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Original Article

Effects of Puerarin on Blood Fluoride, Micro-CT Parameters and Bone Histomorphology in Rats with Fluorosis

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ABSTRACT

Aim: This study aims to examine the effects of puerarin on blood fluoride, micro-Computed Tomography (micro-CT) parameters and bone histomorphology of rats with fluorosis and to investigate whether puerarin has therapeutic effects on skeletal fluorosis. **Materials and Methods:** The 72 rats were randomly divided into the blank control group, model group and puerarin group. All the rats will be administered different doses of drug interventions. Morphological structure of bone tissue was observed by the Hematoxylin and Eosin (H&E) staining. Blood fluoride, micro-CT parameters were monitored. **Results:** Compared with the blank control group, the blood fluoride level in the model group was significantly higher ($p<0.05$). Compared with the model group, the blood fluoride level in the puerarin group were lower ($p<0.05$). Compared with the model group, the micro-CT parameters of the rats in the puerarin group were significantly improved ($p<0.05$). In the model group, the bone cortex became thicker, the trabecular number increased; the trabecular bones were irregularly arranged; the width of medullary cavity was smaller. In the puerarin group, the bone cortex became thinner, the trabecular number decreased, the lamella structure was not clear and the width of the medullary cavity became smaller. **Conclusion:** Fluoride has a dual regulatory effect on bone metabolism, with osteosclerosis and osteoporosis crossing each other. Puerarin regulates bone metabolism in fluorosis rats and plays a therapeutic and osteoprotective role in anti-fluorosis. However, such therapeutic effects are bone region specific and dependent on a drug concentration. Puerarin has a more significant effect on cancellous bone.

Keywords: Puerarin, Fluorosis, Blood fluoride, Micro-CT parameters, Bone histomorphology.

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INTRODUCTION

Endemic fluorosis is a chronic systemic disease that seriously endangers human health. Xinjiang is a high incidence area of skeletal fluorosis due to its special geological environment and climatic characteristics, severe winter, the number of coal-burning ranking among the highest in China and the fluoride content in drinking water higher than the national 1 mg/L standard. In high fluoride areas, people consume excessive fluoride through drinking water or food for a long time, which leads to chronic accumulation of fluorosis in the body.[1] Excess fluoride deposited on the bones, can lead to skeletal fluorosis.

Skeletal fluorosis is a pathological progression of active osteogenesis and accelerated bone transformation, including osteosclerosis, osteochondrosis, osteoporosis, periosteal soft tissue ossification and cartilage and joint degeneration. Therefore, it is a major lesion that causes disability, paralysis and reduced work capacity.[2]

At present, there is no corresponding specific drug treatment for this disease except to improve the living environment of patients. Its chronic course can last for decades and most patients die of chronic nutritional disorders or other serious complications.

Since abnormal bone metabolism is important pathogenesis of skeletal fluorosis, in addition to active symptomatic treatment, it is also a very important means to find drugs that can regulate bone metabolism. Traditional Chinese Medicine (TCM) treatment can significantly relieve limb and joint pain, tetany and other symptoms caused by skeletal fluorosis, improve joint function, improve the quality of life of patients and reduce the family burden of patients.[3,4] Puerarin is an isoflavone derivative isolated from the Chinese herbal medicine puerari, which is mainly found in kudzu root, the dried root of Pueraria lobata.[5] Recent studies have shown that puerarin can play a role in regulating bone metabolism through various signaling pathways such as TRAF6/ROS, MAPK/NF-κB, Wnt and β3 Pyk2/Src/Cbl and so on.[6-8] In

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the past two years, domestic and foreign studies have found that puerarin can inhibit the production of osteoclasts in bone tissue and promote the proliferation and differentiation of osteoblast,[9-11] and there are no reports on puerarin treatment of skeletal fluorosis. In this study, we intend to observe the effects of puerarin on blood fluorine, micro-CT parameters and histomorphology of rats with fluorosis by constructing a rat model of fluorosis and treating them with different concentrations of puerarin, respectively, to clarify that puerarin has a therapeutic effect on skeletal fluorosis and provide new ideas and methods for the clinical application of puerarin in the prevention and treatment of skeletal fluorosis.

MATERIALS AND METHODS

Experimental animals, reagents and materials

The Institutional Animal Ethics Committee approved this study (20210301-50) and the study was performed according the National Research Council's Guide for the Care and Use of Laboratory Animals. 72 healthy SPF-grade female Sprague-Dawley rats, non-pregnant, weighing 110-120 g, were purchased from the Animal Experiment Center of Xinjiang Medical University [license number: SCXK (new) 2018-0002]. They are bred in the animal laboratory of Xinjiang Medical University, with the same feeding conditions: 3 to 4 rats per cage, constant temperature and humidity, artificial alternating light and dark lighting, *ad libitum* water intake and standard chow feeding. Ion meters PXSJ-227L, PF-202-L fluoride ion composite electrodes and JB-10 agitator were purchased from Shanghai Instrument and Electric Scientific Instrument Co, Ltd., Puerarin Injection (Specification: Each vial contains 100 mg puerarin) has been approved by China SFDA (Registration No.: H20033292. Made by: Shandong Fangming Pharmaceutical Group Co, Ltd.,).

Methods

Establish an animal model of fluorosis in rats: According to the results of previous studies the animal model of fluorosis in rats was constructed concerning *ad libitum* water intake, 150 mg/L sodium fluoride *ad libitum* water intake.[12,13] The success rate of modeling was evaluated after 50 days of *ad libitum* water intake and the evaluation criteria for successful modelling: chalky and brown patches could be seen on the enamel, with localised pigmented spots or concave defects.

Grouping and drug treatment

The experiment was divided into a blank control group, model group, low dose puerarin group, medium dose puerarin group, high dose puerarin group and extremely high dose puerarin group, while different treatments were given.

- Blank control group: 1 mL/kg 0.9 NaCl, ih, qd, distilled water.
- Model group: 1 mL/kg 0.9 NaCl, ih, qd, 150 mg/L Sodium fluoride water.
- Low dose puerarin group: 30 mg/kg puerarin injection, ih, qd, 150 mg/L Sodium fluoride water.
- Medium dose puerarin group: 40 mg/kg puerarin injection, ih, qd, 150 mg/L Sodium fluoride water.
- High dose puerarin group: 50 mg/kg puerarin injection, ih, qd, 150 mg/L Sodium fluoride water.
- Extremely high dose puerarin group: 60mg/kg puerarin injection, ih, qd, 150 mg/L Sodium fluoride water.

All Sodium fluoride were dissolved in water to make 150 mg/L solution and *ad libitum* drinking water with solution was given to rats. Puerarin injection was injected into all rats in the puerarin group by subcutaneous injection and once a day. The Sodium fluoride water and puerarin injection was administered continuously for 7 weeks.

Specimen collection

After 7 weeks of drug intervention, the rats were sacrificed by acute exsanguination. Following that, 8 to 10 mL of blood was collected and blood samples were ultracentrifuged at the speed 3000 r/min for 10 min. 1 to 2 mL of serum was transferred in EP tubes. Fluoride ion concentration in serum was measured by a fluoride ion meter. The bilateral femurs were stripped layer by layer, rinsed with saline and fixed in a 10% formaldehyde solution.

Detection of fluoride content in blood serum

All operations were strictly in accordance with the instructions for use of the ion meter. Detecting the serum fluoride concentration was measured in all groups. Measurement of each group was repeated three times and the average was taken.

Detection of the micro-CT parameters of bone tissue in each group. Micro-CT (model no.: skyscan1275, Belgium) was applied to scan the right femur of the rats to be tested with a scanning accuracy of 12 μm and CTVOL software was used for data analysis and three-dimensional reconstruction.

H&E staining was adopted to observe bone morphological changes of bone tissue in each group. It referring to the operation steps of H&E staining methods, which were: preparing paraffin sections, using xylene for dewaxing and hematoxylin for staining; differentiating in 1% hydrochloric acid; dehydrating through alcohol; clearing by xylene, mounting and applying the cover slip. Lastly, histomorphology of bone tissue was examined with the microscope.

Statistical analysis

SPSS 22.0 software was used for analysis. Normally distributed variables were analyzed with the Kolmogorov-Smirnov test and all data were expressed as the Mean±Standard Deviation (SD). $p<0.05$ indicated that the differences were statistically significant.

RESULTS

Fluoride content in the blood serum of rats (Figure 1).

Model group compared with the blank control group: fluoride concentration in the blood serum was increased significantly in the model group rats compared with the blank control group ($p<0.05$).

Comparison between the puerarin group and the model group: serum fluoride concentration was decreased significantly in the high and extremely high dose puerarin groups compared to the model group ($p<0.05$).

Changes of micro-CT parameters of cortical bone in each group (Figure 2).

Changes of cortical bone thickness in each group

The cortical bone thickness in the model group was higher than that in the blank control group ($p<0.05$). Within the puerarin groups, the bone thickness of each puerarin group was lower than that of the model group, but the difference was not statistically significant ($p>0.05$).

Changes of cortical bone density in each group

The cortical bone density of rats in the model group was higher than that in the blank control group ($p<0.05$). Within the puerarin groups, the bone density of the high dose puerarin group was lower than that of the model group, but the difference was not statistically significant ($p>0.05$), while the remaining puerarin groups were still higher than that of the model group.

Changes of cortical bone area in each group

The cortical bone area of rats in the model group was lower than that of the blank control group ($p<0.05$). Within the puerarin groups, the bone area of the extremely high dose puerarin group was higher than that of the model group ($p<0.05$). With the increase of puerarin treatment concentration, the bone area gradually increased, but the difference was not statistically significant ($p>0.05$).

Changes of cortical bone volume in each group

The cortical bone volume of rats in the model group was lower than that in the blank control group ($p<0.05$). Within the puerarin groups, the bone volume in the extremely high dose puerarin group was higher than that in the model group ($p<0.05$). The bone volume gradually increased with the increase of puerarin treatment concentration, but the difference was not statistically significant ($p>0.05$).

Changes in micro-CT parameters of cancellous bone in each group (Figure 3).

Changes in cancellous bone volume fraction (BV/TV) in each group

The cancellous Bone Volume fraction (BV/TV) of rats in the model group was higher than that in the blank control group ($p<0.05$). Within the puerarin groups, except for the extremely high dose puerarin group, all groups were lower than the model group ($p<0.05$). The BV/TV in the extremely high dose puerarin group was significantly higher than that in the other puerarin groups ($p<0.05$).

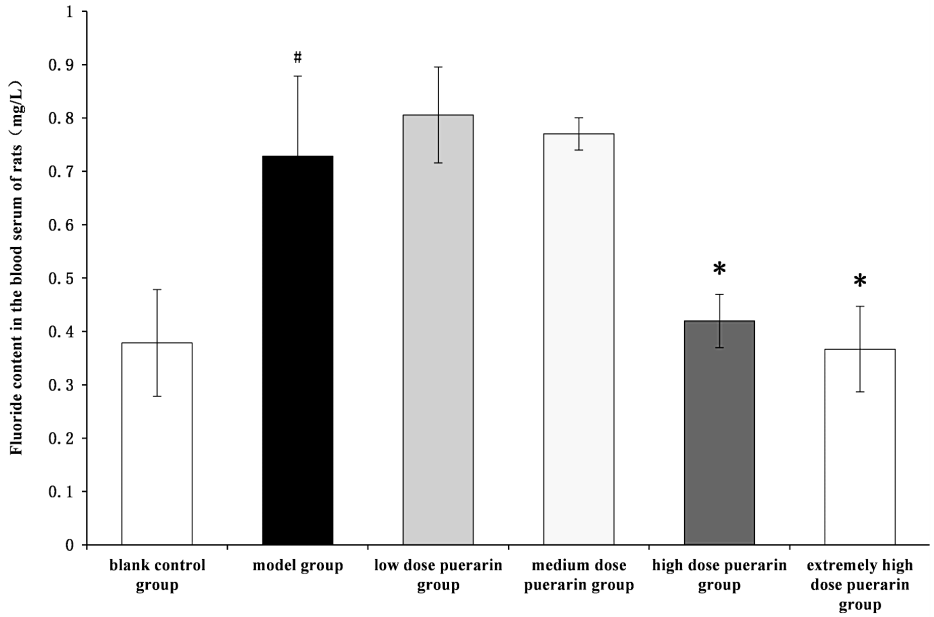

Figure 1: Fluoride content in the blood serum of rats. #Compared with the blank control group, the difference was statistically significant ($p<0.05$). *Compared with the model group, the difference was statistically significant ($p<0.05$).

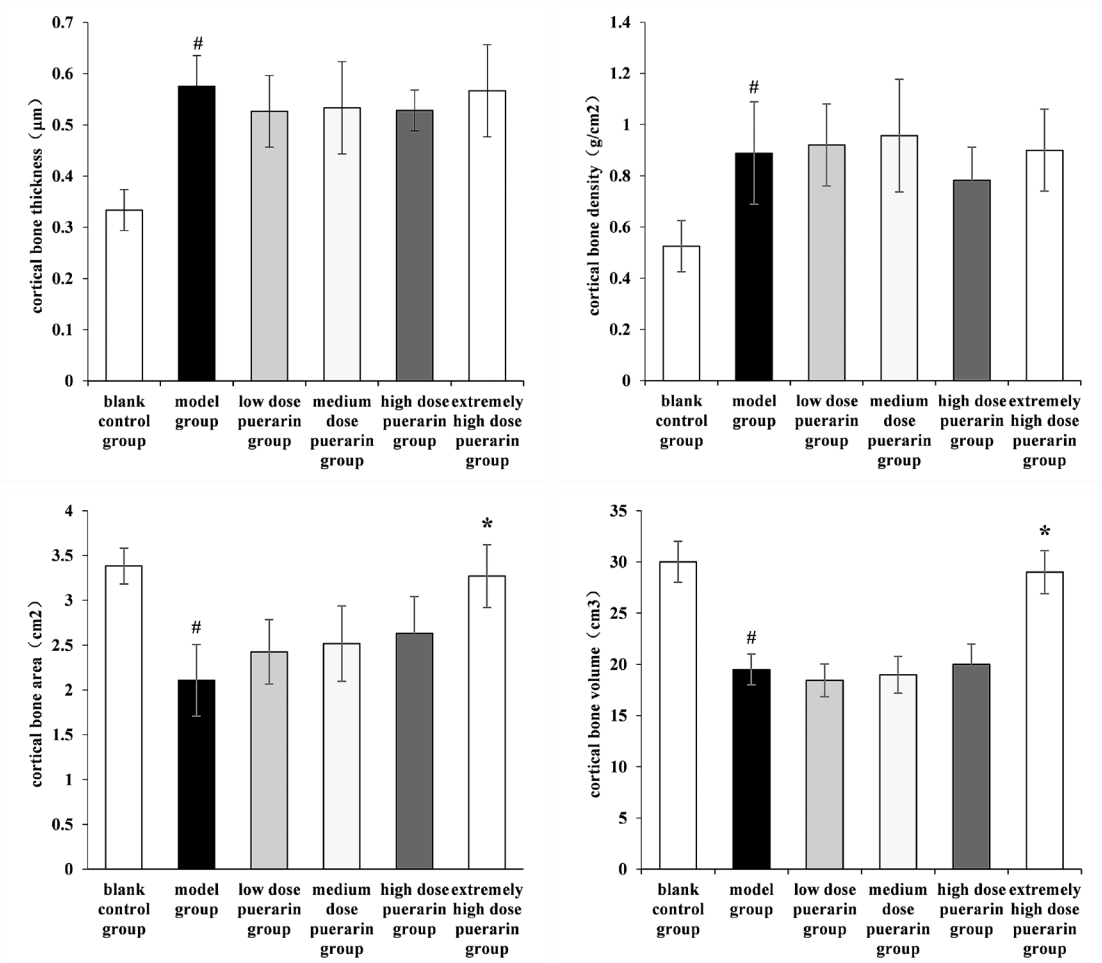

Figure 2: Changes of micro-CT parameters of cortical bone in each group. #Compared with the blank control group, the difference was statistically significant (p<0.05). *Compared with the model group, the difference was statistically significant (p<0.05).

Changes in cancellous Bone Mineral Density (BMD) of rats in each group

The cancellous Bone Mineral Density (BMD) of rats in the model group was higher than that in the blank control group (p<0.05). Within the puerarin groups, all groups were lower than the model group (p<0.05). The BMD of cancellous bone in the extremely high dose puerarin group was significantly higher than that in the other puerarin groups (p<0.05).

Changes in trabecular thickness of cancellous bone in each group

The trabecular thickness of cancellous bone in the model group was higher than that in the blank control group (p<0.05). Within the puerarin groups, all groups were lower than the model group (p<0.05). There is no obvious relationship between the puerarin groups and the concentration of puerarin.

Changes in the trabecular separation of cancellous bone in each group

The trabecular separation of cancellous bone in the model group was higher than that in the blank control group (p<0.05). Within the puerarin groups, the medium and high dose puerarin groups were still higher than the model group (p<0.05). However, the trabecular separation in the extremely high dose puerarin group was significantly decreased (p<0.05).

Changes of cancellous bone structure model index in each group

The cancellous bone structure model index of rats in the model group was higher than that in the blank control group (p<0.05). Within the puerarin groups, the index of cancellous bone structure in each puerarin group was still higher than that in the model group, but the difference was not statistically significant (p>0.05).

Changes in the Bone Surface area to Bone Volume ratio (BS/BV) of cancellous bone in each group

The BS/BV of cancellous bone in the model group was lower than that of the blank control group (p<0.05). Within the puerarin groups, each group was higher than the model group (p<0.05). There was no significant relationship between each puerarin group and the concentration of puerarin.

Changes in the trabecular number of cancellous bone in each group

The trabecular number of cancellous bones in the model group was lower than that in the blank control group ($p<0.05$). Within the puerarin groups, the low, medium and high dose puerarin groups were lower than the model group, but the difference was not statistically significant ($p>0.05$). Meanwhile, the extremely high dose puerarin group was higher than the model group ($p<0.05$).

Changes in the Bone Surface and Tissue Volume (BS/TV) of cancellous bone in each group

The BS/TV of cancellous bone in the model group was lower than that of the blank control group ($p<0.05$). Within the puerarin groups, the low, medium and high dose puerarin groups were still lower than the model group, but the difference was not statistically significant ($p>0.05$). The extremely high dose puerarin group was higher than the model group ($p<0.05$).

Changes of femurs histomorphology in each group (Figure 4).

Blank control group

The lamella of bone cortex was clearly structured and morphological structure was intact. The trabecular bones were thick and were arranged in a regular and orderly manner. The bone cells were clear and intact with normal morphology and hematopoietic stem cells were abundant.

Model group

Bone cortex became thicker; the trabecular numbers were increased with irregular arrangement. The width of the medullary cavity was smaller and the number of hematopoietic stem cells was higher.

Low and medium dose puerarin groups

The bone cortexes were relatively intact, the trabecular numbers were slightly reduced and the arrangement was irregular.

High and extremely high dose puerarin groups

The bone cortex became thinner, the lamella structure was not clear, the trabecular numbers were significantly reduced and the marrow cavity became smaller.

DISCUSSION

Skeletal fluorosis can cause long-term, persistent joint pain, limited range of motion, limb motor dysfunction, reduced work capacity, inability to care for oneself and even paralysis. Previous studies have found that excess fluorine can affect bone metabolism through microRNA[14,15] and related signalling pathways such as oxidative stress,[16] endoplasmic reticulum stress,[17] death receptors,[18] and Transforming Growth Factor β (TGF-β).[19] This study showed that: there are differences in the effects of fluorine on cancellous and cortical bone in rats. Fluorosis can affect the balance between bone anabolism and catabolism and has a dual regulatory effect on bone transformation. It can be manifested as: osteosclerosis, osteoporosis and cross-existence of sclerosis and osteoporosis, which is similar to the findings of Grobler SR and other scholars.[20] Our study found that fluoride level in blood was significantly higher in rats with fluorosis compared to normal rats. The BV/TV of cancellous bone, bone density and trabecular thickness of cancellous bone were elevated. The bone density and thickness of cortical bone were increased. These indicate that bone anabolism was greater than bone catabolism. However, the trabecular number, BS/BV, BS/TV and cortical bone area and volume decreased and the trabecular separation increased and the structural model index was closer to three. These indicate that the ratio of plate structure and rod structure in the composition structure of trabeculae was affected. The bone catabolism was

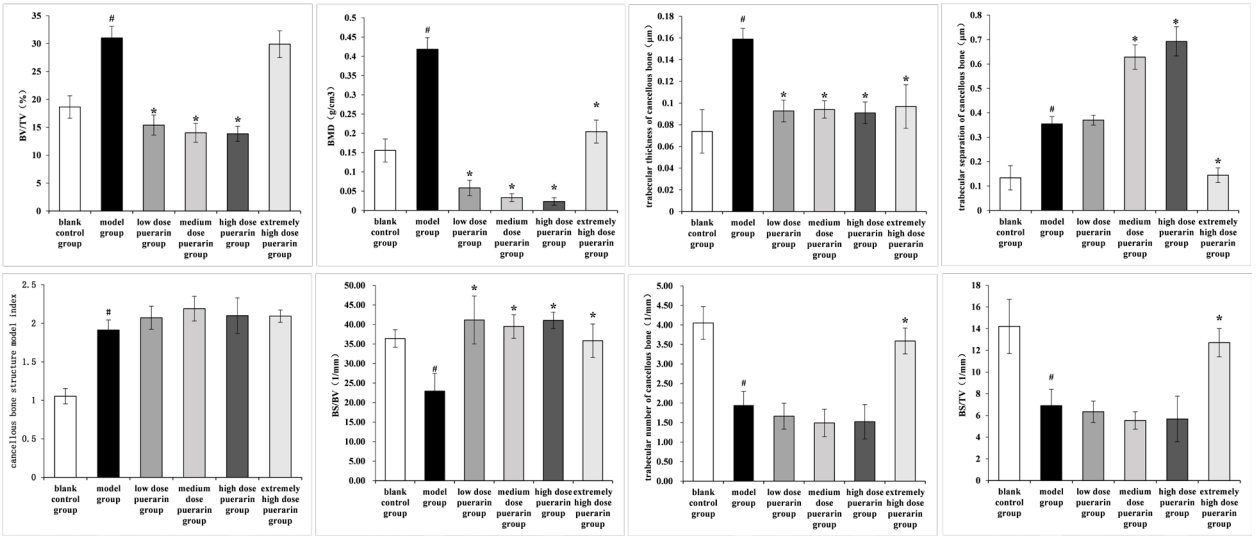

Figure 3: Changes in micro-CT parameters of cancellous bone in each group. #Compared with the blank control group, the difference was statistically significant ($p<0.05$). *Compared with the model group, the difference was statistically significant ($p<0.05$).

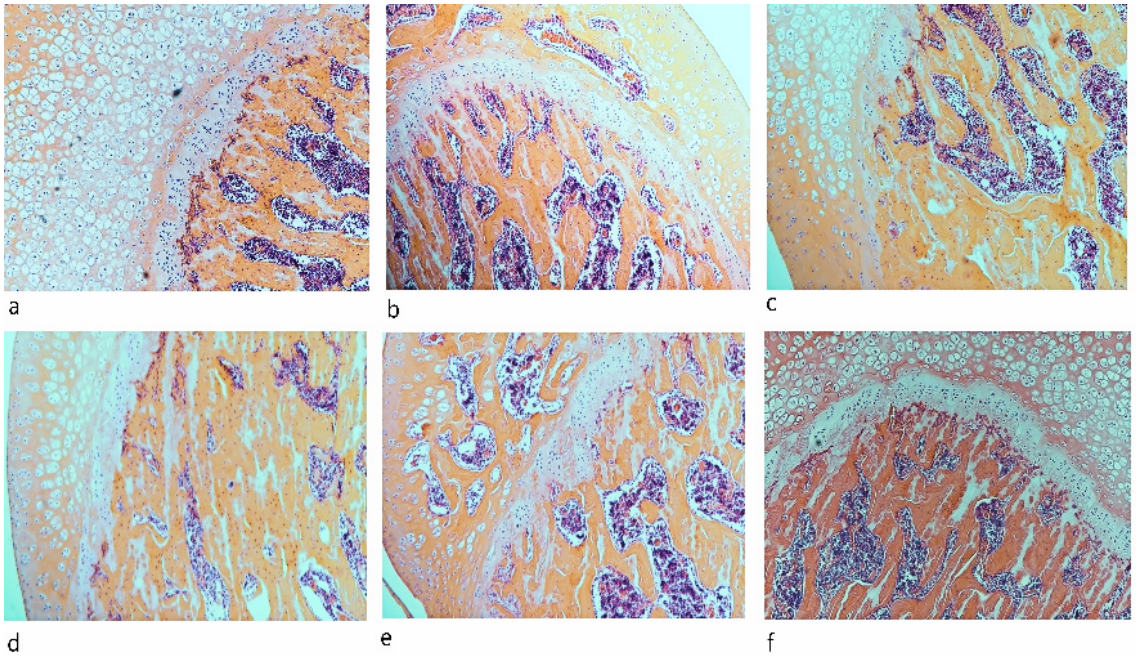

Figure 4: Changes of femurs histomorphology in each group. a: blank control group, b: model group, c: low dose group, d: medium dose group, e: high dose group, f: extremely high dose group.

greater than bone anabolism and fluorosis resulted in the main rod-like structure of rat trabeculae.

This study shows that puerarin can partially reverse the effect of fluorosis on bone metabolism balance in rats and has a certain therapeutic effect, but the therapeutic effects are dependent on specific bone regions and drug concentrations. The experiment showed that the improvement of cancellous bone indexes in the puerarin group was better than that in cortical bone. It demonstrates that the therapeutic effect of puerarin on fluorosis is dependent on specific bone regions. Meanwhile, cancellous bone is more affected, probably because the structure of puerarin is similar to that of estradiol, which can improve oxidative stress indexes and exert estrogen-like physiological effects through estrogen receptors and PI3K/AKT signaling pathways.[21,22] Moreover, the distribution of estrogen receptors at the tissue level was mainly related to cancellous bone and other related mechanisms. It was found that the blood fluoride concentration in high and extremely high dose puerarin groups decreased. Low, medium and high dose puerarin groups could make the cancellous bone volume fraction, cancellous bone mineral density, trabecular thickness, cancellous bone surface-volume ratio, cortical bone area and cortical bone thickness of fluorosis rats tend to normal rats. There were obvious differences between the extremely high dose puerarin group and other puerarin groups in the indexes of cancellous bone volume fraction, cancellous BMD, trabecular separation, trabecular number and bone surface and tissue volume of cancellous bone. It indicates that a drug concentration range is critical for puerarin to exert its therapeutic effects on bone metabolism. Puerarin concentrations lower than the lower end of this range has no effect. The concentration of puerarin within this range has therapeutic effects on bone metabolism, which was greater than that of fluorosis on the balance of osteogenesis and osteoclast. Drug concentration above the upper end of this range shows reduced therapeutic effect. The existence of this concentration range may be related to the effect of puerarin itself on bone metabolism and the metabolic effect of the body on puerarin and the specific mechanism needs further research.

In addition, this study found that for some cancellous bone micro-CT parameters, the extremely high dose puerarin group was significantly different from other puerarin groups and the improvement effect was shown for the first time in the extremely high dose puerarin group. But this feature was not seen in cortical bone. This may be related to the difference in the therapeutic effect of puerarin on cortical and cancellous bone. The specific concentration of puerarin in the treatment of fluorosis-poisoned rats needs further study.

By measuring fluoride level in blood, bone tissue H&E staining and micro-CT, the changes produced by the body in response to fluorosis can be detected early. As well as changes in histomorphology, bone density, trabecular number and other indicators. These results further improve the diagnostic criteria of fluorosis, while facilitating early detection, diagnosis and treatment of fluorosis.

In conclusion, puerarin can exert anti-skeletal fluorosis effects by affecting the balance of bone catabolism-anabolism and improving bone micro-CT parameters and bone tissue structure. However, this study is mainly conducted in animal experiments and there was a lack of clinical cases, lack of large samples and

multi-center controlled clinical trials. At the same time, the specific mechanism of puerarin affecting bone metabolism in the treatment of skeletal fluorosis is still unclear and further research is needed.

CONCLUSION

Fluoride has a dual regulatory effect on bone metabolism, with osteosclerosis and osteoporosis crossing each other. Puerarin regulates bone metabolism in fluorosis rats, reduces blood fluoride level and leads to improved micro-CT parameters and histomorphological structure of fluorosis bone. Therefore, it plays a therapeutic and osteoprotective role in anti-fluorosis. However, such therapeutic effects are bone region specific and dependent on a drug concentration. Puerarin has a more significant effect on cancellous bone, but the effects on cortical bone are weaker than that of cancellous bone. Puerarin concentration specifically within this range exerts obvious therapeutic effects.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

H&E staining: Hematoxylin and eosin staining; **TCM:** Traditional Chinese Medicine; **TRAF6:** Tumor necrosis factor receptor associated factor 6; **NF-*k*b:** Nuclear factor kappa-B; **MAPK:** Mitogen-activated protein kinases; **micro-CT:** Micro computed tomography; **ih:** Hypodermic injection **qd:** Quaque die; **SD:** Standard deviation; **BV:** Bone Volume; **TV:** Tissue Volume; **BMD:** Bone Mineral Density; **BS:** Bone Surface; **TGF-β:** Transforming Growth Factor; **β PI3K:** Phosphoinositide 3-kinase.

SUMMARY

Osteofluorosis is a global disease, which seriously affects the quality of life of patients. Signal pathways such as TGF-β play an important role in the occurrence and development of this disease. This study shows that puerarin has a certain therapeutic effect on fluorosis rats, but its effect on TGF-β and other signal pathways is not clear. The specific mechanism of puerarin's therapeutic effect and its therapeutic effect on clinical patients need further study.

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