration partly reduced the positive action induced by fluoride treatment instead of inhibition of IGF1 signaling.

The Intracellular Signaling in Femur of Rats Exposed to Fluoride with or Without Streptozotocin

The transforming growth factor-beta 1 (TGFβ1) as well as β-catenin and Wnt10 signaling are essential pathway of bone metabolism. It indicated that treatment with 10 mg/kg.day of fluoride significantly enhanced the expression of TGFβ1 in femur of rats compared to that in the untreated rats; however, the 20 mg/kg.day of fluoride treatment obviously inhibited the TGFβ1 expression. Moreover, the STZ administration further reduced gene level of TGFβ1 in femur, and co-treatment of fluoride and STZ similarly decreased its expression. Moreover, It was found the stimulating action of fluoride on β-catenin and Wnt10 expression, and the 10 mg/kg.day of fluoride treatment significantly induced the expression of β-catenin and Wnt10 compared to that in the control. Actually,

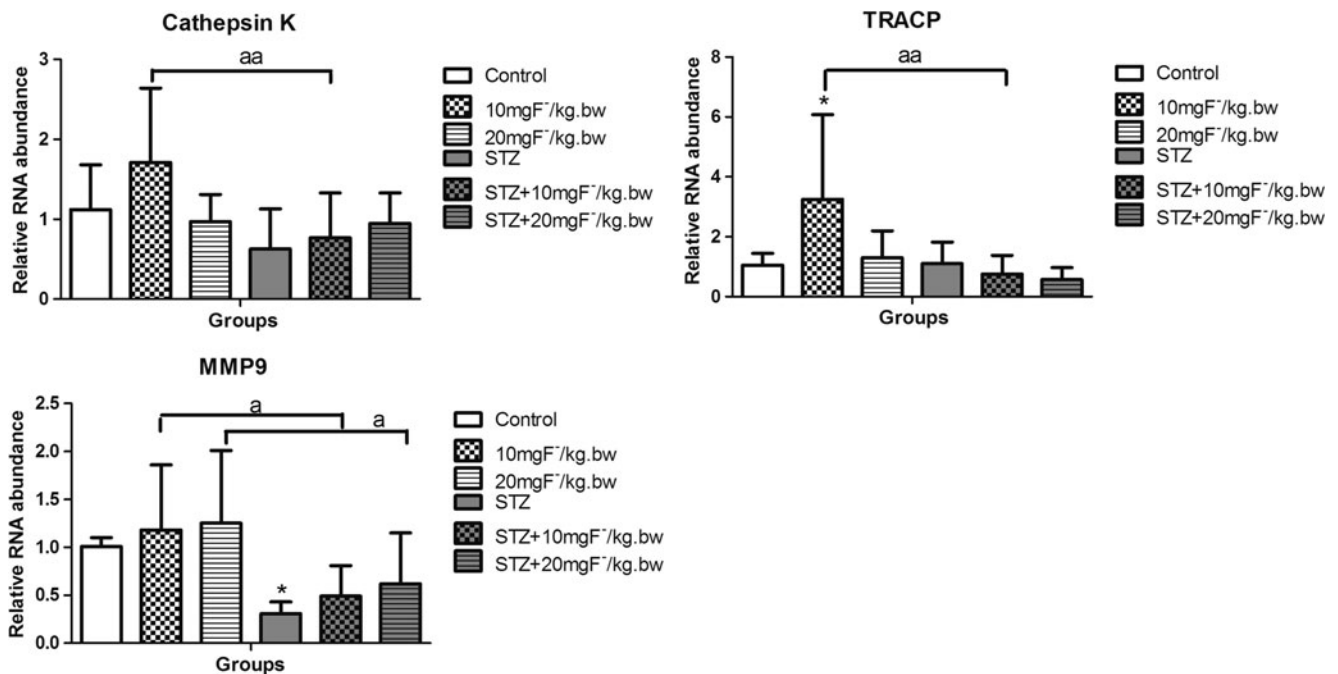


Fig. 3 Relative RNA abundance of osteoclast markers genes in femur of fluoride-treated rats with or without Streptozotocin. The Wistar rats were treated with fluoride by gavage and Streptozotocin by intraperitoneal injection. Quantitative analysis of cathepsin K, matrix metalloproteinase (MMP9) and tartrate-resistant acid phosphatase (TRACP) gene

expression levels was performed via real-time RT PCR. Results are expressed as mean ± SD (n = 3). It indicated the significant changes (*, P < 0.05; **, P < 0.01) compared to the control group, and the significant changes (a, P < 0.05; aa, P < 0.01) between two groups

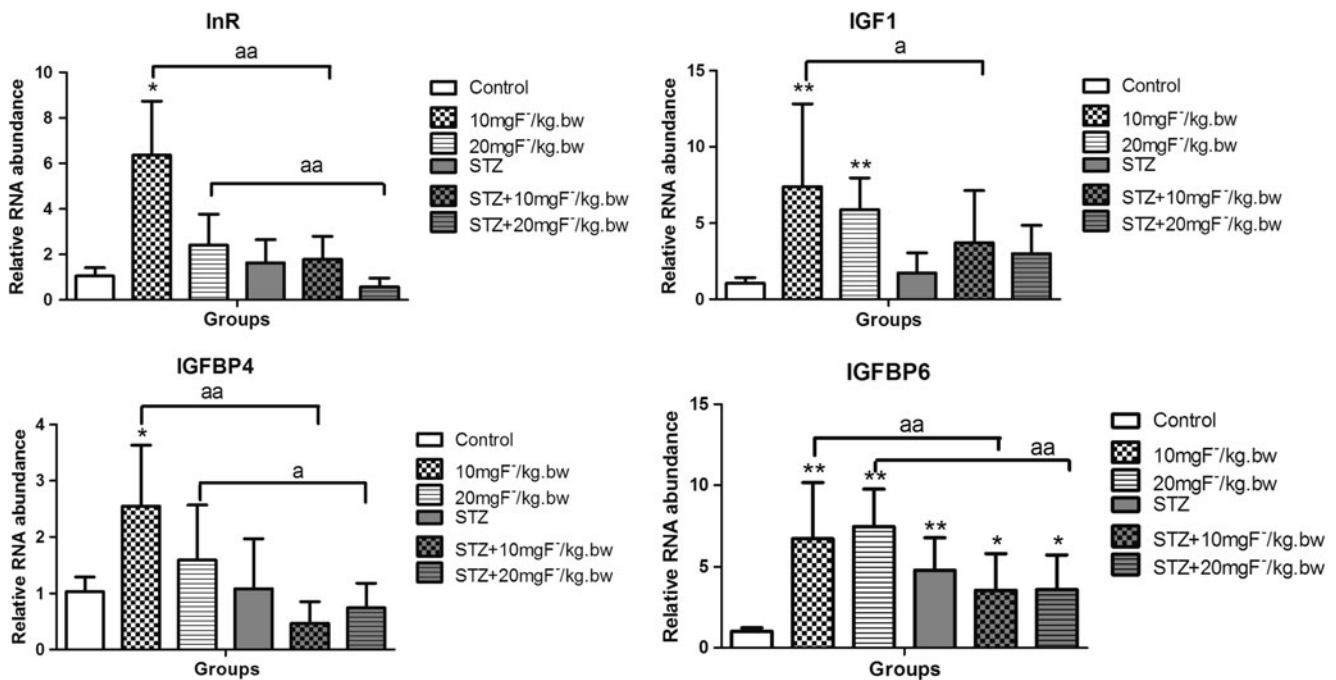


Fig. 4 Relative RNA abundance of osteoclast markers genes in femur of fluoride-treated rats with or without Streptozotocin. The Wistar rats were treated with fluoride by gavage and Streptozotocin by intraperitoneal injection. Quantitative analysis of insulin receptor (InR), insulin-like growth factor 1 (IGF-1), insulin-like growth factor binding protein 4

(IGFBP4) and insulin-like growth factor binding protein 6 (IGFBP6) gene expression levels was performed via real-time RT PCR. Results are expressed as mean \pm SD (N = 3). It indicated the significant changes (*, $P < 0.05$; **, $P < 0.01$) compared to the control group, and the significant changes (a, $P < 0.05$; aa, $P < 0.01$) between two groups

the STZ administration hardly influenced the expression of β -catenin and Wnt10 except that the expression of β -catenin was significantly lower in rats co-treated with fluoride and STZ than that in single same dose of fluoride treatment (Fig. 5). These results suggested that low dose of fluoride obviously induced bone formation and enhanced TGF β 1 production, and STZ administration completely inhibited TGF β 1 expression but partly influenced the β -catenin/Wnt10.

The Intracellular Insulin Signaling in MC3T3-E1 Cells Transfected with Insulin Receptor siRNA

The MC3T3-E1 cells as pre-osteoblast model in vitro were transfected with insulin receptor (InR) siRNA in order to knock down the gene level of InR. After transfected with siRNA for 2 days, it indicated the significant decrease of InR expression in the siRNA transfection group (positive control), which corroborated the effectiveness of InR siRNA in the MC3T3-E1 cells. Based on the effect of InR siRNA, the MC3T3-E1 cells were exposed to varying concentrations of fluoride. It showed the stimulating action of fluoride on InR expression and the 8 mg/L of fluoride significantly induced its expression compared to that in the positive control. Due to inhibition of InR siRNA in cells, the gene level of InR in all of fluoride-exposed groups was lower than that in the control. The insulin receptor substrate 1 (IRS1) as an intracellular downstream of InR and IGF1 pathways also indicated the

decrease at gene level, and co-exposure of cell to 1, 2 mg/L of fluoride obviously increased its expression compared to the positive control. The negative modulators of InR signaling pathway also was investigated in this study, such as Esp and fork head box protein O1 (FoxO1). The expression of Esp showed significantly upregulation in all cells transfected with InR siRNA. Among them, the positive control and co-treated with 1 mg/L of fluoride and InR siRNA significantly increased Esp expression. On the other hand, the expression of FoxO1 was hardly influenced in cells transfected with siRNA InR except that cells co-exposed to 2 mg/L of fluoride and InR siRNA condition (Fig. 6). These results suggested that fluoride still induced the IRS1 expression though siRNA InR significantly inhibited its gene level in MC3T3-E1 cells. Accordingly, the gene level of Esp was enhanced in response to inhibition the intracellular insulin receptor.

The Osteogenesis Factors in MC3T3-E1 Cells Transfected with Insulin Receptor siRNA

This study investigated the transcription factors and marker associated with bone formation in MC3T3-E1 cells transfected with InR siRNA. The expression of Runx2 and osterix indicated completely opposed changes. Cells transfected with InR siRNA promoted the expression of Runx2, but 1, 2 mg/L of fluoride and InR siRNA co-exposure reduced its expression by comparison with the

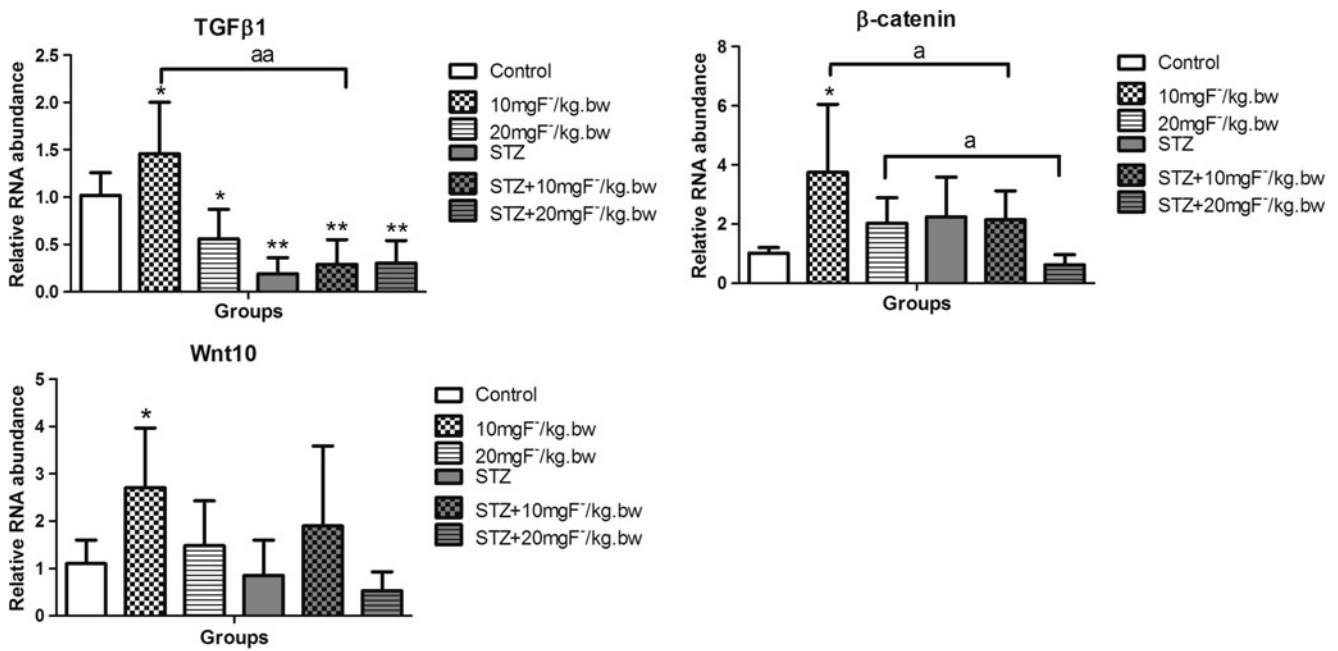


Fig. 5 Relative RNA abundance of cellular factors in femur of fluoride-treated rats with or without Streptozotocin. The Wistar rats were treated with fluoride by gavage and Streptozotocin by intraperitoneal injection. Quantitative analysis of transforming growth factor-beta 1(TGFβ1), β-catenin, and Wnt10 gene expression levels was performed via real-time

RT PCR. Results are expressed as mean ± SD (N = 3). It indicated the significant changes (*, $P < 0.05$; **, $P < 0.01$) compared to the control group, and the significant changes (a, $P < 0.05$; aa, $P < 0.01$) between two groups

positive control. Whereas the expression of osterix was completely inhibited in cells transfected with InR siRNA with or without fluoride, and showed significant decrease

compared to that in the control. The OCN as a late bone mark also showed the significant decrease at gene level in cells of positive control and 2, 8 mg/L of fluoride groups, which

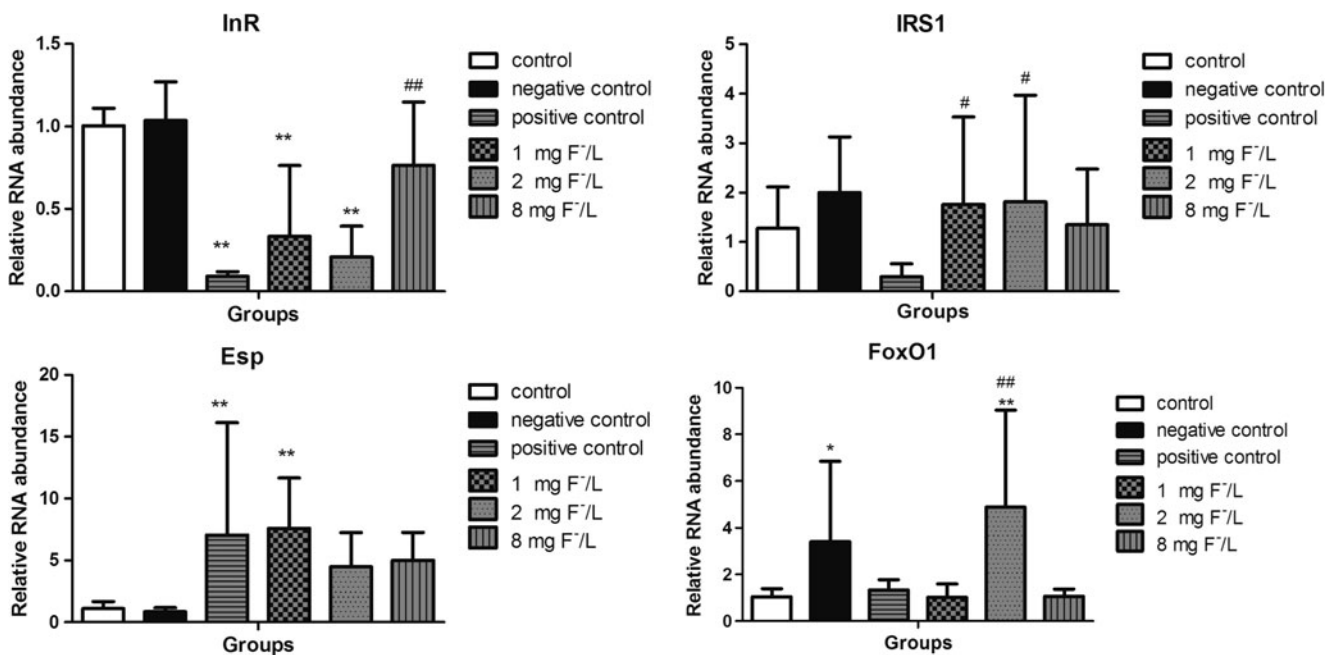


Fig. 6 Effects of fluoride on insulin receptor signaling factors expression in MC3T3-E1 cells transfected with insulin receptor siRNA. The MC3T3-E1 cells were treated with α-MEM containing 1, 2 and 8 mg/L of fluoride and transfected with 50 nM of insulin receptor (InR) siRNA for 2 days. Quantitative analysis of InR, Esp, insulin receptor substrate

1(IRS1) and forkhead box protein O1(FoxO1) gene expression levels was performed via real-time RT PCR. Results are expressed as mean ± SD (n = 3). It indicated the significant changes (* $P < 0.05$, ** $P < 0.01$) compared to the control group, and the significant changes (#, $P < 0.05$; ##, $P < 0.01$) compared to the positive control group

suggested the InR siRNA obviously affected the OCN expression in osteoblast (Fig. 7). These results showed that intracellular InR signaling was closely associated with osterix and OCN instead of Runx2 in osteoblasts exposed to fluoride.

The Osteoclastogenesis Factors in MC3T3-E1 Cells Transfected with Insulin Receptor siRNA

This study indicated significant increase of RANKL expression in positive control compared to that in the control, and markedly reduced its expression in cells co-exposed to 1, 2 mg/L of fluoride and InR siRNA compared to the positive control. The OPG as decoy ligand of RANKL yet indicated obviously decreased of gene level in cells transfected with siRNA InR, and its expression was significantly lower in cells of the positive control and co-exposure to fluoride groups by comparison to that in the control (Fig. 8). These results indicated that siRNA InR obviously influenced the OPG expression in osteoblasts exposed to fluoride.

Discussion

It is well established that ingestion of higher amounts of fluoride causes metabolic disorders, by interacting with various cellular and molecular processes such as gene expression, cell cycle, proliferation and migration [8]. To corroborate the

previous finding that fluoride-induced bone lesion involved high bone turnover [9], we firstly observed the osteogenesis and osteoclastogenesis related genes expression. Results showed that low dose of fluoride treatment significantly enhanced the expression of the bone formation and resorption markers, such as ALP and TRACP, which implied the stimulating action of fluoride on osteoblast and osteoclast differentiation. The key transcription factor of bone formation Runx2 indicated the marked increased in rats treated with low dose of fluoride. Moreover, the key transcription factors for bone resorption RANKL and M-CSF expression markedly enhanced in low and high doses of fluoride, but OPG expression slightly increased. Similarly, the osteoclast marker MMP9 showed mild increase in rats treated by low and high doses of fluoride. The previous work had observed that the low dose of fluoride obviously decreased the bone mineral density, which suggested the imbalance of bone mass metabolism [7]. These results demonstrated there appeared an active bone turnover induced by fluoride, and the osteoblast was more sensitive to toxic action of high dose of fluoride than osteoclast. Meantime, a previous study found that fluoride could increase the level of serum insulin, which was consistent with Lupo et al's research [5, 7]. Fulzele et al. [10] concluded the close relation between bone and insulin, and recognized the important role of insulin in bone metabolism. In this study, the STZ was administered once to inhibit the function of insulin-producing beta cells of the pancreas and investigated the effect

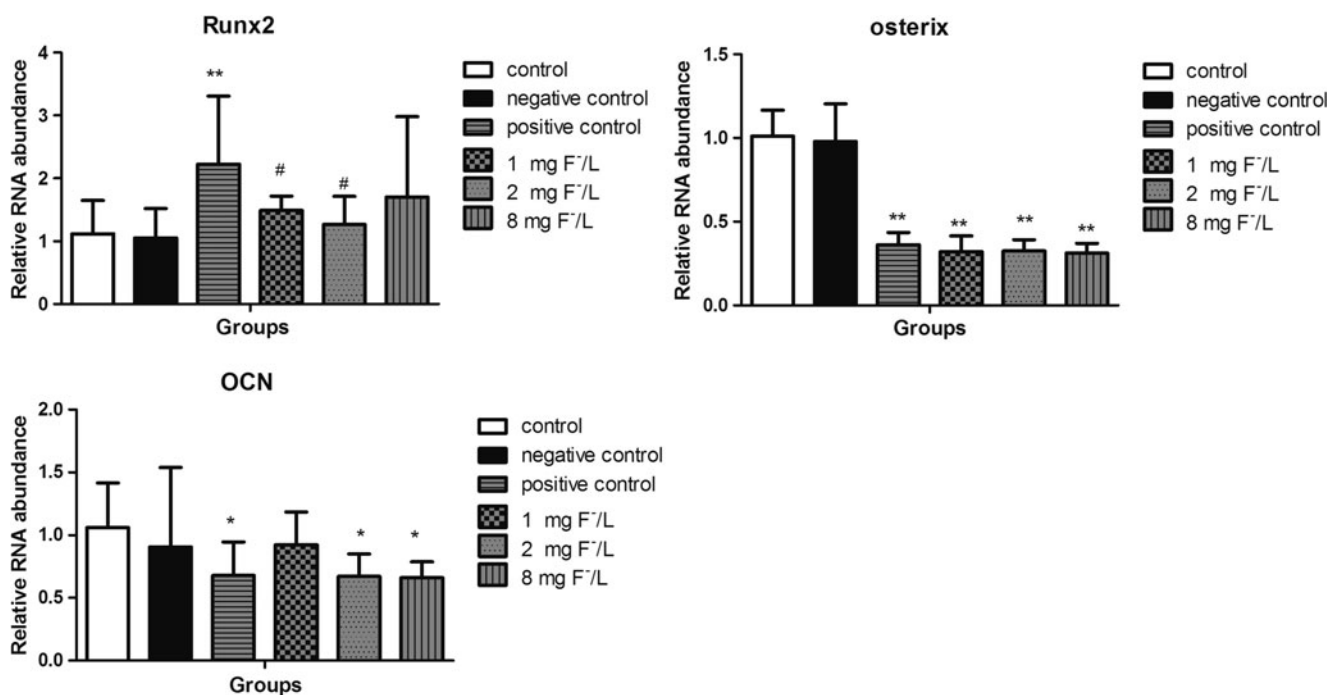


Fig. 7 Effects of fluoride on osteogenesis signaling factors expression in MC3T3-E1 cells transfected with insulin receptor siRNA. The MC3T3-E1 cells were treated with α -MEM containing 1, 2 and 8 mg/L of fluoride and transfected with 50 nM of insulin receptor (InR) siRNA for 2 days. Quantitative analysis of *runx2* (Runx2), osterix

and osteocalcin (OCN) gene expression levels was performed via real-time RT PCR. Results are expressed as mean \pm SD ($n = 3$). It indicated the significant changes (* $P < 0.05$, ** $P < 0.01$) compared to the control group, and the significant changes (#, $P < 0.05$) compared to the positive control group

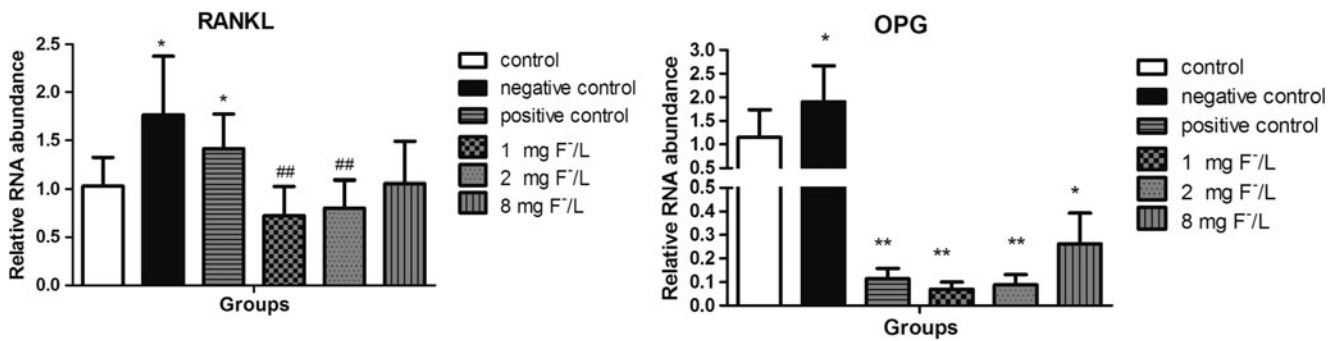


Fig. 8 Effects of fluoride on osteoclastogenesis signaling factors expression in MC3T3-E1 cells transfected with insulin receptor siRNA. The MC3T3-E1 cells were treated with α -MEM containing 1, 2 and 8 mg/L of fluoride and transfected with 50 nM of insulin receptor (InR) siRNA for 2 days. Quantitative analysis of receptor activator of NF- κ B

ligand (RANKL) and osteoprotegerin (OPG) gene expression levels was performed via real-time RT PCR. Results are expressed as mean \pm SD ($n = 3$). It indicated the significant changes (* $P < 0.05$, ** $P < 0.01$) compared to the control group, and the significant changes (##, $P < 0.01$) compared to the positive control group

of an impaired insulin function on bone turnover in the present study. Our results showed a markedly decrease of gene expression in bone formation markers, such as ALP and OCN, and decrease also occurred in bone resorption markers, such as cathepsin K and MMP. These changes of genes expression reflected the possible role of insulin in bone lesion induced by excessive amount of fluoride. However, the bone resorption related transcription factors, such as RANKL and OPG indicated an increase of gene expression in rats administered with STZ, which demanded to be further studied.

This study showed that fluoride enhanced the intracellular InR, IGF1 and IGF1BPs, which suggested that insulin mediated bone turnover by these factors during fluoride induced bone lesion. Osteoblasts express functional InR and respond to exogenous insulin [11], and IGFs in circulation are bound with IGF1BPs, which function to stabilize and regulate IGFs action on osteoblast. However, Lau et al. found that aluminum fluoride but not that of NaF stimulated the expression of IGF1 and IGF1BPs [12]. The converse results yet demanded to be clarified. In this study, the co-treatment with STZ and fluoride partly reduced InR, IGF1 and IGF1BPs expression other than completely inhibited them. Insulin signaling in osteoblasts is important for normal bone acquisition and stimulates OCN predication [13]. These results implied that intracellular InR signaling was activated by high level of insulin, but insulin might not be the only way to activate them in the process of bone turnover induced by fluoride. In this study, there still existed high expression of IGF1 and IGF1BPs induced by fluoride, which probably activated the InR signaling instead of insulin. Therefore, fluoride activated the InR and IGF1 signaling in femur of rats, and insulin was important not the only element involved in the mechanism of activating InR in the process of fluoride induced bone turnover.

The bone turnover is coupling between bone formation and bone resorption, and recent studies recognized TGF β as secreted contributors to coupling linkage [14]. In this study, the low dose of fluoride stimulated the active bone turnover

accompanied with significant increase of TGF β 1 expression; however, the STZ administration obviously inhibited the TGF β 1 expression. Likewise, the changes of Wnt10/ β -catenin indicated that the low dose of fluoride increased the β -catenin and Wnt10 expression. Nevertheless, the stimulating action on β -catenin induced by fluoride was stunted by STZ administration. The important roles of Wnt/ β -catenin signals in osteoblastogenesis and osteoclastogenesis have been found [15, 16]. Thus, changes of factors reflected that Wnt/ β -catenin and TGF β 1 was involved in bone turnover caused by fluoride, and insulin probably influenced the TGF β 1 and β -catenin to play a role on bone turnover during the development of bone lesion.

To define specific actions of InR in bone lesion induced by fluoride, we determined the transcription factors related with osteoblastic and osteoclastic differentiation after pre-osteoblast knocked down the InR gene. The InR expression was effectively inhibited and Esp gene increased in this study, which reflected the role of Esp as a negative regulator of intracellular InR signaling in osteoblasts [17]. The IRS1 as the downstream of InR and IGF1 still was lower in osteoblast, but increased after cells treated with fluoride. The earlier researcher reviewed that IGF1, IGF1 receptor and its main signaling molecule IRS1 are pivotal for the fetal growth [18]. Thus, we speculated that fluoride might induce the IGF1, which activated IRS1 instead of InR. Base on the downregulation of InR expression, the obvious inhibition of osterix and OCN expression in osteoblasts was observed, and fluoride hardly improved two gene levels under this condition. Likewise, the knock-down expression of InR significantly decreased the OPG expression, and it was still low in osteoblast co-treated with fluoride. Thraikill and Fulzele [13, 19] clarify that insulin receptor expression in osteoblasts is critically important for proper bone development and osteoblast proliferation, survival, and differentiation in vitro. The concomitant decrease of OPG, osterix and OCN with inhibition of InR expression in osteoblast exposed to fluoride suggested that

fluoride probably stimulated the expression of OPG, osterix and OCN through InR signaling, but fluoride induced the expression of Runx2 and RNAKL by other pathway instead of InR.

These changes of genes expression reflected the important role of insulin in bone lesion induced by excessive amount of fluoride. IGF1 also was involved in the mechanism underlying fluoride induced bone turnover. Meanwhile, insulin probably influenced the TGF β 1 and β -catenin to play a role on bone turnover during the development of bone lesion. Furthermore, fluoride probably stimulated the expression of OPG, osterix and OCN through InR signaling. To conclude, insulin played the important role in bone lesion induced by excessive amount of fluoride through mediating InR receptor signaling, and IGF1 probably modulated InR or its downstream to exert action on bone turnover caused by overdose of fluoride.

Acknowledgments This work was supported by grant for skeletal fluorosis research from National Natural Science Foundation of China [81072249], the Norman Bethune Program of Jilin University [2012222] and Doctoral Fund of Ministry of Education of China [20130061110084].

Compliance with Ethical Standards

Conflicts of Interest The authors declare that they have no competing interests.

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