# Prevention and treatment of osteoporosis caused by oestrogen shortage in rats

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**Abstract**: Through castrated rats osteoporosis models, this study aims to explore whether the intraperitoneal injection of *Scutellaria baicalensis* extract could prevent the occurrence of osteoporosis and slow down/reverse osteoporosis or not, and further explore the influence on BMMSCs extracted by *Scutellaria baicalensis* extract. *Scutellaria baicalensis* extracts on the prevention and treatment of oestrogen shortage and osteoporosis. After the screening of appropriate concentration, in the model of castrated female rats with osteoporosis, rats in prevent group were given intraperitoneal injection of *Scutellaria baicalensis* extract within 1 week after the castration, and the rats in treatment group were injected *Scutellaria baicalensis* extract at the abdominal cavity in two months of castration. It required 2 times per week, 12 weeks in total, Micro CT scanning was performed respectively at 6th and 12th week on one side of the femur to compare the differences in bone mass and bone trabecular quantity, in order to evaluate the prevention and treatment of *Scutellaria baicalensis* extract in osteoporosis. After intraperitoneal injection of *Scutellaria baicalensis* extract, Micro CT results showed the animal bone mass and bone trabecular quantity in prevention and treatment group were increased compared to control group, while, it only reduced bone loss and restored bone mass to a certain extent. In this study, the small molecular compound with higher content of flavonoids through leaching, extraction, enrichment method from Traditional Chinese medicine radix scutellariae and could be utilized on the prevention and treatment of osteoporosis caused by rats castration.

**Keywords**: *Scutellaria baicalensis* extract, osteoporosis, prevention, treatment.

## INTRODUCTION

Osteoporosis is a kind of whole body metabolic bone disease characterized by bone loss, loss of trabecular bone, bone cortex thins, destroyed bone microstructure, resulting in ostepsathyrosis increases and higher incidence of fracture (Gensens and Lems, 2011). At present, more than 200 million people have been beset by osteoporosis disease. Osteoporosis is divided into primary osteoporosis and secondary osteoporosis, and primary osteoporosis with a higher proportion, 85%~90% and it can be also divided into postmenopausal women by the lack of estrogen as a result of osteoporosis and age-related osteoporosis. Secondary osteoporosis is caused by a number of internal secretions, liver and kidney diseases (Sun *et al.,* 2014). Overall, female osteoporosis occurred in women after menopause estrogen sectretiondecline (Bian *et al.*, 2011). The osteoporosis will shows a series of clinical symptoms as the different course of the disease, such as pain, decrease bone length, fracture, weak respriatory function, and so on. The previous clinical commonly used to treat osteoporosis drugs mainly are estrogen replacement, bisphosphonates, thyroxine fragment, calcitonin, calcium and vitamin D. the first four medicine had side effect in different degrees, such as breast tumor, osteonecrosis, cardiovascular disease, etc. The last two drugs must be used at the same time, while, it still has limited efficacy (Zeng *et al.*, 2012). Therefore, it urgently needs a safe, effective osteoporosis treatment method (Zou *et al.*, 2014). The traditional Chinese medicine had a profound culture. People have also been exploring try to use Chinese medicine to treat osteoporosis, because of its emphasis on the concept of a entirety, to be able to mobilize internal cause, and ACTS on the body associated with multiple links. Existing research shows that the core of Chinese medicine in the treatment of osteoporosis is its natural polysaccharide composition (Tang *et al.*, 2010). *Scutellaria baicalensis* is a traditional Chinese medicine which widely used in China with a long history. It is rich in favonoids and more researched baicalin, baicalein, wogonoside, wogonin, *et al*. (Zhang *et al.*, 2006). In the past, the *Scutellaria baicalensis* pharmacological research focused on antiviral, anti-inflammatory, anti-tumor, antioxidant activity and cardiovascular protection, and less research on anti-osteoporosis.

Small molecule compounds are extracted from scutellaria root by technological process. The solid powder extraction is performed some physical property analysis, along with relative detection, such as its stability, security, toxicity, etc., in order to determine the extract is accord with the application of biological safety within the scope of topical there may be slight irritation, without toxic ingredients. This extraction was used for live animal models to explore its prevention and treatment in castrated rats with estrogen shortage

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***Material***

*Main kit instruments*

Θ-MEM medium (Gibco, USA)
Fetal bovine serum (Gibco, USA)
Glutamine (Sigma, USA)
Dexamethasone (Sigma, USA)
β-glycerinsodium phosphate (Sigma, USA)
Stretomycin (Sigma, USA)
Ascorbic acid (Sigma, USA)
MTT (Sigma, USA)
0.1% Pentobarbital sodium (Sigma USA)
0.25% trypsin (Sigma, USA)
Iodivolt (Tianjin Chemical Reagent Co., China)
75% ethanol (Tianjin Chemical Reagent Co., China)
Isopropyl alcohol (Xi'an Chemical Reagent Factory, China)
Dimethyl sulfoxide (Xi'an Chemical Reagent Factory, China)
SGB-E (Tissue Program Center of The Forth Military Medical University, China)
Culture dish (Falcon, USA)
96-hole culture plate (Falcon, USA)
Scanning electron microscope (Forma, USA)
Comosite optical microscope and photographic system (Olympas, Japan)
Micro CT (GE, Germany)
Carbon dioxide incubation box (Forma, USA)
Centrifuge (Falcon, USA)
Enzyme-linked immunoassay (BioTEK Instruments USA)
YJ-875 ultra-clean table (Zhengzhou purification equipment factory, China)

***Rats model***

The rats model were 6 weeks old female SD rats from Animal Experiment Center of The Forth Military Medical University, China.

***Methods***

*Experimental grouping*

This experiment explored the prevention and treatment of scutellariabaicalensis extract on osteoporosis caused by oestrogen shortage in rats. The experimental rats were randomly divided into two group: prevention group and treatment group, then each group was randomly divided into three sub-groups, 3 rats in each sub-group, which were named Sham control group (Sham group), OVX-scutellaria injection group (also named SGB-E group), and OVX control group (OVX group).

***Model building methods***

For osteoporosis research at present is mainly the animal osteoporosis model. This experiment used ovariotomy model to simulate the osteoporosis women lack of estrogen after postmenopausal period (Zeng *et al.*, 2012).

***SGB-E group and OVX group modeling methods***

1) 1% pentobarbital sodium was injected into the abdominal cavity under the dose of 3 mg/kg.
2) Then fixed rates with limbs and head.
3) Hair shaving with area larger than the scope of surgical incision.
4) Spread the sterilized sheet, iodine and cotton ball disinfection, and alcohol tampon deiodine.
5) Determined the position of surgical incision: approximately 1 cm distance to costal margin and 1 cm distance to the spine.
6) Longitudinal incision, with a length of about 1cm, incision the skin, fascia and muscle layer in turn.
7) Find a mass of adipose tissue, and the adipose tissue was clipped out gently, then found the ovarian tissue.
8) The blood pipe clamp was placed in the ovarian tissue, and the ovaries were cut off.
9) The check the safety of the ligament and return the remaining tissue to the abdominal cavity without hemorrhage.
10) Layered suture: used tweezers to lift the muscle layers with stitching the muscle layer, so as not to sew the tissue organs inside the abdominal cavity.
11) Gently wiped the wound with iodivolt cotton ball again.
12) After anesthesia, awaken rates were placed into the cage, with 50~55% humidity, 22$^{o}$C, could eat and drink by its self.

***Sham group modeling method***

Anesthesia, the shaving, disinfection, incision, stitching method etc., were all the same, the only difference was that ovaries were not removed, and removed the adipose tissue and then replaced the ovaries to abdominal cavity.

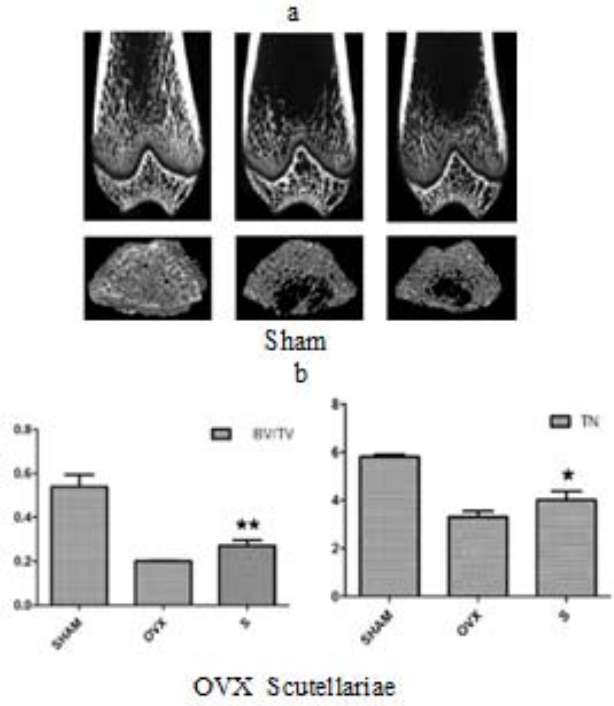
***Methods of administration***

*Cultivation of normal rats BMMSCs*

1) Kill the rats with dislocating neck. 2) Soak for around 5 minutes with 75% ethanol. 3) The femur and tibia of the rats were taken from the super clean bench. 4) Place the femur and tibia in a glass dish with sterilized PBS and quickly removed the muscle, fascia and other tissues on the ice bag; constantly replace the Petri dish with sterilized PBS wash and soak until the muscles and fascia were removed. 5) With a sterile scissors to cut tibia and the femur epiphyseal into two pieces, with sterile syringes, with α-MEM nutrient solution (10% PBS, 100μM/Lascorbic acid, 0.292mg/mL glutamine, 100 unit/ml penicillin/streptomycin, if not special instructions, below are specifications for the medium), rinse repeatedly marrow cavity until becoming white, the rinsed water are evenly and gently shaken. 6) Collect the cell suspension in the centrifuge tube, and after the balance, 800g, centrifuge 5 minutes. 7) Discard the supernatant liquid, then re-suspend the cultivation, and mix gently, and divide evenly and transferred to two sterile plastic Petri dishes, and add the infusion medium to 10ml. 8) In 30$^{o}$C, 5%$CO_2$ and saturated humidity, marker for $P_0$ generation, when cell fusion to 0%-9-%, 0.25% trypsin digest in batches, extend the cell in order for $P_1$ generation, $P_2$ generation.

***The SGB-E contained medium with concentration gradient***

50mg SGB-E powder is dissolved in 200ul DMSO, and a solution of 250mg/ml concentration, then the concentration is diluted to 1000ug/ml, 500ug/ml, 250ug/ml, 100ug/ml, 50ug/ml, 10ug/ml, 5ug/ml, 1ug/m.



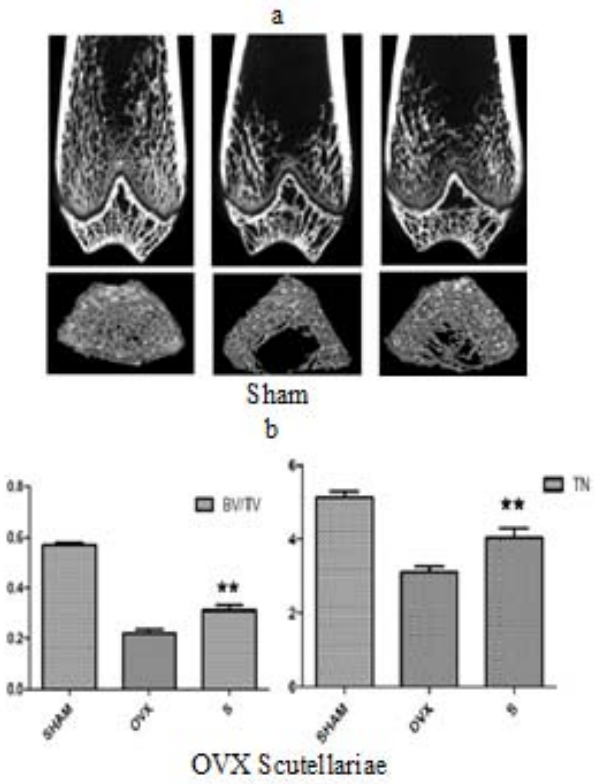

Pic a. Prevention group at the end of 6th week, Micro CT 3D image of reconstructed rat femoral head in Sham group, OVX group and SGB-E group.

Pic b. Prevention group at the end of 6th week, the rats femoral bone volume/total volume, bone trabecular quantity difference statistical analysis. *For comparison with OVX group, $p<0.05$, ** is compared with OVX group, $p<0.01$.

**Fig. 1**: Comparison of the morphological parameters of the epiphysis of the backbone in prevention group at the end of 6th week.

*Drug concentration of MTT colorimetric screening*

1) normal rats with a good growth status of $P_1$ generation BMMSCs, which are digested by trypsin during the logarithmic period and are blown into individual cells.2) after cell counting, the single-cell suspension was inoculated into 96-orifice plate with 7000/foramen, and then added to the end volume of 180ul, in α-MEM medium, and the edge hole was filled with sterile PBS.3) after the inoculation of 24 hours, the cell attached the wall, and the culture medium containing SGB-E should be replaced, and each hole should be added in accordance with the specified concentration of the design.4) 24hours per hole and 20ul MTT(5mg/ml), in 37°C, 5% $CO_2$ incubation and saturated humidity condition.5) to fully reflect the training of 4 hours, then end the cultivation, carefully absorb the cleaning, avoid the absorption of the bottom of the board, add DMSO 200ul, aboid the micro-quake about 10minutes, make the precipitation fully dissolved.6) Enzyme league immune detection, choose 490nm wavelength, test each hole absorbance value, under the same concentration of the cell average, to represent the average absorbance value of indirect cell volume, the abscissa denotes the different drug concentrations, absorbance values for the vertical curve plotting. 7) under the microscope, the growth state of the cells was observed and the optimum concentration was screened.




Pic a. Prevention group at the end of 12th week, Micro CT 3D image of reconstructed rat femoral head in Sham group, OVX group and SGB-E group

Pic b. Prevention group at the end of 6th week, the rats femoral bone volumn/total volume, bone trabecular quantity difference statistical analysis. ** is compared with OVX group, $p<0.01$.

**Fig. 2**: Comparison of the morphological parameters of the epiphysis of the backbone in prevention group at the end of 12th week.

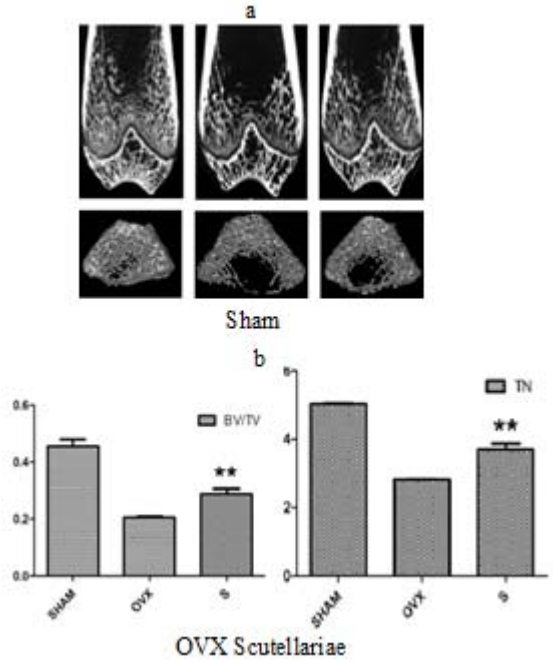
***Action mode***

The methods is intraperitoneal injection.

***Injection dose***

Sceening SGB-E BMMSCs suitable 30ug/ml, the drug concentration will be applied to cells when 3-5 times. This research choose 4 times. Thus, the injection concentration was 120ug/ml. For the convenience of weighing, set according tot he 120mg/kg compound injection, injection take SGB-E powder and dissolved in DMSO with 200mg/ml. SGB-E group, 0.6ml/kg is chosen

for intraperitoneal injection. The Sham group and OVX group, the rats were weighted, and DMSO intraperitoneal injection were chosen and according to 0.6ml/kg. Thus, SGB-E powder can be precipitated out, so it should be used before each injection.




Pic a. Treatment group at the end of 6th week, Micro CT 3D image of reconstructed rat femoral head in Sham group, OVX group and SGB-E group

Pic b. Treatment group at the end of 6th week, the rats femoral bone volume/total volume, bone trabecular quantity difference statistical analysis, ** is compared with OVX group, $p<0.01$.

**Fig. 3**: Comparison of the Micro CT morphological parameters of the epiphysis of the backbone in treatment group at the end of 6th week

***Injection cycle***

The drug intraperitoneal injection is performed on prevention group one week after castration, and is on treatment group within two months after the castration. The injections were respectively 12 weeks, and performed related indicators detection respectively at the end of 6th weeks end and the 12th weeks end.

***Drug frequency***

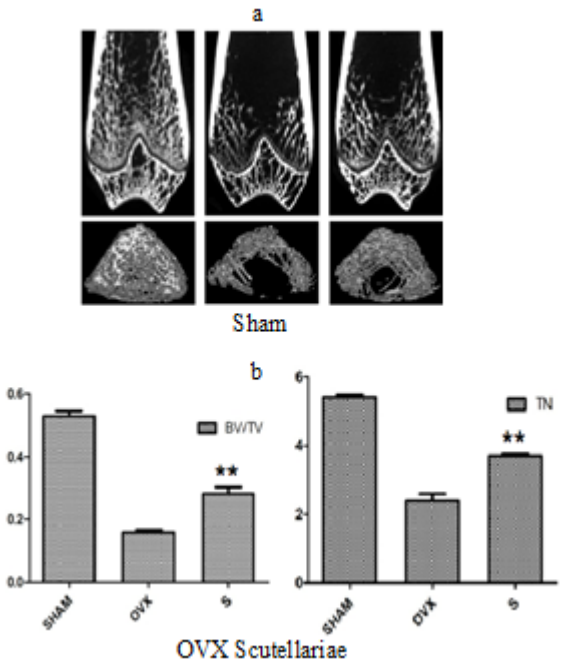
2 times per week.

***Test indexes***

Bone volume fraction, bone trabecular quantity, the femur of rats was scanned by Micro CT.

## STATISTICAL ANALYSIS

Bone volume ratio, bone trabecular quantity and other experimental data were expressed as mean± standard (x ± S) and SPSS 22.0 software for processing, SGB-E group and OVX group use independent sample T test to compare differences between groups.




Pic a. treatment group at the end of 12th week, Micro CT 3D image of reconstructed rat femoral head in Sham group, OVX group and SGB-E group

Pic b. treatment group at the end of 12th week, the rats femoral bone volume/total volume, bone trabecular quantity difference statistical analysis, ** is compared with OVX group, $p<0.01$.

**Fig. 4**: Comparison of the Micro CT morphological parameters of the epiphysis of the backbone in treatment group at the end of 12th week.

## RESULTS

***General conditions after injection***

After the operation, the rats in each group recovered from normal anaesthesia, with good spirit, activity, no obvious abnormality of appetite and behavior, and there is no infection or death. The rats weight was weighted before each injection, and the weight of each group increased normally. The rats slightly struggled during the injection, but no abnormal behaviour observed after injection. The appetite and the spirit is good, the skin was normal, and there was no infection and death.

***Analysis of Micor-CT results of rats in the prevention group***

Prevention group at the end of 6th group and 12th weeks end, and one side of femoria line were detected through CT determent detection. Femoral reconstruction showed bone cortex difference is no big, but the quantity of rabecular bone volume fraction and the trabecular bone etc in SGB-E group were higher than that in OVX group, with difference being statistically significance, but not as high as in Sham group. That is, after a period of

intraperitoneal injection, SGB-E can partially improve the loss ob bone loss caused by the castration of rats, but can not prevent the loss of bone mass.

***Analysis of Micro-CT results of rats in the treatment group***

In treatment group, one side femoral was respectively removed at the end of $6^{th}$ week and $12^{th}$ week and performed Micro CT detection, femoral reconstruction showed bone cortex difference was not big, while, the number of trabecular bone volume fraction and the trabecular bone etc in SGB-E group had increased compared to that in OVX group, with difference being statistical difference, but not as much as in Sham group. It can be further indicated that the castration model had caused rats osteoporosis in rats, SGB-E intraperitoneal injection for a period of time could partly recover the osteoporosis in rats, and increased bone mass and trabecular bone, while could not reverse the osteoporosis and return back normal level.

## DISCUSSION

In the first part of the experiment, this study had extracted molecules of solid powder mixture from scutellaria roots, and detected the proportion of its containing flavonoids as high as 8%~10%, and the extraction process was was also easier. Chinese medicine in the treatment of osteoporosis is the main active material natural polysaccharide components, mainly flavonoids (Zeng *et al.*, 2012). A lot of Chinese traditional medicine enriching with favonoids had a good curative effect. The existing literature reports including epimedium, eucommia bark, rhodiola, bone repair, etc (Yang *et al.*, 2013). While, scutellari baicalensis is is rich in flavonids, whether it had advantage in the application of anti-osteoporosis or not was reported very rare. This study explored the use of it. In this experiment, rats model with ovaries removed stimulated women with osteoporosis caused by oestrogen shortage (Zeng *et al.*, 2012), and observed the effects of scutellaria baicalensis extract on bone mass, bone volume ratio and trabecular bone, and provide animal experiment evidence for new medicine invention and usage.

From the experimental results, the long-term use of scutellaria baicalensis extract could improve the ovary osteoporosis of rats, for the happened osteoporosis, SGB-E could recover bone mass in a certain degree, but cannot return back to normal level, but had increased than control group without any treatment, the bone volume ratio and trabecular quantity also obviously increased, while, it had certain distance to normal level. SGB-E was a mixture, which contain baicalin, radix scutellariae and other compounds, the content was about 8%~10%, compared to monomer, the composition of complex compounds treatment effect was limited, of course, because the purity cannot be guaranteed, content ratio of flavonoid fractions was also different in different harvesting period of stulellariae baicalensis (Zhao *et al.*, 2012). In additional, this kind of small molecular extract basic cannot dissolve in normal saline, and could be dissolved in propylene glycol, but the solubility was not high. But if the test used in the injection concentration when they do with propylene glycol solvent precipitation of solid material and chose the good solubility dimethyl sulfoxide, dimethyl sulfoxide was toxic, however, it requires strict injection dosage and closely observe the reaction of animals after each injection, such as spirit, behavior, changes in activity.

This part of the experiment discussed the radix scutellariae whether can be used as preventative medicine of psteoporosis. Different to therapeutic effect part of the experiment, two months after the ovaries removal, rats already had osteoporosis then the intraperitoneal injection was performed for experimental treatment. In this experiment, the rats with ovaries removed and did not had ostoporosis and SGB-E were intraperitoneal injected, after a period of time, the same femur was removed for Micro-CT, it was discovered that, the SGB-E injected group had more bone mass, bone volume and trabecular bone than control group. It indicated the use of sculellariae against anti-ostoporosis. In the absence of oestrogen in the body and the osteoporosis has not formed, the timely use of scutellaria, as a preventive treatment can effectively prevent the loss of bone mass and the destruction of bone trabeculae in the disease of osteoporosis. However, it was only to a certain extent to slow the loss of bone mass and bone srabecular process, but it doesn't stop the trend, the bone mass and trabecular bone volume ratio of SGB-E bone mass injection group still exist the difference in normal group.

## CONCLUSION

This study confirmed the prevention and treatment of scutellaria baicalensis georgi in osteoporosis in the rats model experiment. It could improve the rats bone mass after menopause and bone trabecular quantity, etc, although it could not completely prevent the loss of bone mass and reverse the condition of osteoporosis. But *Scutellaria baicalensis* was available, safe and effective and could be long-term used traditional Chinese medicine, its medicinal value or to be further explored. This study laid the foundation of animal experiment to further develop more safe and effective drugs for prevention and treatment of osteoporosis in postmenopausal women. However, the mechanism of osteoporosis was still needed to be further studied and discussed.

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