In vivo and in vitro action mechanism of treatment of glucocorticoid-induced osteoporosis by regulation of osteoprotegerin/receptor activator of nuclear factor-κB pathways by denshensu

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ABSTRACT

The research aimed to discuss the action mechanism of the treatment of glucocorticoid-induced osteoporosis (GIOP) by denshensu. In the research, 60 rats were purchased and divided into a control group, model group, estradiol group, and denshensu treatment group. Except for the control group, GIOP models were established for all other groups, and then the structural changes of osseous tissues as well as osteoprotegerin (OPG), expression of receptor activator of nuclear factor-κB ligands (RANKL) were detected. Besides, the changes in osteoclasts were observed by bone marrow-derived mononuclear phagocytes in vitro. The results showed that the micro-structure of bone trabeculae, bone mineral density (BMD), and bone metabolic markers of rats in the denshensu treatment group were enhanced significantly, while trabecular separation and structural model index were reduced (P<0.05). OPG messenger ribonucleic acid (mRNA) and protein levels in the hypothalamus and femur tissues were increased, while RANKL content was remarkably decreased (P<0.05). In addition, in vitro experiments revealed that denshensu inhibited the differentiation of positive osteoclasts, and osteoclast-related genes were reduced (P<0.05). To conclude, denshensu might inhibit the expressions of OPG and RANKL and further play a role in treating GIOP.

Introduction

As metabolic osteopathy, osteoporosis is commonest among middle-aged and elderly people. The main symptoms of this disease include the abnormalities of patients’ trabecular structure, a decrease in bone content, and a reduction in bone strength. Osteoporosis patients are at extremely high risk of pathological fracture (1,2). In clinical practice, the treatment of inflammation and immune suppression by considerable glucocorticoids results in an increase in the number of patients with glucocorticoid-induced osteoporosis (GIOP). GIOP becomes a severe sequela caused by the adoption of massive glucocorticoids. According to relevant studies and statistics, about 40% of patients in the population treated with glucocorticoids suffer from osteoporosis (3-5). The action of glucocorticoids on the body causes various injuries, including the reduction in bone content, osteoporosis, the deterioration of bone micro-structure, osteonecrosis, and fracture resulting from the increased osteopathogenesis (6-8). Among different types of osteoporosis diseases, the incidence of GIOP is ranked in third place following postmenopausal women osteoporosis and senile osteoporosis (9,10). The clinical manifestations of GIOP patients include whole-body osteodinia, a decrease in height, fracture, and radiography. There are a series of changes in osseous tissue in patients’ bodies, including a significant reduction in bone content, the increased osteopathy. As a result, the incidence of fracture and even disability is extremely high. In particular, the fracture of weight-bearing bones in patients’ bodies, such as femurs and lumbar vertebrae, results in huge damage to the living quality and families of patients themselves as well as society (11-14).

In recent years, multiple studies demonstrate that two significant pathogenic mechanisms of GIOP are oxidative stress and bone marrow adipose differentiation. When the human body is in a state of suffering from particular diseases, senility, or taking some drugs, the differentiation of numerous fat in bone marrow causes the continuous accumulation of fat in the medullary cavity. Meanwhile, reactive oxyradicals in the human body also aggregate. Consequently, osteoporosis comes into being (15-17). These studies demonstrate that the search for drugs that can inhibit fat differentiation in the bone marrow and resist oxidative activity is the direction of the prevention and treatment of GIOP (18,19). At present, the drugs that can specifically prevent and treat GIOP are not discovered. Hence, GIOP patients are still treated with drugs targeting primary osteoporosis (20,21). Some studies showed that denshensu plays a role in anti-oxidation as a polyphenols small molecule compound, which can promote the differentiation and proliferation of osteoblasts. According to relevant studies, the application of denshensu in rat models can prevent and treat the symptoms of scleratin loss and bone marrow adipose differentiation (22-24).

The Osteoprotegerin (OPG)/receptor activator of the nuclear factor-κB (RANK) signal pathway is the classical signal pathway of body bone metabolism and plays a great role in bone metabolism and bone cell differentiation.
role in sclerotic balance and osteoporosis (25,26). NF-κB is a transcription factor, which is derived from B lymphocytes. Relevant studies proved that the transcription factor plays an essential role in promoting bone formation and inhibiting bone resorption, and OPG/RANK signal pathway can regulate the activity and functions of NF-κB (27-29). The receptor activator of nuclear factor-κB ligand (RANKL) is the ligand of RANK with two expression forms, including soluble proteins and transmembrane proteins. They are usually distributed on the surfaces of osteoclasts, osteoblasts, and lymphocytes (30). After considerable RANKL existing on the surface of osteoclasts is combined with RANK, NF-κB is activated and then gather inside nuclei, which promotes the activation and expression of osteoclast-related genes, resulting in the continuous differentiation of osteoclasts, and causes the resorption of massive bone (31). However, OPG can competitively block the combination between RANKL and RANK to inhibit the proliferation and differentiation of osteoclasts and prevent sclerotic loss and osteoporosis (32). Some previous studies indicate that inadequate RANKL causes osteosclerosis and OPG gene deletion results in osteoporosis. The results showed that the existence of OPG can help reduce the damage to sclerotic (33).

In the research, 60 female Sprague Dawley (SD) rats were purchased from The Fifth Hospital of Xiamen and divided into a control group, model group, estradiol treatment group, and denshensu treatment group. In each group, there were 15 rats. Except for the control group, dexamethasone was injected into rats in all other groups, and GIOP models were established to detect bone mineral density (BMD), bone metabolic markers, micro-structure of bone trabeculae, apoptosis at distal bone, and the expressions of OPG as well as RANKL. Besides, the changes of osteoclasts were observed by rat bone marrow-derived mononuclear phagocytes in vitro to discuss the action mechanism of the treatment of GIOP by the regulation of the OPG/RANK signal pathway by denshensu.

Materials and Methods

Experimental animals

All SD rats included in the research were purchased from The Fifth Hospital of Xiamen and females with 300g in weight. Totally 60 rats were divided into 4 groups, including a control group, model group, estradiol treatment group, and denshensu treatment group. Each group included 15 rats fed in a ventilated and tidy animal experimental base. During the experiment, all processing for SD rats was strictly implemented in accordance with national experimental animal norms. The implementation of the animal experiment had been approved by Experimental Animal Ethics Committee.

Main reagents and instruments

Dexamethasone injection, physiological saline, and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) reaction liquid were purchased from Shanghai Yuanye Biotechnology Co., Ltd. Denshensu, RANKL, and tartrate-resistant acid phosphatase (TRAP) staining kits were purchased from Sigma Company. Bone alkaline phosphatase (BALP) enzyme-linked immunosorbent assay (ELISA) kits, core binding factor-α1 (CBF-α1) kits, c-terminal type I collagen telopeptide (CTX-I) ELISA detection kits, procollagen type I amino-terminal peptide (PINP) ELISA detection kits, and osteocalcin (OC) ELISA detection kits and reverse transcription kits were purchased from Shanghai Qiuyuan Biotechnology Co., Ltd. Polymerase chain reaction amplification reagents were all purchased from Shanghai Fantei Biotechnology Co., Ltd. The main instruments were a computed tomography (CT) imaging system (German Siemens with the type of Inveon) and a BMD instrument (South Korea with the type of EXA-PRESTO).

Establishment of rat GIOP models

The muscles of rats in the model group, estradiol treatment group, and denshensu treatment group were injected into dexamethasone injection, and the injection dose was 2.5 mg/kg. The injection was carried out once at a fixed time each Tuesday and Thursday for 4 weeks. With the same feeding conditions, rats in the control group were injected with the same dose of physiological saline at a fixed time.

Treatment methods

After the successful establishment of GIOP, drug treatment was carried out. Rats in the control group and model group were both performed with the gastric infusion of sterilized physiological saline (50μg/kg). Rats in the estradiol treatment group were performed with a gastric infusion of estradiol solution (50μg/kg). Rats in the denshensu treatment group were given a gastric infusion of denshensu solution (50μg/kg). The gastric infusion was offered at a fixed time once every day, and the treatment intervention lasted for 3 months. Besides, SD rats in 4 groups were fed in the same environment.

In vivo experiment observation indexes

Rat femur specimens were taken and micro-CT was adopted to analyze osseous tissue-related quantitative indexes, including BMD, relative bone volume, bone trabecular thickness, bone trabecular separation, trabecular number, connectivity density, and structural model indexes. The detection process of bone metabolic marker content was as follows. 2mL of rat blood was collected and centrifuged. After that, the supernatant was extracted. Next, ELISA was utilized to detect the contents of BALP, CBF-α1, CTX-I, OC, and PINP in the serum of rats in each group.

The detection process of the distal apoptosis of rats' femurs was as follows. Rats' femur tissues were taken and processed in formalin. After that, the specimen embedded in paraffin was cut into sections with 5μm in thickness in a slicer and then stained with TUNEL. Next, paraffin sections were processed with xylene, ethanol, water, trypsin K, phosphate buffer solution (PBS), and TUNEL reaction liquid. Then, it was placed in a wet box to guarantee certain humidity. In the wet box, it was incubated at 37°C for 1 hour. After the incubation, it was washed with PBS 3 times and then processed with diaminobenzidine (DAB) stain for 30 minutes. Next, it was washed with PBS 3 times and re-stained in hematoxylin in a staining solution for 10 minutes. After alcohol gradient dehydration, it was rinsed. Finally, the apoptosis in the femur tissues of rats in each group was observed by microscope.

The detection process of OPG and the expressions of RANKL mRNA and proteins were as follows. The total
RNA in the hypothalamus in the femur tissues of rats in each group was extracted. Besides, complementary deoxyribonucleic acid (cDNA) was obtained by reverse transcription kits, and OPG and RANKL mRNA contents were detected by amplification using OPG and RANKL primers. After that, rats’ hypothalamic and femur tissues were taken and the total proteins in tissues were extracted for Western blotting. After the incubation by first-antibody and second-antibody, the total proteins were performed with color observation, and then the relative expression level of the proteins was measured by quantitative software.

Cell culture in vitro and index detection

Mononuclear phagocytes in the bone marrow of rats’ femurs were separated and then inoculated into 96-well plates for culture. After that, RANKL was utilized to induce the osteoclast differentiation of mononuclear phagocytes. Besides, different concentrations of denshensu were added to carry out in vitro intervention. The culture lasted for 8 days until mature osteoclasts were formed. Next, TRAP kits were adopted to test cell staining, detect the number of mature osteoclasts quantitatively, and assess the role of denshensu in RANKL. The expression of related genes in the formation of osteoclasts was detected 0, 24, and 48 hours after the intervention of mononuclear phagocytes by denshensu, including cathepsin K (CTSK), proto-oncogenes c-fos (c-fos), nuclear factor activated T c1 (NFATc1), and TRAP.

Statistical methods

Statistical product and service solution (SPSS) was utilized in data statistics and analysis. Data that conformed to normal distribution were expressed by mean±standard deviation (mean±S), measurement data were expressed by t-test and enumeration data were expressed by chi-square ($\chi^2$) test. $P<0.05$ indicated that the differences showed statistical significance.

Results

Detection results of the bone trabecular structure of rats in each group

BMD, relative bone volume, bone trabecular thickness, bone trabecular separation, number of bone trabeculae, connectivity density, and structural model indexes of femur tissues of rats in four rats were detected. The results demonstrated that relative bone volume, number of bone trabeculae, bone trabecular thickness, and connectivity density of rats’ femurs in the model group were all significantly increased compared with those in the control group ($P<0.05$). Besides, relative bone volume, the number of bone trabeculae, bone trabecular thickness, and connectivity density of rats’ femurs in the estradiol treatment group and denshensu treatment group were enhanced compared with those in the model group. In contrast, bone trabecular separation and structural model indexes were both decreased compared with those in the model group ($P<0.05$). Figure 1 illustrated the comparison of the micro-structure of bone trabeculae of rats in each group below.

Detection results of BMD and bone metabolic markers of rats in each group

BMD and the contents of bone metabolic markers in the serum of rats in each group were detected, including BALP, CBF-α1, CTX-I, OC, and PINP. The results showed that the contents of BALP, CBF-α1, CTX-I, OC, PINP, and BMD of rats in the model group, estradiol treatment group, and denshensu treatment group were significantly lowered compared with those in control group ($P<0.05$). Besides, the contents of BALP, CBF-α1, CTX-I, OC, PINP, and BMD were enhanced compared with those in the model group ($P<0.05$). Figure 2 presented the comparison of BMD and bone metabolic markers of rats in each group below.

Apoptosis in the femur of rats in each group

According to the observation of femur tissues of rats in each group after TUNEL staining, surviving nuclei were stained blue, and nuclei were stained green after apoptosis. The results showed that there were few apoptotic cells in rats in the control group, while numerous apoptotic...
cells emerge among rats in the model group. Compared with those in the model group, apoptotic cells in the femur tissues of rats in the denshensu treatment group were reduced. Figure 3 displayed the staining results of rats’ femur tissues by TUNEL below.

Results of expression levels of OPG as well as RANKL mRNA and proteins

OPG and RANKL mRNA expression levels of the hypothalamus and femur tissues of rats in each group were detected. The results demonstrated that OPG mRNA contents in the hypothalamus and femur tissues of rats in the model group were significantly decreased compared with those in the control group, while RANKL mRNA content was increased (P<0.05). Besides, OPG mRNA contents in the hypothalamus and femur tissues of rats in the denshensu treatment group were significantly enhanced compared with those in the model group, while RANKL mRNA content was reduced (P<0.05). Figure 4 showed the comparison of the expression levels of OPG and RANKL mRNA in the hypothalamus (A) and femur tissues (B) of rats in each group below.

The expression levels of OPG and RANKL proteins in the hypothalamus and femur tissues of rats in each group were detected. The results demonstrated that the contents of OPG protein in the hypothalamus and femur tissues of rats in the model group were significantly reduced while the contents of RANKL proteins were remarkably enhanced (P<0.05) compared with those in the control group. Besides, the contents of OPG protein in the hypothalamus and femur tissues of rats in the denshensu treatment group were significantly increased, while the content of RANKL proteins was decreased compared with those in the model group (P<0.05). Figures 5 and 6 showed the expression levels of OPG and RANKL proteins in the hypothalamus (A) and femur tissues (B) with the detection by Western blotting and the results of the comparison of the expression levels of OPG and RANKL proteins in the hypothalamus (A) and femur tissues (B) of rats in each group, respectively.

Results of detection in positive osteoclasts

After the differentiation induced by RANKL, the generation of positive osteoclasts was observed under the intervention by different concentrations of denshensu. The results demonstrated that a higher concentration of denshensu meant fewer positive osteoclasts. Compared with that in the non-intervened group, the number of positive osteoclasts in the intervened group was significantly decreased with the intervention of 25μmol/L, 50 μmol/L, and 75 μmol/L of denshensu (P<0.05). Figures 7 and 8 demonstrated the results of staining by TRAP and the results of the influences of different concentrations of denshensu in RANKL-induced osteoclast differentiation, respectively.
Results of detection in mRNA levels of osteoclast-related genes

The expression levels of CTSK, c-fos, NFATc1, and TRAP in osteoclasts 0, 1, and 2 days after RANKL-induced differentiation was detected. The results showed that the expression levels of osteoclast-related genes, including CTSK, c-fos, and TRAP, were all significantly reduced (P>0.05), and the expression levels of NFATc1 showed no statistical differences (P>0.05). Figure 9 showed the influences of denshensu in relevant genes of RANKL-induced osteoclasts below.

Discussion

Osteoporosis is a metabolic osteopathy common among middle-aged and elderly people. It is classified into senile osteoporosis, women postmenopausal osteoporosis, and GIOP (34). In clinical treatment, the incidence of the disease is constantly growing. At present, it becomes one of the ten commonest diseases worldwide (35). As a drug, glucocorticoid is usually utilized to treat multiple chronic diseases, such as chronic kidney disease, leukemia, and rheumatoid arthritis. Long-term use of glucocorticoids by patients with chronic diseases causes negative influences on the normal metabolism of osseous tissues and the incidence of osteoporosis. In this case, patients undergo a series of changes, such as osteopenia, osteoporosis, deterioration of bone micro-structure, osteonecrosis, and increased osteopathsathyrosis often causing fracture (36). With the development of diseases, a series of changes occur in osseous tissues of GIOP patients’ bodies, including significant osteopenia, increased osteopathsathyrosis, and high risks of fracture and even disability, especially the fracture of weight-bearing bones in bodies, such as femurs and lumbar vertebrae. The fracture of weight-bearing bones causes huge damage to the living quality, longevity, and families of patients themselves as well as society (37). Therefore, osteoporosis becomes a significant public health issue in China, and it is imperative to prevent and treat this disease. As a polyphenols small molecule compound, denshensu plays a role in anti-oxidation and can promote the differentiation and proliferation of osteoblasts. It is crucial to research the treatment of GIOP by its action pathway and mechanism (38). OPG/RANK signal pathway is the classical signal pathway of body metabolism and plays a significant role in bone balance and osteoporosis. OPG is a secreted protein that can regulate bone resorption. In addition, it expresses in osteoblasts, B cells, as well as dendritic cells and is rich in osseous tissues (39). RANK is a homologous trimer transmembrane protein that has 616 amino acid sequences. According to relevant studies, it is formed in osteoblasts or precursor cells of osteoclasts, such as mononuclear phagocytes, and then plays roles (40). The objective of the research was to discuss the action mechanism of the treatment of GIOP by denshensu and whether its action pathway is closely related to OPG/RANK signal pathway. In the research, a total of 60 female SD rats were purchased and divided into a control group, model group, estradiol treatment group, and denshensu treatment group. Each group included 15 rats. Except for the control group, dexamethasone was injected into rats in all other groups and GIOP models were established. Besides, BMD, bone metabolic markers, bone trabecular micro-structure, bone distal apoptosis, and the expression levels of OPG and RANKL were detected. Meanwhile, the changes in osteoclasts were observed by rats’ bone marrow-derived mono-
nuclear phagocytes in vitro.

In the research, BMD, relative bone volume, bone trabecular thickness, bone trabecular separation, number of bone trabeculae, connectivity density, and structural model indexes of femur tissues of rats in 4 groups were detected by the rat in vivo experiment. The results revealed that relative bone volume, number of bone trabeculae, and connectivity density of rats’ femurs in the model group were significantly reduced, while bone trabecular separation and structural model indexes were both remarkably enhanced compared with those in the control group (P<0.05). Besides, relative bone volume, the number of bone trabeculae, bone trabecular thickness, and connectivity density of rats in the estradiol treatment group, and denshensu treatment group were increased, while bone trabecular separation and structural model indexes were both significantly reduced compared with those in model group (P<0.05). The contents of markers, including BALP, CBF-α1, CTX-I, OC, and PINP, as well as BMD of rats in the model group, estradiol treatment group, and denshensu treatment group were lowered (P<0.05). Besides, the contents of markers, including BALP, CBF-α1, CTX-I, OC, and PINP, as well as BMD were significantly enhanced compared with those in the model group (P<0.05). According to the observation after the staining of the femur tissues of rats in each group by TUNEL, there were only a few apoptotic cells among rats in the control group, while considerable apoptotic cells appeared among rats in the model group. Compared with that in the model group, the number of apoptotic cells in the femur tissues of rats in the denshensu treatment group was reduced. A series of rat in vivo experiments demonstrated rat GIOP models were successfully constructed in the research, and rats’ osteoporosis was effectively improved with the action of denshensu. Therefore, the drug showed therapeutic effects on osteoporosis. The expression levels of OPG and RANKL mRNA, as well as proteins in the hypothalamus and femur tissues of rats in each group, were detected, and the results showed that the contents of OPG mRNA and proteins in the hypothalamus and femur tissues of rats in the model group were significantly decreased, while the contents of RANKL mRNA and proteins were remarkably increased compared with those in the control group (P<0.05). In addition, the contents of OPG mRNA and proteins in the hypothalamus and femur tissues of rats in the denshensu treatment group were significantly enhanced, while the contents of RANKL mRNA and proteins were reduced compared with those in the model group (P<0.05). The results demonstrated that mRNA and protein levels of OPG and RANKL were both changed correspondingly after the treatment by denshensu, which might play its role in treating osteoporosis by regulating OPG/RANKL. After the differentiation induced by RANKL, the generation of positive osteoclasts was observed with the intervention of different concentrations of denshensu in vitro. The results showed that a higher concentration of denshensu indicated fewer positive osteoclasts. Compared with that in the non-intervened group, the number of positive osteoclasts in the intervened group was significantly decreased with the intervention of 25μmol/L, 50 μmol/L, and 75 μmol/L of denshensu (P<0.05). What’s more, the expression levels of CTSK, c-fos, NFATc1, and TRAP in cells 0, 1, and 2 days after RANKL-induced differentiation were detected, and the results showed that the expression levels of osteoclast-related genes, including CTSK, c-fos, and TRAP, were all significantly lowered with the intervention of denshensu (P<0.05). In contrast, the expression level of NFATc1 showed no statistical differences (P>0.05). A series of in vitro experiments demonstrated that denshensu inhibited the expression of RANKL and further inhibited the differentiation of osteoclasts, and the role of denshensu in treating osteoporosis might be related to the RANKL pathway. In the study, Doustimotlagh et al. (41) adopted denshensu to inhibit the differentiation of osteoclasts induced by RANKL and found out that the drug could down-regulate phosphorylation level and then affect RANKL-induced osteoclast differentiation, which was consistent with the result of the research.

Conclusion

In the research, a total of 60 female SD rats were purchased and divided into a control group, model group, estradiol treatment group, and denshensu treatment group. Each group included 15 rats. Except for the control group, dexamethasone was injected into rats in all other groups and GIOP models were established. Besides, BMD, bone metabolic markers, bone trabecular micro-structure, bone distal apoptosis, and the expression levels of OPG and RANKL were detected. Meanwhile, the changes in osteoclasts were observed by rats’ bone marrow-derived mononuclear phagocytes in vitro to discuss the action mechanism of the treatment of GIOP by denshensu and whether its action pathway was closely related to OPG/RANK signal pathways. Rats’ in vivo experiments indicated that rat GIOP models were successfully constructed in the research, and rats’ osteoporosis was effectively improved with the action of denshensu. As a result, the drug showed therapeutic effects on osteoporosis. After the treatment by denshensu, the expression levels of mRNA and proteins of OPG and RANKL were both changed correspondingly. Denshensu might play its role in treating osteoporosis by regulating OPG/RANKL. In vitro experiments showed that denshensu inhibited the expression of RANKL and further inhibited the differentiation of osteoclasts, and the role of denshensu in treating osteoporosis might be related to the RANKL pathway. In the research, the close relationship between the treatment of GIOP by denshensu with the OPG/RANK pathway was only investigated preliminarily. In subsequent research, the specific mechanism that it adopted to affect the signal pathway needed to be profoundly explored. Besides, whether the therapeutic role of denshensu depended merely on OPG/RANKL pathway also should be investigated to provide a more practical and effective theoretical basis and reference values for the treatment of GIOP.

References


