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TITLE:

Preparation of Gushukang (GSK) Granules for In Vivo and In Vitro Experiments

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KEYWORDS:

Traditional Chinese medicine, Gushukang granule, drug-containing serum, serum pharmacology, osteoclastogenesis, ovariectomized mice, osteoblastogenesis, in vivo, in vitro

SUMMARY:

This article provides a detailed protocol for preparing a working solution of Gushukang granules for animal studies and GSK granule containing serum for in vitro experiments. This protocol can be applied to pharmacological investigations of herbal medicines as well as prescriptions for both in vivo and in vitro experiments.

ABSTRACT:

Traditional Chinese herbal medicine plays a role as an alternative method in treating many diseases, such as postmenopausal osteoporosis (POP). Gushukang (GSK) granules, a marketed prescription in China, have bone-protective effects in treating POP. Before administration to the body, one standard preparation procedure is commonly required, which aims to promote the release of active constituents from raw herbs and enhance the pharmacological effects as well as therapeutic outcomes. This study proposes a detailed protocol for using GSK granules in in vivo and in vitro experimental assays. The authors first provide a detailed protocol to calculate the animal-appropriate dosages of granules for in vivo investigation: weighing, dissolving, storage, and administration. Second, this article describes protocols for micro-CT scanning and

the measurement of bone parameters. Sample preparation, protocols for running the micro-CT machine and quantification of bone parameters were evaluated. Third, serum-containing GSK granules are prepared, and drug-containing serum is extracted for in vitro osteoclastogenesis and osteoblastogenesis. GSK granules were intragastrically administered twice per day to rats for three consecutive days. Blood was then collected, centrifuged, inactivated, and filtered. Finally, serum was diluted and used for performing osteoclastogenesis and osteoblastogenesis. The protocol described here can be considered a reference for pharmacological investigations of herbal prescription medicines, such as granules.

INTRODUCTION:

Traditional Chinese medicine (TCM) is one of the important complementary and alternative approaches to treat osteoporosis[1,2]. Water decoction is the basic and most commonly used form of the formula[3]. However, drawbacks also exist: bad taste, inconvenience for carriage, short shelf life and inconsistent protocols, limiting the uses as well as the curative effects. To avoid the above disadvantages as well as to pursue better effects, granules were developed and have been widely used[4]. Although many studies have explored the pharmacological mechanisms of one or more effective components from the granules[5–7], the exact mechanisms and underlying pharmacological processes are still difficult to identify. This is because too many effective components from one granule may simultaneously exert similar or opposed effects[4]. Therefore, the development of one standard protocol to prepare the granules before delivering to the body not only would have a great impact on the therapeutic outcomes but is also required for both in vivo and in vitro assays.

Moreover, the curative effects of granules in the clinic are difficult to confirm and exactly identify using in vitro or ex vivo studies, which creates a challenge because the pharmacological mechanisms are too complex. To resolve this, the preparation of drug-containing serum was first proposed by Tashino in 1980s[8]. From then on, numerous researchers applied drug-containing serum to herbal medicine, including granules[9–11]. Currently, the choice of drug-containing serum for in vitro investigations is regarded as one strategy that closely mimics physiological conditions.

Gushukang (GSK) granules were developed to treat postmenopausal osteoporosis (POP) based on clinical practice in light of the theory of TCM. GSK granules prevent bone loss in ovariectomized (OVX) mice in vivo, inhibit osteoclastic bone resorption, and stimulate osteoblastic bone formation[4]. Consequently, Li et al.[12] found that GSK granules have bone protective effects in OVX mice by enhancing the activities of calcium receptor to stimulate bone formation. To confirm the bone-protective effects as well as the pharmacological effects of GSK granules, the authors here provide a detailed procedure for preparation of working solutions and drug (GSK granule)-containing serum. Moreover, this article describes the application of GSK granules in an OVX-induced osteoporotic mouse model and GSK granule-containing serum for in vitro osteoclastogenesis/osteoblastogenesis.

GSK granules are composed of several herbs[13,14] and can be completely dissolved in saline easily. Therefore, saline serves as the vehicle. Sham-operated mice (Sham) and OVX mice were

administered the same volume of saline as the granule-administered mice. The equivalent doses of GSK granules for the mouse were calculated based on the Meeh-Rubner equation[15]. This equation not only has the advantage of obtaining safe dosages but also guarantees pharmacological effects[15]. The three dosages of GSK granules were generated as the follows: (1) GSKL: OVX + low-dose GSK granules, 2 g/kg/day. (2) GSKM: OVX + medium-dose GSK granules, 4 g/kg/day. (3) GSKH: OVX + high-dose GSK granules, 8 g/kg/day. Mice in the GSKL, GSKM and GSKH groups were intragastrically administered GSK granules. Calcium carbonate (600 mg/tablet) with vitamin D3 (125 international unit/tablet), for example, in a mature and marketed product (e.g., Caltrate [CAL]) for treating and preventing osteoporosis, was used as a positive control.

PROTOCOL:

All of the experimental procedures were performed with the approval of Institutional Animal Care and Use Committee of the Shanghai University of TCM (SZY201604005).

1. Preparation and administration of GSK working solution

1.1. Calculate the equivalent doses of GSK granules for mouse.

1.1.1. Calculate body surface based on the Meeh-Rubner equation[15]: body surface = K x (body weight$^{2/3}$)/1000, where the K values are 10.6 for human and 9.1 for mouse. Assuming a human body weight of 70 kg, then human body surface (m^2) = 10.6 x ($70^{2/3}$)/1000 = 1.8 m^2. Assuming a mouse body weight of 20 g (0.02 kg; e.g., 1 month old, female, C57/BL6), then mouse body surface (m^2) = 9.1 x ($0.02^{2/3}$)/1000 = 0.0067 m^2.

1.1.2. Based on the calculated body surface, calculate the body transform ratio for human and mouse. Human: 70 kg/1.8 m^2 = 39. Mouse: 0.02 kg/0.0067 m^2 = 3. GSK granule = 20 g/70 kg x 39/3 = 3.72 g/kg $\approx$ 4 g/kg.

1.1.3. Based on a body weight of 20 g per mouse, calculate the equivalent dosage for mouse: 4 g/kg x 0.02 kg = 0.08 g.

1.1.4. Calculate three equivalent doses of GSK granules based on 20 mice per group and an intervention lasting for 3 months (90 days): (1) GSKL (OVX + low-dose GSK granules [2 g/kg/day]): 0.04 g mouse/day x 20 mice x 90 days = 72 g. (2) GSKM (OVX + medium-dose GSK granules [4 g/kg/day]): 0.08 g mouse/day x 20 mice x 90 days = 144 g. (3) GSKH (OVX + high-dose GSK granules [8 g/kg/day]): 0.12 g mouse/day x 20 mice x 90 days = 216 g.

NOTE: Prepare an additional 20% of GSK granules in practice to offset the loss.

1.2. Calculate the volume of GSK granule per mouse based on the body weight[15]: e.g., volume (V) = 0.24 mL/mouse/day.

NOTE: The volume for intragastric administration for mouse is 0.12 mL/10 g.

1.3. Weigh 10-days' worth of three doses of GSK granules. Weigh 8 g, 16 g, and 24 g of GSK granules and serve as GSKL, GSKM, and GSKH, respectively.

1.4. Calculate the equivalent dose of calcium carbonate with vitamin D3 (CAL) for mouse based on the Meeh-Rubner equation[15] as in steps 1.1.1 and 1.1.2: CAL dosage = 2 tablet/70 kg x 39/3 = 0.372 tablet/kg $\approx$ 0.4 tablet/kg.

1.5. Based on a body weight of 20 g per mouse (e.g., 1 month old, female, C57/BL6), calculate the equivalent dosage of CAL for mouse: 0.4 tablet/kg x 0.02 kg = 0.008 tablet. Then calculate the equivalent dose of CAL based on 20 mice per group and an intervention lasting for 3 months (90 days): 0.008 tablet x 20 x 90 = 14.4 tablets. Weigh 10-days' worth of CAL (1.6 tablets).

1.5. Dissolution

1.5.1. Place 8 g of GSK granules into a 50 mL tube. Add 48 mL of saline and shake tube to completely dissolve.

NOTE: The standard for complete dissolution is the absence of sediment. Complete dissolution can be further confirmed if a gavage needle can draw up the working solution and then expel it smoothly.

1.5.2. Repeat step 1.5.1 with 16 g and 24 g of GSK granules.

1.5.3. Place 1.6 tablets (10-days' worth) of CAL into a 50 mL tube. Add 48 mL of saline and shake tube to completely dissolve.

NOTE: The working solutions can be stored at -4 °C and prepared every 10 days.

1.6. Intragastric administration

1.6.1. Grasp the back of the mouse (1 month old, female, C57/BL6) with the mouse facing forward and ensure that it stays firmly in that position. Keep the mouse calm for 2–3 min before administration.

NOTE: Ensure that the researcher can clearly see the front of the mouse. Wear gloves to prevent mouse bites, particularly for new researchers.

1.6.2. Place the gavage needle (size: #12, 40 mm) in the working solution of GSK granules and draw 0.24 mL of the working solution.

1.6.3. Put the gavage needle into the mouse through one side of its mouth until the gavage needle reaches the stomach.

NOTE: To confirm the gavage needle has reached the stomach: (1) The gavage needle encounters the feeling of resistance. Meanwhile, the mouse shows the action of swallowing before the gavage needle passes the physical narrowing of the esophagus. (2) Inject approximately 0.5 mL of the working solution into the mouse and wait for 1 min. If there is no solution coming out of the mouse, this means that the gavage needle has reached the stomach.

1.6.4. Inject the working solution of GSK granule (0.24 mL/mouse) into the stomach and then draw out the gavage needle. Return the mouse into its cage.

1.6.5. Repeat step 1.6.4 with the CAL solution and inject 0.24 mL of CAL solution per mouse.

NOTE: The volume of CAL solution is calculated as in step 1.2.

2. Micro-CT scanning

2.1. Tibia harvesting and preparation

2.1.1. Intraperitoneally anesthetize mouse with 300 mL/100 g of 80 mg/kg ketamine the day following the 90 day's intervention. Use a needle pinch of the toes to confirm whether the mouse is completely anesthetized. No response indicates successful anesthesia. Then kill the mouse with cervical dislocation.

2.1.2. Fix the mouse with both the arms and legs on foam with tacks.

2.1.3. Cut off the skin with scissors (size: 8.5 cm) and tweezers (size: 10 cm) of the legs from the proximal to the distal end and then harvest tibias.

2.1.4. Immediately put the tibias into 70% ethyl alcohol and wash for 3 times.

2.2. Wrap the left tibia of the mouse with sponge foam and put it into a sample tube (35 mm diameter, 140 mm length).

NOTE: The long axis of the specimen should be along with that of the sample tube. Ensure the proximal end of the tibia points upwards.

2.3. Running the micro-CT 80 scan machine

2.3.1. Start the micro-CT 80 scan machine at room temperature.

2.3.2. Set the sample tube into micro-CT 80 and start cross-section scanning with the following scanning parameters: pixel size 15.6 µm, tube voltage 55 kV, tube current 72 µA, integration time 200 ms, spatial resolution 15.6 µm, pixel resolution 15.6 µm, and image matrix 2048 x 2048.

NOTE: The cancellous bone is distinguished from the cortical bone by pre-scanning. The scan area of the tibia is defined as the cancellous bone area from 5 mm below the tibial plateau to the distal end.

2.4. Quantification of bone parameter

2.4.1. After completing cross-section scanning, obtain the images of the left tibias.

2.4.2. Set the density threshold to 245−1000. Use the micro-CT evaluation program V6.6 to measure the following bone parameters: bone mineral densities (BMD), bone volume over total volume (BV/TV), trabecular bone number (Tb.N), trabecular bone thickness (Tb.Th), as well as bone trabecular bone separation (Tb.Sp).

3. Preparation of blood serum for in vitro experiments

3.1. Calculation

3.1.1. Based on a rat body weight of 0.2 kg (1 month old, female, Sprague-Dawley), calculate the dosage of GSK granule: human dosage/day x body weight of human x K/body weight of rat = 20 g/70 kg/day x 70 kg x K (K = 0.018) /0.2 kg = 2 g/kg/day.

NOTE: K is the pharmacological transformation coefficient between human and mouse[15] (K = 0.018).

3.1.2. Repeat step 3.1.1 and calculate the following dosages.

3.1.2.1. Calculate dosage of GSKL: 10 g/70 kg/day x 70 kg x K/0.2 kg = 1 g/kg/day.

3.1.2.2. Calculate dosage of GSKM: 20 g/70 kg/day x 70 kg x K/0.2 kg = 2 g/kg/day.

3.1.2.3. Calculate dosage of GSKL: 40 g/70 kg/day x 70 kg x K/0.2 kg = 4 g/kg/day.

3.1.2.4. Calculate dosage of CAL: 2 tablet /70 kg/day x 70 kg x K/0.2 kg = 0.2 tablet/kg/day.

3.1.3. Calculate the total dosage of GSK granule and CAL.

3.1.3.1. Calculate the total dosage for GSKL: 1 g/kg/day x 0.2 kg x 6 rats x 3 days = 3.6 g.

3.1.3.2. Calculate the total dosage for GSKM: 2 g/kg/day x 0.2 kg x 6 rats x 3 days = 7.2 g.

3.1.3.3. Calculate the total dosage for GSKH: 4 g/kg/day x 0.2 kg x 6 rats x 3 days = 14.4 g.

3.1.3.4. Calculate the CAL dosage = 0.2 tablet/kg/day x 0.2 kg x 6 rats x 3 days = 0.72 tablet.

NOTE: A total of 10 mL of GSK granule-containing serum is needed to prepare 100 mL culture medium (20% GSK granule-containing serum). Each rat (6 rats/group) is expected to provide 1.5−2 mL of GSK granule-containing serum after centrifugation.

3.1.4. Calculate the volume of GSK granules applied per rat based on body weight[15]: e.g., volume (V) = 2 mL/rat/day.

NOTE: The volume for intragastric administration for rat is 0.1 mL/10 g.

3.2. Weigh 3-days' worth of three doses of GSK granules. Weigh 3.6 g, 7.2 g, and 14.4 g of GSK granules and serve as GSKL, GSKM, and GSKH, respectively. Weigh 0.72 tablet for the CAL group.

3.3. Place 7.2 g of GSK granules into a 50 mL tube. Add 36 mL of saline and shake tube to completely dissolve. Repeat this with 3.6 g and 14.4 g of GSK granules.

3.4. Repeat section 1.6 for intragastric administration with 2 mL of GSK working solution.

NOTE: Administer the same volume of saline (2 mL per rat) to prepare serum and serves as a blank control group for in vitro assays.

3.5. Preparation of the GSK-containing serum

3.5.1. Intraperitoneally anesthetize the rats with 300 mL/100 g of 80 mg/kg ketamine 1 h after the last administration of GSK granules. Use a needle pinch of the toes to confirm whether the rat is completely anesthetized. No response indicates successful anesthesia.

3.5.2. Expose the abdomen to the bottom of the thorax of rats using straight operating scissors after incising the skin and peritoneum.

NOTE: The surgical instrument must be sterilized at high temperatures and high pressures prior to use. The surgical area must be sterilized with 70% ethanol during blood collection.

3.5.3. Remove the connective tissue of the abdominal aorta with tissue paper to expose the vessel clearly.

3.5.4. Draw blood from the abdominal aorta using a 10 mL, 22 G syringe. Then remove the needle and transfer the blood to a 15 mL sterile tube. Usually, 6−8 mL of blood can be obtained from one rat.

NOTE: Each rat must be kept living when drawing blood. One indicator is that the abdominal aorta pulsates when the rat is alive. The rat is dead after blood draw.

3.5.5. Keep the tube upright at room temperature for 30−60 min until the blood is clotted in the

tube. Then centrifuge the tube at 500−600 x *g* for 20 min. Transfer all the supernatant (serum) from one group (6 rats) to one 50 mL sterile tube and shake to mix.

3.5.6. Inactivate the serum by incubating in a 56 °C water bath for 30 min. Filter the serum using a 0.22-µm-pore-size hydrophilic polyethersulfone syringe filter. Store at -80 °C for long-term usage (less than 1 year).

NOTE: The filtered serum can be used for in vitro osteoclastogenesis and osteoblastogenesis.

3.6. Application

3.6.1. In vitro osteoclastogenesis

3.6.1.1. Dilute the three dosages of the GSK-containing serum (GSKL, GSKM, GSKH) at the ratio of 1:4 with minimum Eagle's medium (α-MEM) containing L-glutamine, ribonucleosides, and deoxyribonucleosides.

NOTE: Ensure that the final concentration of GSK-containing serum for in vitro osteoclastogenesis and osteoblastogenesis is 20%.

3.6.1.2. Add the diluted GSK-containing serum (200 µL/well) from step 3.6.1.1 to bone marrow macrophages (BMMs) from 4−6 week old C57BL/6 mice for osteoclastogenesis and stimulate BMMs with macrophage colony-stimulating factor (M-CSF, 10 ng/mL) and receptor activator for nuclear factor-κB ligand (RANKL, 100 ng/mL) as previously described[2].

3.6.2. In vitro osteoblastogenesis

3.6.2.1. Repeat step 3.6.1.1.

3.6.2.2. Add the diluted GSK-containing serum (2 mL/well) to bone mesenchymal stem cells (BMSCs) from 4−6 week old C57BL/6 mice to generate osteoblast as previously described[16].

REPRESENTATIVE RESULTS:

Micro-CT scanning results indicated that the OVX mice showed significant bone loss compared to saline control mice (**Figure 1A**). The intervention (90 days) of GSK granules greatly increased the BMD, particularly in the GSKM group (**Figure 1B**). The bone structure parameters, such as BMD, BV/TV, Tb.N, and Tb.Th, were quantified. GSK granule treatments led to increased BMD, BV/TV, Tb.N and Tb.Th but decreased Tb.Sp (**Figure 1C**).

Tartrate resistant acid phosphatase (TRAP) staining showed an increase in the number of osteoclasts in OVX mice compared to control mice (**Figure 2A**). GSK granule treatments decreased TRAP-positive osteoclasts compared to the OVX group. These findings were confirmed by calculating the ratio of TRAP-positive area to trabecular bone surface (OCs/BS%) and the ratio of osteoclast number to bone area (OCs/mm^2). These quantitative results showed

a significant decrease in the number of osteoclasts in GSK groups compared to the OVX group (**Figure 2B,C**).

The GSK granule-containing serum was administered to bone marrow macrophages (BMMs) from 4−6 week old C57BL/6 mice to generate osteoclast and the number of osteoclasts was analyzed by TRAP staining. The results showed that GSK granule-containing serum decreased the number of TRAP-positive osteoclasts in GSK groups compared to the control group (**Figure 3A,B**).

Alkaline phosphatase (ALP) staining showed that GSK granule-medicated serum exerted stimulatory effects on osteoblastogenesis with MSCs from C57BL/6 mice. ALP staining showed that all three groups of GSK granule-medicated serum had increased the activity of ALP (**Figure 4A,B**) compared to the control group.

FIGURE LEGENDS:

Figure 1: GSK granule prevents bone loss in OVX-induced mice. (**A**) Mice were treated with GSK granules for 3 months and left tibias were harvested to perform micro-CT analysis. Representative three-dimensional (3D) reconstruction images of the trabecular bone of left tibias were shown. Scale bar = 0.5 mm. (**B**) Bone mineral density (BMD) was measured and quantified. (**C**) Bone parameters of left tibias, such as the trabecular bone number (Tb.N), bone volume over total volume (BV/TV), trabecular bone thickness (Tb.Th), and trabecular bone separation (Tb.Sp), related to the trabecular bone structure in all the groups were shown. GSKL, GSKM, and GSKH groups were compared with control (Con; sham+ saline) and the OVX group (n = 6, **P* < 0.05, versus control; **P* < 0.05, versus OVX). CAL: Calcium carbonate with vitamin D3.

Figure 2: GSK granules suppress the number of osteoclasts in OVX mice. (**A**) TRAP staining was performed on lumbar vertebra 3 (L3) after the GSK-treated mice were harvested. TRAP results from control (sham + saline), OVX (OVX + saline), CAL (OVX + Caltrate), GSKL (OVX + low dose GSK, 2 g/kg/day), GSKM (OVX + medium dose GSK, 4 g/kg/day), and GSKH (OVX + high dose GSK, 8 g/kg/day) were measured and analyzed. Scale bar = 100 μm (top images) or 50 μm (bottom images). (**B**) Quantification of osteoclast-covered surface over bone surface. (**C**) Osteoclast number. Values were expressed as mean ± standard error of the mean (SEM). **P* < 0.05, OVX versus control (Con); **P* < 0.05, the groups of CAL or GSKL/GSKM/GSKH versus the OVX group. All the assays were repeated with at least 3 mice.

Figure 3: GSK granule medicated-serum decreases osteoclastogenesis from bone marrow macrophages (BMMs). (**A**) BMMs from C57BL/6 mice (4−6 week old) were harvested, and cultured with M-CSF (10 ng/mL) and RANKL (100 ng/mL) (control), M-CSF and RANKL plus GSK, or CAL medicated serums. Osteoclastogenesis was assessed at day 4−6 by TRAP staining. Scale bar = 100 μm. (**B**) The number of osteoclasts was quantified. **P* < 0.05, the groups of GSKL/GSKM/GSKH versus control.

Figure 4: GSK granule-medicated serum promotes osteoblastogenesis. (**A**) Bone mesenchymal

stem cells (MSCs) from C57BL/6 mice (4−6 week old) were isolated and treated with GSK or CAL medicated serum. ALP staining was performed at day 7 to assess osteoblastogenesis. Scale bar = 100 μm. (**B**) The number of osteoblasts was quantified. *P < 0.05, the groups of CAL or GSKL/GSKM/GSKH versus control. All the assays were repeated with at least 3 mice or 3 times.

DISCUSSION:

Granules of TCM agents have become one of the common choices for formulations or prescriptions. GSK granules are composed of several herbal medicines based on clinical experiences or the TCM theory, and they exert better curative effects with fewer side effects[4]. Compared with water decoction, the granules have these advantages: good taste, convenience of delivery, long-term storage, standard protocol and consistent curative effects, as well as higher productivity. Currently, granules are one of the most commonly used pharmacy formations in TCM. However, the underlying mechanisms of pharmacological effects are still rarely studied. It is necessary to determine the critical steps in the preparation of granules to investigate the underlying pharmacological mechanisms.

In the past decades, one or more representative effective components from herbal medicine have usually been used to perform molecular assays and pharmacological outcomes due to their structural clarity. Many investigations have been performed to understand the curative effects with effective components from TCM herbs[5−7]. However, it is still difficult to mimic what will happen in a patient due to the complex environment, with many effective components working together. To resolve this problem, investigations with granules can explore pharmacological processes and are one choice in performing molecular studies compared to investigations with effective components.

Preparation of working solutions for granules contains four basic steps. The first step is dissolution. Granules are commonly mixed in saline after stirring to complete dissolve before further investigations. The quantity and property of granules affects the time and stability of granules during the dissolution process. The variation in dissolution time and stability depends on the herbs, due to their physical, chemical, and pharmacological characteristics[17]. Proper shaking and higher temperature usually promote and ensure complete dissolution of granules. The next step is concentration. The proper volume of gavage administration for animals is carefully considered and is determined by the volume of the working solution. Oral gavages at high concentrations, such as 10 mL/kg or more, can lead to several absorption-related problems. Rapid shunting of the working solution of granules into the duodenum is one common problem. Other problems, such as aspiration pneumonia, due to the passive reflux of the working solution of granules into the esophagus, are also observed[18]. Filtration is the third step, which helps the gavage needle to decrease in volume and prevents it from being clogged with herbal granules, as well as aids the digestion of granules. The fourth step is storage. The storage of working solutions of granules at low temperature (-20 °C) guarantees better outcomes.

The approach to calculate the animal bioequivalent dose is important to determine the effects of granules in the practice of TCM. The body weight (mg/kg) and species are commonly

considered. The body surface area (mg/m^2) is frequently used to perform the calculation[19] because the metabolic rate is related to the size of the individual animal. It is common sense to consider both body surface area and body weight, and therefore, the Meeh-Rubner equation was used, which is common in in vivo investigations in pharmacological studies[19,20].

Several kinds of animals are chosen for drug-containing serum preparation, such as rabbits, guinea pigs, rats, and mice. For in vivo investigations, the same species is preferred. Rats were selected because they not only provide more serum than mice but are also closer to mice in terms of evolution than other animals. The dose equivalent in vivo (rat: 7-fold of the equivalent dose) and clinical usage for patients are also recommended. Ten times the equivalent dose of the serum-provided animals is not commonly applied for in vivo investigations because treated cells or organs can lead to potential toxic reactions[21]. Methods such as injection, skin administration, and inhalation are the commonly used administration procedures in accordance with in vivo administrations. Oral administration by gavage needles was chosen in the present study. The granule administration frequency varies from once to twice per day, and the intervention period is 3−14 days. The final collection of blood is usually performed within 2 h after the last administration[22,23], when the concentration of granules in blood is relatively stable and at the peak level according to a previous study[24].

Drug-containing serum for in vitro assays before use is still controversial. Some researchers hold that it may result in unexpected reactions or side effects, which affect the results because of the presence of numerous active components in serum, including enzymes, hormones, antibodies, and complements[25]. However, some researchers hold the opposite opinion that active components might also be removed by the inactivation process[26]. To reach a middle ground, the serum in this study was inactivated before incubation in a water bath at 56 °C for 30 min. Moreover, a blank serum group was included, in which the serum from saline-treated animals is used, to rule out potential side effects. Therefore, drug-containing serum may serve as a potential method to investigate the pharmacological mechanisms or therapeutic outcomes.

Compared to similar methods, the protocol here has the following advantages: (1) Comprehensiveness. Both in vitro and in vivo methods are used simultaneously and can mutually support each other in pharmacological effects. (2) Suitability. Only mice and rats are included because they are closely related. (3) Repeatability. Both mice and rats are easily purchased at low cost, and the methods can be easily repeated. (4) Low cost. The OVX-induced osteoporotic mouse model is commonly used and reliable[27−28] and can be easily made or purchased. Therefore, the protocols here are more suitable compared to other methods for studying the pharmacological effects of herbal medicine, such as granules.

However, there are several limitations to the protocols with GSK granules. First, three dosages were administered, although the granules showed no significant dose-dependent tendency for in vivo investigations. The reason may be that dosages for animal studies are not sensitive and the intervention time is not sufficiently long, which requires further testing. Next, a longer period of intervention is needed for in vitro parallel investigations. The drug-containing serum,

although inactivated, may cause side effects after prolonged intervention. Third, only one volume of working solution is used for animal administration, which can be modified in future studies. Finally, animal species chosen for the preparation of drug-containing serum and the administration routines can be changed and will be tested in further studies.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

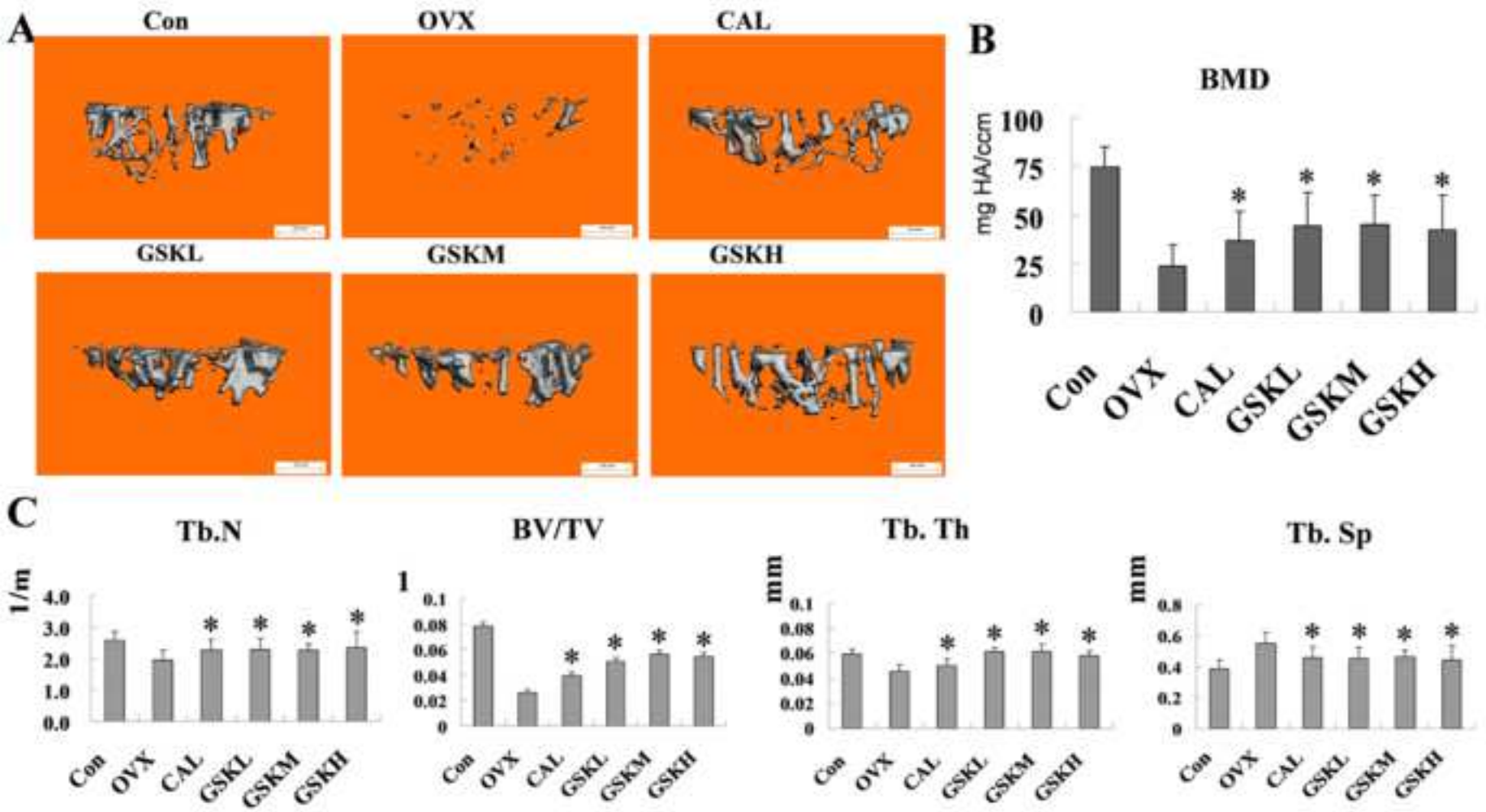


Figure 2

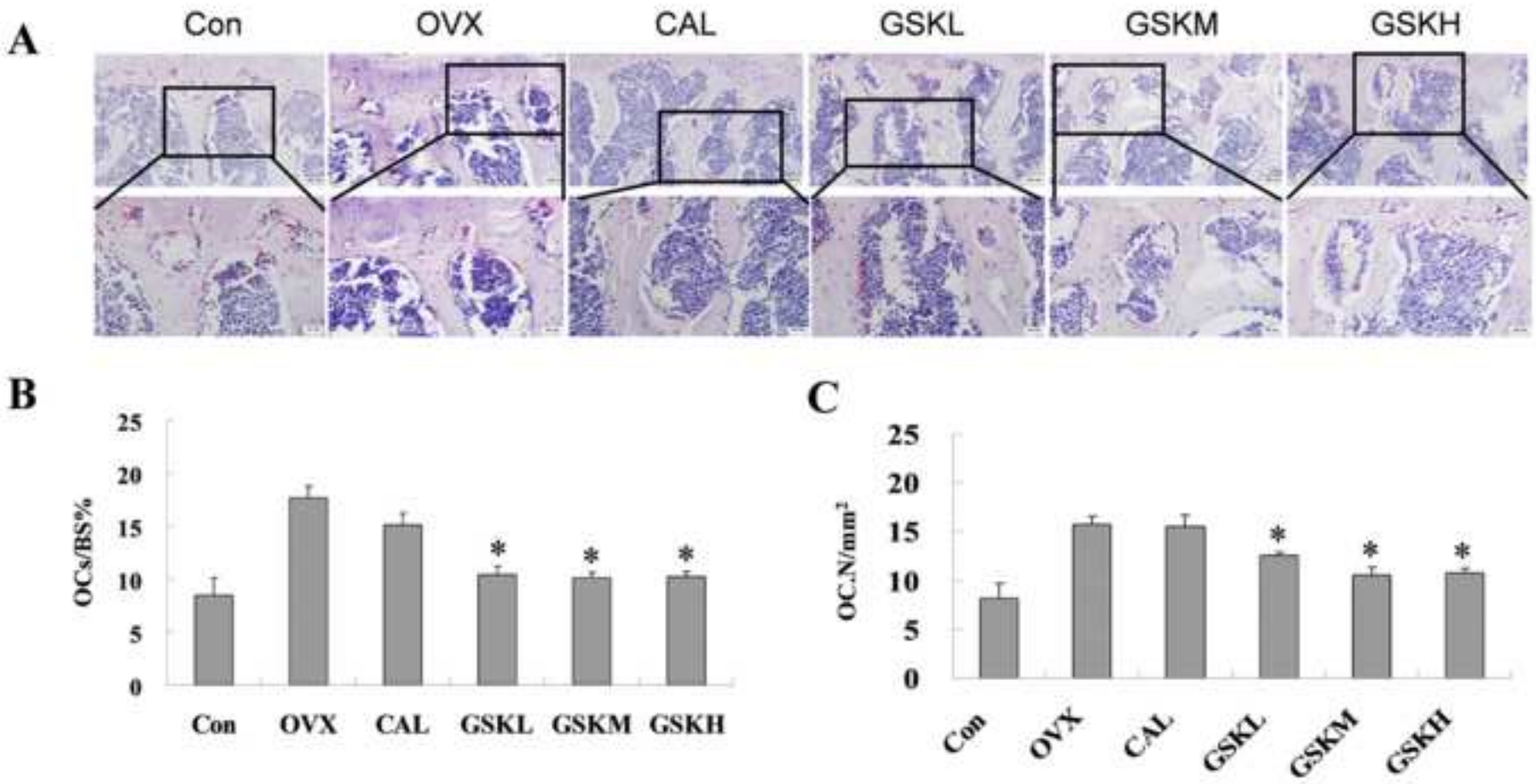


Figure 3

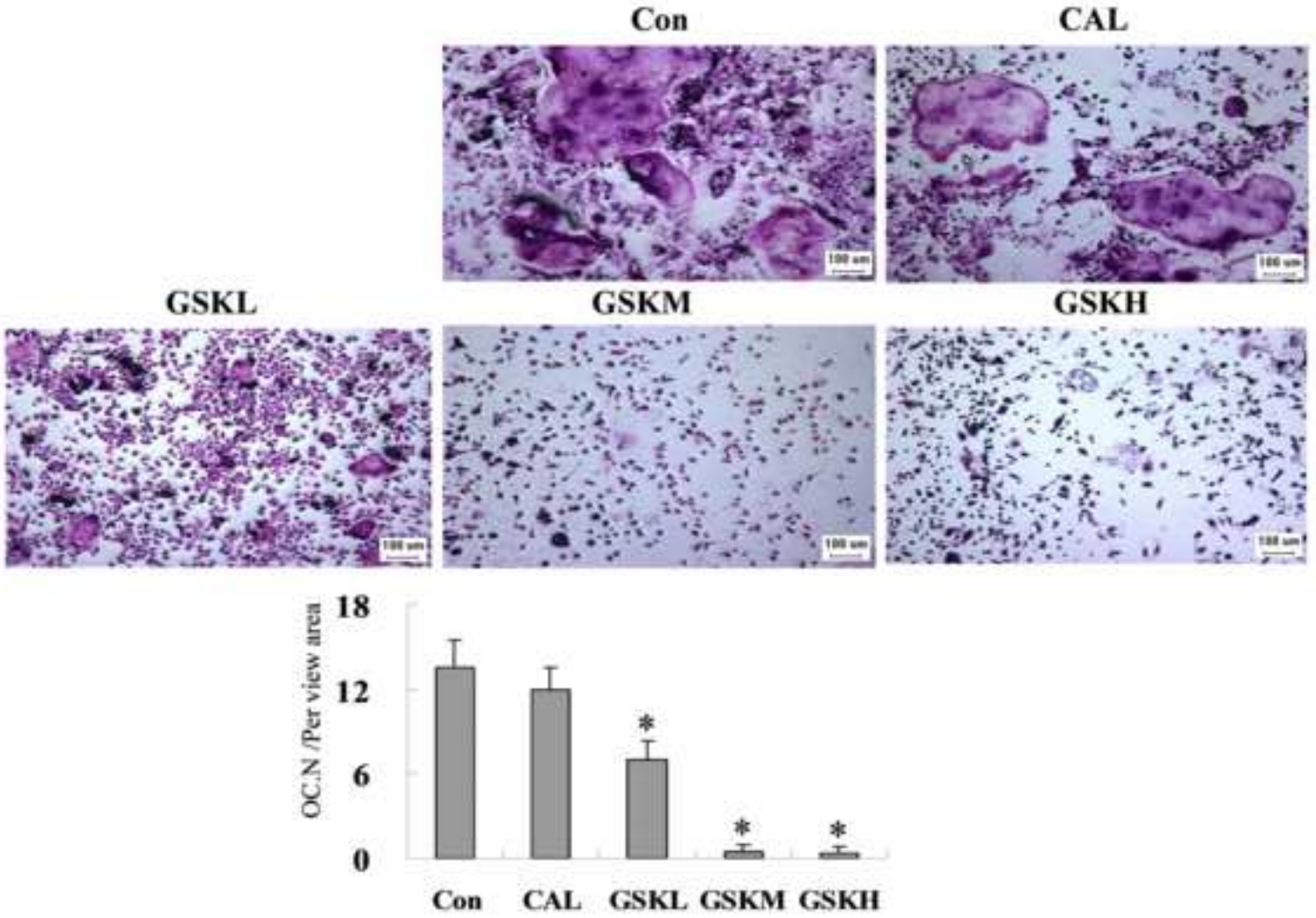
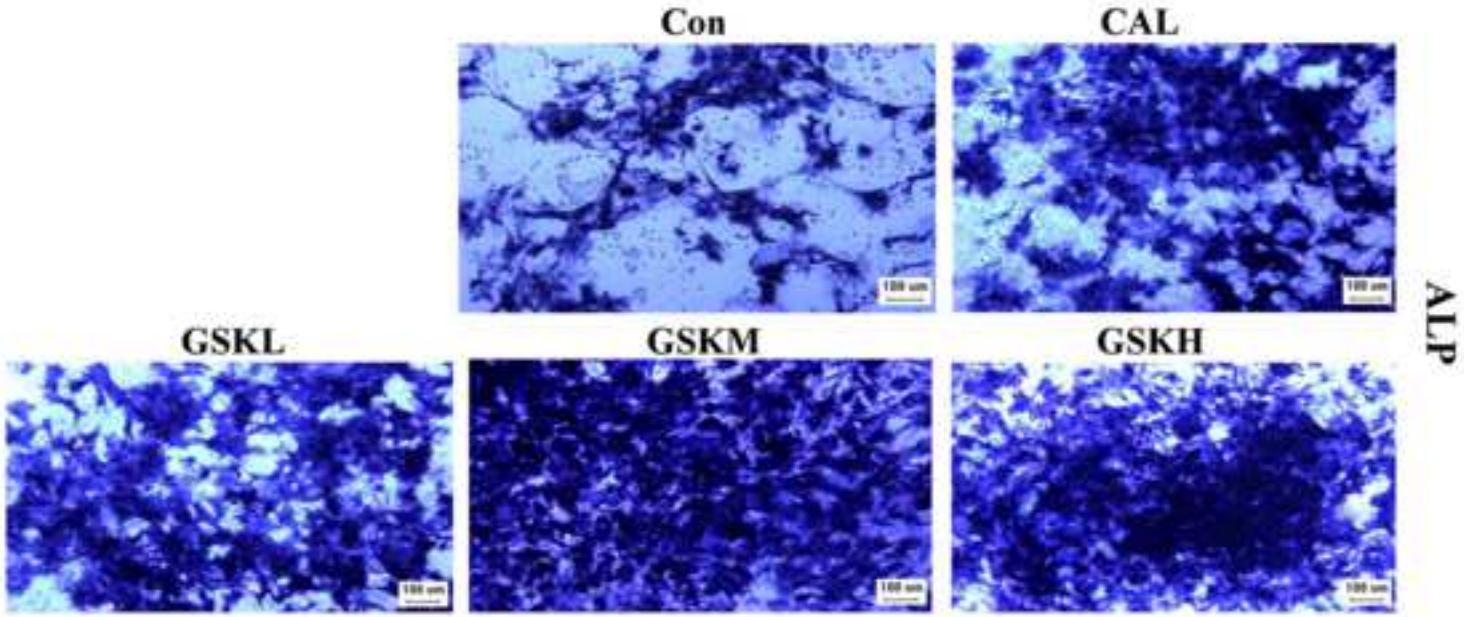
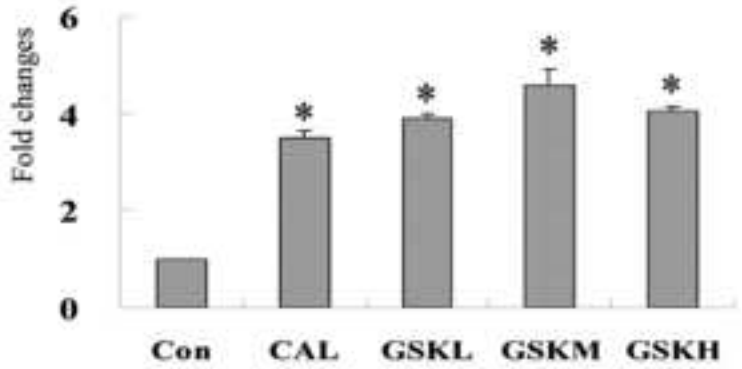


Figure 4

A



B



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
α-MEM	Hyclone laboratories	SH30265.018	For cell culture
β-Glycerophosphate	Sigma	G5422	Osteoblastogenesis
Caltrate (CAL)	Wyeth	L96625	Animal interventation
C57BL/6 mice	SLAC Laboratory Animal Co. Ltd.	Random	Ainimal preparation
Dexamethsome	Sigma	D4902	
Dimethyl sulfoxide	Sigma	D2438	Cell frozen
(EDTA)	Sangon Biotech	60-00-4	Samples treatmnet
Fetal bovine serum	Gibco	FL-24562	For cell culture
Gushukang granules	kangcheng companyin china	Z20003255	Herbal prescription
Light microscope	Olympus BX50	Olympus BX50	Images for osteoclastogenesis
L-Ascorbic acid 2-phosphate sequinagneium slat hyclrate	Sigma	A8960-5G	Osteoblastogenesis
Microscope	Leica	DMI300B	Osteocast and osteoblast imagine
M-CSF	Peprotech	AF-300-25-10	Osteoclastogenesis
Micro-CT	Scanco Medical AG	μCT80 radiograph microtomograph	Bone Structural analsysis
RANKL	Peprotech	11682-HNCHF	Osteoclastogenesis
Sprague Dawley	SLAC Laboratory Animal Co. Ltd.	Random	Blood serum collection
Tartrate-Resistant Acid Phosphate	Sigma-Aldrich	387A-1KT	TRAP staining

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Author(s): Yongjian Zhao, Qiang Wang, Shufeng Liu, Bing Shu, Yongjun Wang, Dongfeng Zhao

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Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

In the revised, all the changes are in red color through the manuscript.

1. Please revise lines 118-123, 145-160, 224-228, 233-235, 242-246, 247-253 to avoid previously published text.

Answer: Thanks for your reminding and the text has been edited.

2. The manuscript is written in poor English and should be edited to be clear and free from grammatic mistakes.

Answer: The manuscript has been edited by Elsevier and the certificate for Language Editing Services was also attached (Registration No. 331566771).

3. Please expand the Summary to briefly describe the applications of this protocol.

Answer: Thanks.

The authors added the applications of this protocol to the summary part (line 31-33).

4. Please define all abbreviations before use.

Answer: Thanks for the editorial's suggestions, and the authors defined the abbreviations through the manuscript.

5. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Answer: Thanks. The authors added animal care guidelines number (SZY201604005.Line 100-102) in the revised manuscript.

6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

Answer: We adjusted the numbering of the protocol to follow the JoVE Instructions and only one protocol was kept.

7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Answer: Thanks. The personal pronouns have been deleted.

8. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the

imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Answer:

Thanks. The protocol has been edited and “Note” was added if necessary.

9. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

Answer:

The authors have added more details to the protocols in the revised version. Meanwhile, the references were added to make the protocol more clear.

10. Lines 99-100: These numbers do not make sense based on definitions of these treatments in lines 83-84 [2g/kg/day (GSKL), 4g/kg/day (GSKM), 8g/kg/day(GSKH)]. Please double check and revise.

Answer:

Thanks and we have defined the abbreviations and checked the manuscript (Line 93-95 and line 123-126).

11. Lines 99-100, 129-130: What is considered to be proper position? Grasp the mice for how long?

Answer:

Thanks for your reminding and we have revised the manuscript to address this issue clearly (Line 159-161).

12. Line 112: How to make sure that the needle will reach the stomach?

Answer:

Thanks, and we have added more details the manuscript to address this step (Line

173-177).

13. Steps 1-4 of Protocol I and steps 1-5 of Protocol II are very similar except that the calculated rat-equivalent dose is different. Please consider combining these two parts.

Answer:

The authors agreed with the suggestions, and these two parts have been combined.

14. 6.1: Please mention how proper anesthetization is confirmed.

Answer:

The method for confirming proper anesthetization has been added (Line 251-252).

15. 6.2: Are the surgical area and surgical instrument sterilized prior to incision?

Answer:

Yes, the surgical area and surgical instrument need to be sterilized prior to incision (Line 257-259).

16. 6.5: Please specify the serum from how many animals are mixed.

Answer: There are 6 rats in each group, and serum of these rats is mixed together (Line 273).

17. 7.1: Please provide the composition of MEM medium.

Answer:

The authors have added the information of the composition of MEM medium (Minimum Eagle's medium) containing) in the revised version (Line 284).

18. Step 7: Please describe the actions being performed here in the imperative tense. Please provide sufficient details so that these steps can be replicated.

Answer: Thanks and we have rewritten these sentences in the revised version.

19. Please describe Micro-CT analysis in the protocol because such data are presented in the Representative Results.

Answer: The authors have added the Micro-CT analysis to the protocol (line 182-219).

20. Figure 1: Please describe panel C in the figure legend.

Answer: The figure legend of "Figure 1C" has been added (line 330).

21. Figures 2-4: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend.

Answer: Thanks for your reminding, and we have added the scale bars (line 329-330, line 341, line 349 and line 354-355).

22. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique.

Answer: Thanks for your reminding. We have checked the discussion part and added necessary information to the discussion part.

23. References: Please do not abbreviate journal titles.

Answer: Thanks and we have checked the references.

24. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.

Answer: Thanks and the authors have checked the alphabetical orders according to the Name of Material/ Equipment.

Reviewers' comments:

Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Reviewer #1:

Manuscript Summary:

The authors provided the detailed protocol of the calculation of the animal-appropriate dosages of the granule in vivo and prepared drug-contained serum to explore the pharmacological mechanisms of the treatment of GSK on POP. The results demonstrated that GSK may treat osteoporosis through prompting osteoblast and inhibiting osteoclast. The MS failed to reveal the related signaling pathway and the effect of GSK on osteoporosis has been presented in a published paper (J Bone Miner Metab. 2018 May 2.). Thus, the MS isn't of sufficient quality for publication.

Answer: Thanks, we have notice this article and shown in reference 12. Our manuscript is that we want to show one standard protocol for in vitro and in vivo experiment in stead of the molecular investigations. Our further research will focus on the molecular mechanism including related signaling pathway.

12. Li,X.L.,Wang,L.,Bi,X.L.,Chen,B.B, Zhang,Y. Gushukang exerts osteopreserve effects by regulating Vitamin D and Calcium metabolism in ovariectomized mice. Journal of bone Mineral metabolism. 2018. DOI: 10.1007/s00774-018-0924-1.

Major Concerns:

1. The topic of this MS is of significance to explore the pharmacological mechanisms of GSK. However, the similar topic has been published (J Bone Miner Metab. 2018 May 2.).

Answer: Thank you.

The purpose of our study is to show the detailed protocols of evaluating the effects of Traditional Chinese medicine (TCM) in ovariectomized induced bone loss in rat in stead of exploring the pharmacological mechanisms. The advantages of the protocol were to show the methods of both the *in vivo* and *in vitro* methods comparing to the published papers (J Bone Miner Metab. 2018 May 2, reference 12).

2. The MS failed to study the underlying pathway when explore the mechanisms of GSK, The authors are advised to further explore the possible molecular mechanisms.

Answer: Thanks for the reviewer's suggestions. The manuscript only shows the

methods for *in vivo* and *in vitro* investigations. We may further explore the possible molecular mechanisms in our ongoing work.

Minor Concerns:

1. The MS is full of textual and grammatical mistakes, which are enumerated as follows:

(1) Summary: "the'".

(2) Introduction: "The water decoration is the basic and most common used formation of the formula3, however,…", "the exactly mechanism", "dugs contained serum".

(3) Dissolving and concentration: "…and the Shake the tube to…".

Answer: Thanks. We have checked the manuscript to remove the textual and grammatical mistakes.

Reviewer #2:

Manuscript Summary:

The current MS described a protocol of how to prepare GSK granule and how to make medicated serum as well as how to determine the effect of GSK using in vivo and in vitro assays. The methods are reliable and repeatable. It also provides useful information on how to test the effects of the TCM compounds in cell experiments.

Minor Concerns:

(1) There are some spelling mistakes and grammar mistakes in the MS. Such as "line 60, the exactly mechanism";

Answer: Thanks and we have checked the manuscript to remove the spelling and grammatical mistakes.

(2) What is the meaning of "halt in the working solutions"?

Answer: The description has been edited and replace with: “draw up the exact volume of working solution” (Line 166-168)

(3) The rats should be included in the materials.

Answer: Thanks and the information of rats has been added to the materials.

Reviewer #3:

Manuscript Summary:

In this manuscript the authors endeavor to describe protocols of calculation of the animal appropriate dosages of Gushukang granule in vivo, and the application of the GSK-containing serum on osteoclast and osteoblast. Although this is a very important for administration of Chinese medicine, the method described lacked many details necessary to allow not only a scientific assessment of the representative results shown, but more importantly, allow it to be reproduced. Additionally, the manuscript requires extensive copy-editing, which made several areas unclear.

Answer: Thanks. The revised manuscript was edited by Elsevier and the certificate for Language Editing Services was also attached (Registration No. 331566771).

Major Concerns:

1. The Gushukang granules has been used to treat osteoporosis rats. The authors have to compare their procedure with other similar methods, clearly. Why this method is more suitable than other one?

Thanks. The manuscript described how to evaluate the effects of the GSK granules with *in vivo* and *in vitro* investigations. It also contained the methods of how to prepare compound medicine for both *in vivo* and *in vitro* assays. Therefore, the protocols in this MS are more comprehensive as well as suitable for readers to follow comparing to other similar methods (Line 426-433).

2. There was no description of preparing OVX mice, but it was shown in the results.

Answer: The OVX mice are commonly used animal model of osteoporosis, and are one matured method. Therefore, the reference has been added (refs 26-28, line 430-432).

3. There was no mention of μCT scanning protocols.

Answer: Thanks. We have added the μCT scanning protocols in the revised version (line 182-219).

4. What is the merit of the Meeh- Rubner equation? Please emphasis it.

Answer: Thanks. We have added necessary information to Meeh-Rubner equation

(line 108-109).

Body surface (m^2)= K×(Body Weight$^{2/3}$)/1000

5. Line 83-84: please specify how to calculate "the equivalent doses of 2g/kg/day(GSKL), 4g/kg/day (GSKM), 8g/kg/day(GSKH)". Please add Meeh-Rubner equation.

Answer: Thanks. We have added necessary information of how the equivalent doses of GSK were calculated (line 108-109 and 112-117).

6. Please clarify the reason for choosing caltrate as positive control, and how to calculate the dose of caltrate.

Answer: Thanks. Caltrate, which contains calcium and vitamin D_3, is one marketed product and severed as one supplementary agent in the managements of osteoporosis, which is easily to be purchased (line 137-142).

7. Line 166-174: Please describe the concentration of RANKL and M-CSF, and protocols of TRAP staining and ALP staining. concentration of RANKL and M-CSF,

Answer: Thanks and we have added the concentrations of RANKL and M-CSF as well as the protocols of TRAP stain and ALP staining (Line 290-291).

8. There was no description of histology methods, but results shown.

Answer: We have added the reference of histology methods which is commonly used.

9. Histological analysis of osteoblast is needed for the assertion the effect of GSK on OVX mice.

Answer: Thanks for your suggestion. What is mean of the reviewer's comments of that Histological analysis of osteoblast? Do the author want see the activity of osteoblast (bone formation) or the number of osteoblast?

1). For the activity of osteoblast (Bone formation), the hard tissue machine is needed for the histological analysis to assess the activity of osteoblast, however, there is no tissue machine in our lab and therefore we did not perform this assay to identify the effect of GSK granules on OVX mice.

2). For the numbers of osteoblast, the standard osteoblast in OVX mice is varied due to the size, and shape of osteoblast is difficult to identify and the results is subjective.

Therefore, this method is rarely seen and we did not carried out the assays for the assertion the effect of GSK granules on OVX mice.

10. In the in vitro assays, blank serum group should be added to exclude the effect of serum on osteoblast and osteoclast.

Answer: Yes, blank serum group has been added (line 244-46).

11. The authors should provide experimental evidences that drug concentration in blood is at its peak level at time point of 1- and 2- hour after the last administration.

Answer: The peak level at time point of 1- and 2- hour after the last administration have been confirmed by many previous investigations and it is commonly regulation, the authors have added reference (line 293-295, discussion page 9).

12. This manuscript is suitable for publication in JOVE. However, the english language is poor and should be enhance.

Answer: Thanks. The language manuscript has been edited by Language Editing Services (Registration No. 331566771).

Reviewer #4:

This article describes the protocols for the preparation of Gushukang (GSK) granule for in vivo and in vitro experiments. Bone protective effects of GSK granule on bone mineral densities of OVX mice have been already reported in several papers. Although authors suggested and discussed the protocols for the preparation of GSK granules, it is not clear this protocol is unique compared to other herbs used in vivo experiments. Therefore, I could not find any novel results in this manuscript

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To whom it may concern

The paper "Preparation of Gushukang (GSK) granules for in vivo and in vitro experiments" by Yongjian Zhao, Qiang Wang, Shufen Liu, Yongjun Wang, Bing Shu, Dongfeng Zhao was edited by Elsevier Language Editing Services.

Kind regards,

Elsevier Webshop Support

Response to editorial comments:

A1: Based on the numbers for 90 days in step 1.1.4, 10-days's values corresponding to 8, 16, and 24. Please double check.

Response: Thanks. We check the values and the 10-days's values are corrected and the values are 8, 16, and 24.

A2: Please describe how to obtain this value. It is unclear.

Response: we added information on line 138-139 and 1.4.1-1.4.2 to show how to obtain this value.

A3: Please describe how to obtain this value. It is unclear.

Response: The information added on line 138-139 and 1.4.1-1.4.2 to make this more clearly.

A4: Please move this to section 1.5 (i.e., add as 1.5.3) which describes how to prepare CAL solution.

Response: We move this part to 1.5.3, aiming to make this clearer.

A5: Inject?

Response: Yes, the word put should b replaced with inject.

A6: Comes out from what?

Response: The sentence should be: come out from the mouse.

A7: Please add step 1.6.5 to describe the injection of Caltrate solution.

Response: Yes, 1.6.5 was added.

A8: Is this done immediately after step 1.6.

Response: This is done at the next day after the 90 day's intervention.

A9: Is this done immediately after step 1.6.4?

Response: This is done at the next day after the 90 day's intervention.

A10: Please specify surgical tools used. Is the tibia washed?

Response: Thanks. Please see 2.1.3 and 2.1.4 to address these questions.

"2.1.3. Cut off the skin with scissor (8.5 cm) and tweezers (10 cm) of the legs from the proximal to the distal end and then harvest tibia.

2.1.4. immediately put the tibia into 70% ethyl alcohol and wash for 3 times."

A11: It is unclear how these values are obtained: 1/60, 90/100, 60. Please explain.

Response: the calculation has been updated in 3.1.1to 3.1.3 (Lines 237-250) to make the calculation clearer.

A12: Should 1/3 appear as superscript (exponent)?

Response: the calculation has been updated in 3.1.1to 3.1.3 (Lines 237-250) to make the calculation clearer.

A13: What is this value used for? The following calculation is based on 2 g/kg/day.

Response: the calculation has been updated in 3.1.1 to 3.1.3 (Lines 237-250) to make the calculation clearer.

A14-16: How to obtain?

Response: The exact calculation has been added from 3.1.1 to 3.1.3 (Lines 237-250).

A17: These steps are essentially the same as step 1.3 and 1.5 for mice. Then what is the point of the calculations in the previous steps for rats? Please explain.

Response: the calculation has been updated in 3.1.1to 3.1.3 (Lines 237-250) to make the calculation clearer. Reference！！！

A18: In section 1.6, 24 mL of working solution is injected, not 2 mL. Please clarify.

Response: the calculation has been updated. The averages volume is 2 mL per mouse and the total volume (6 rats) is 12 mL.

A19: 2 mL or 24 mL?

Response: The averages volume is 2 mL per mouse and the total volume (6 rats) is 12 mL.

A20: Is the blood transferred from the syringe to a tube?

Response: Yes, we transfer the blood with syringe (10 mL) to a tube (3.5.5).

A21: What is the GSK-containing serum used for? It is not mentioned in the protocol.

Response: the GSK-containing serum used for in vitro experiments and the “3.6. Application”: *In vitro* osteoblastogenesis and *in vitro* osteoclastogenesis

“3. Preparation of blood serum for in vitro experiments”.

A22: Do you mean diluted GSK-containing serum?

Response: Yes, GSK-containing serum should be diluted with α-MEM before performing in vitro experiments, such as In vitro osteoblastogenesis and in vitro osteoclastogenesis

A23-24: What are these and how are they obtained? from 4-6 week old C57BL/6 mice?

Response: This protocol is one mature method that harvest bone marrow macrophages from 4-6 week old C57BL/6 mice as shown in reference 2.

A25: Micro-CT scanning is done immediately after the GSK injection. The editor is not convinced that the medicine takes effect immediately. Please explain.

Response: Micro-CT scanning is done after the finish of the 90 day's intervention as described in 2.1.1", not on immediately after the last injection.

"2.1.1. Intraperitoneally anesthetize mice with 300 mL/100 g of 80 mg/kg ketamine at the next day of after the 90 day's intervention."

A26: These experiments are done on rats, not on mice, based on section 3 of the protocol, correct?

Response: No, these experiments are done on mice.

The effects of GSK granules on osteoclast were identified by TRAP staining. TRAP staining was performed by both in vivo experiments (OVX mice, Figure 2) and in vitro experiments (osteoclastogenesis, Figure 3). The results in Figure 2 aiming to show the inhibitory effects of

GSK granules on OVX mice while the results in Figure 3 (section 3 of the protocol) show the effects of GSK-containing serum (prepared by rats) on osteoclastogenesis with BMMs from mice (4-6 week old C57BL/6)