

Original Article

The effect of Migu capsule on osteoporotic fracture healing: a micro-computed tomography and biomechanical study in a mouse model

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Received March 17, 2017; Accepted April 21, 2017; Epub June 1, 2017; Published June 15, 2017

Abstract: Migu Capsule (MG), an oral Chinese herbal preparation, is made with the water extract of Bushenfang (BSF). BSF is a traditional Chinese herbal formula which was used for prevention and cure degenerative skeletal disease. Prior studies have reported MG could shorten the healing duration of osteoporotic fracture in patients. The present study aimed to investigate the effects of MG on bone healing in a mouse model of osteoporotic fracture. Thirty-six C57BL/6J mice (12 mice in each group) were randomly divided into three groups. Mice in Migu Capsule intervention group (MG group) and ovariectomized control group (OVXC group) received ovariectomy operation. The sham operated control group (SOC group) received a sham operation. Three weeks after the first surgery operation, each mouse withstood right femur osteotomy and internal fixation. SOC and OVXC groups were given 0.9% normal saline which acts as vehicle. MG group were given the mixture of 0.9% normal saline and MG. All the treatments were given via oral gavages once a day for 42 days. Mice were euthanized at a time point of 42 days post femur osteotomy. The results indicated that the SOC and MG groups exhibited a significantly lower mean value of TV than the OVXC group ($P < 0.05$). As to the vBMD, the SOC and MG groups showed a significantly higher mean value ($P < 0.05$). Regarding the maximum load, the SOC and MG groups exhibited obviously higher values than the OVXC ($P < 0.05$). In summary, the results shown here demonstrated that bone healing in a postmenopausal osteoporotic mouse model was significantly improved via treatment with MG as revealed by micro-CT and biomechanical methods. Further studies are needed to clarify the underlying mechanism of action of MG on bone healing.

Keywords: Bone, healing, osteoporosis, mouse

Introduction

Osteoporosis (OP) is considered to be the most frequent multifactorial systemic osteometabolic disease in humans, especially the geriatric and postmenopausal female. The disease is a systemic osteometabolic disorder characterized with substantial loss of bone mass and architectural microdamage of cancellous bone, both of which are associated with weakened bone strength and increased bone fracture risk, particularly of the distal forearm, proximal femur and vertebral body of postmenopausal female. According to a global investigation of osteoporosis among postmenopausal female,

the proportion of incident hip fractures among incident major fractures increased more than five-fold with age, from 6.6% among women 55 to 59 years of age to 34% among those ≥ 85 years of age [1]. As a result of the global aged tendency of population, medical and socioeconomic impact of osteoporosis and fragility fractures due to osteoporosis is increasing further. In addition to the increasing incidence of fragility fractures, it is also associated with an increased rate of orthopaedic implant failure and hinders motor functional recovery [2]. Moreover, in vivo animal studies also have testified that osteoporotic bones suffer prolonged and impaired healing process [3]. Now, much

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attention has been paid to prevention of fragility fractures due to osteoporosis, however, relatively less attention were paid to the healing process of osteoporotic fracture. Furthermore, treatment of osteoporosis, either by antiresorptive medication or anabolic agents could improve the outcome of osteoporosis, but their effect on the osteoporotic fracture healing process is not fully understood as well [4-6].

The bone tissue healing process is a particular form of wound tissue healing. According to research findings of Cruess and Simmons [7, 8], the conventional process of bone healing can be divided into three different phases: I, the reactive phase, including trauma, haematoma and inflammation whereby the initial inflammation is regarded as an initiation factor of bone tissue healing [9]; II, the reparative phase, characters of this phase are callus formation and lamellar bone deposition, and III, the remodeling phase, recreating the original bone contour and marrow cavity. Although the healing phases in histological aspects are well known, the molecular and cellular procedures of bone healing are still largely unknown. And then, osteoporotic bone fractures healing process is further complicated.

In consideration of these complexities and ethical issues, experimental animals are deemed to be appropriate to study the effects of drugs on the osteoporotic bone healing [10]. Osteoporosis due to estrogen withdrawal following menopause is labelled as primary osteoporosis Type I (Postmenopausal osteoporosis). Ovariectomized (OVX) mice may serve as an appropriate model for postmenopausal osteoporosis [11, 12]. Moreover, mice are also widely applied in research of fracture healing, and considered as the ideal model animal for bone regeneration study [13-16].

Biomechanical in vitro testing has been applied extensively in bone healing studies and is deemed the golden standard of healing evaluation since it quantitative detect stiffness and strength of a material, two structurally dependent parameters that indicate whether a material or bone easily fails or not [13]. Patterns of biomechanical testing such as three-point bending [17], four-point bending [18], and torsion [19] have each been utilized in vitro testing. It was well known that bone and callus biomechanical properties are determined by a

combination of tissue material properties and bone architecture [20]. In recent decade, micro-computed tomography (micro-CT) were applied extensively to quantitative assess bone trabecular architecture in human and animal bone tissue [21], also has been verified to be a reliable and feasible technique of evaluating the mice bone callus [13].

Traditional Chinese medicine has been extensive applied in healthcare in East Asia for thousands years. In the recent decades, Chinese herbal formulas have been attracting more and more attention for their Clinical effectiveness. Migu Capsule (MG), an oral Chinese herbal preparation, was awarded china invention patent in 2008. MG is made with the water extract of Bushenfang (BSF). BSF is a traditional Chinese herbal formula which was used for prevention and cure degenerative skeletal disease for decades, its main ingredients include of seven component herbs: Yinyanghuo (*Epimedium rhizomatosum* Stearn), Heshouwu (*Polygonum multiflorum* Thunb), Huangqi (*Astragalus mongholicus* Bunge), Shihu (*Dendrobium nobile* Lindl), Roucongrong (*Cistanche deserticola* Y.C.Ma), Gusuibu (*Davallia mariesii* T. Moore ex Baker), and Juhua (*Chrysanthemum morifolium* Ramat). Previous study have indicated MG could enhance bone mineral density and bone biomechanical properties in osteoporotic rats, and improve bone mineral density in the femoral neck and vertebrae without significant side effects in woman with postmenopausal osteoporosis [22, 23]. Further clinic research showed MG could shorten the healing duration of osteoporotic fracture in senile patients [24]. MG's potent multiple functions and long history without adverse health effects make it a possible substitute for therapeutic agent for osteoporotic fracture.

The present study aimed to investigate the effects of MG on bone healing in a mouse model of osteoporotic fracture.

Materials and methods

Preparation of MG

MG's main ingredients include of seven component herbs as follows: Yinyanghuo (*Epimedium rhizomatosum* Stearn) 15 g, Heshouwu (*Polygonum multiflorum* Thunb) 12 g, Huangqi (*Astragalus mongholicus* Bunge) 30 g, Shihu

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(*Dendrobium nobile* Lindl) 12 g, Roucongong (*Cistanche deserticola* Y.C.Ma) 12 g, Gusuibu (*Davallia mariesii* T. Moore ex Baker) 24 g, and Juhua (*Chrysanthemum morifolium* Ramat) 12 g. The raw herbs bought from Shanghai Medicinal Materials Company in China (Shanghai, China). Their voucher specimens have been deposited in Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine (Shanghai, China). According to Shi's method [25], the total of 107 g of seven herbs were milled to a fine powder and then was decocted twice in 2 L of ultrapure water for 2 hours. In the next step, the water extract was filtered through a Whatman No. 1 filter paper, and the filtrate was dried to achieve about 26.5 g powder. Then the powder was kept drying preservation at 4°C, analyzed for composition by high performance liquid chromatography (HPLC), in which major peaks were identified as the marker compounds to their originating individual herbs [25].

Animals and treatment

Adult specific pathogen-free female C57BL/6J mice (20-25 g) obtained from Shanghai University of Traditional Chinese Medicine Laboratory Animal Centre (Shanghai, China) were used in this study. Thirty-six mice (12 mice in each group) were randomly divided into three groups. Mice in Mi-Gu Capsule intervention group (MG group) and ovariectomized control group (OVXC group) received ovariectomy operation referring to established protocol. The sham operated control group (SOC group) received a sham operation in which a similar incision was made but ovariectomy was not performed. Three weeks after the first surgery operation for ovariectomy, every mouse withstood right femur osteotomy and internal fixation. SOC and OVXC groups were given 0.9% normal saline which acts as vehicle. MG group were given the mixture of 0.9% normal saline and MG, in which the dose of MG was 630 mg/kg/day mouse weight. All the treatments were given via oral gavages once a day for 42 days.

In this experiment, all mice housed in cages of a central animal house with climate-controlled conditions (21°C room temperature, 60% humidity, and 12 hours day-night light condition) and were fed a standard laboratory diet. The animal care and experiment protocol were approved by the Animal Experiment Ethics

Committee of the Shanghai University of Traditional Chinese Medicine.

Fracture method

Three weeks after the first surgical procedure for ovariectomy, the right-sided femurs of all animals were fractured according to established protocol [27]. Briefly, after anesthesia, the lateral thigh skin was shaved and disinfected. In the second step, a 6-7 mm skin incision from hip to knee was made on the lateral side of the right thigh, and the mid-femur was exposed by using blunt dissection of the fascia, muscles and periosteum. Then the femur was stabilized and a middle transverse osteotomy was performed with a saw. After the mid-diaphyseal osteotomy, a stainless steel needle (0.45-mm-diameter) was inserted into the marrow cavity of femur for fixation. Then, the wound was irrigated with 0.9% normal saline and closed by layers. X-ray imaging (Model MX-20; Faxitron X-Ray, Wheeling, IL) was used to confirm the positions of the steel needles. After incision antisepsis, the mice were remanded to their cages. Analgesic (paracetamol 50 mg/kg/day) was given for three days.

Mice were euthanized at a time point of 6 weeks post femur osteotomy. After sacrifice, the right femurs were then harvested, surrounding muscle tissues and stainless steel needles were removed carefully. Only femurs were used for further analysis. The femurs were packed with gauze dipped in 0.9% normal saline, kept at -20°C in frozen tubes [28].

Micro-CT measurement

The femurs were detected in a micro-CT device (eXplore Locus SP, GE Healthcare, London, Ontario, Canada) with a 12 µm spatial resolution protocol with 75 kV, 90 µA, and 2960-milliseconds exposure time. During detecting procedure, the samples were immersed in phosphate buffered saline. Bone mineral density was standardized with an acrylic calibration phantom in which serial densities equivalent to air, water, and bone. The 3D reconstruction and quantitative analysis of scanned three-dimensional image was performed using Microview software (Version 2.3, GE Healthcare, London, Ontario, Canada). The region of interest (ROI) was defined as a 5-mm-long polygonal region centered at the fracture zone as described pre-



Figure 1. Representative micro-CT images at 6 weeks post-fracture. SOC: sham-operated control group. OVXC: OVX control group. MG: OVX+MG treatment group. A: 3D reconstruction from micro-CT images. B: Coronal section micro-CT images. C: Transverse section micro-CT images.

viously [29]. The measured parameters in ROIs included total callus volume (TV), bone callus mineral content (BMC), and volumetric bone callus mineral density (vBMD).

Biomechanical testing

The biomechanical properties of the femurs after surgery were analyzed using three point bending test. The biomechanical testing was performed using a mechanical testing device (Instron 5543, Canton, MA, USA), after micro-CT image scanned. At the night before three-point bending testing day, the samples were slowly thawed in an 4°C refrigerator and kept packed in saline-soaked gauze. At one hour prior to testing, the samples were got out from the refrigerator and kept in room temperature.

As previously described protocol, the samples of femur was placed horizontally on the test apparatus with neutral position (anteroposterior direction), centered on the supporting bars (span = 8 mm); and the fracture zone was positioned directly under the loading bar. A load was applied with a loading rate of 8.0 mm/min until breakage. Biomechanical parameters including maximum load, stiffness and energy to failure were calculated according to the load-deformation curve using built-in computer software.

Statistical analyses

Analysis of data was performed using statistics software package SPSS 17.0 for Windows (SPSS, Chicago, IL, USA). Normality and homogeneity of the data were confirmed before analysis of variance (ANOVA). Differences among the means were analyzed by one-way ANOVA followed by Tukey's HSD tests. A *p*-value of less than 0.05 was considered to be statistically significant. All graphical values were presented as bar graphs with median and standard deviation (SD).

Results

Micro-computed tomography

Representative three-dimensional micro-CT images of specimens are displayed in **Figure 1**. TV represents the volume of the total callus site, and vBMD represents the volumetric density of mineral in callus site in terms of mgHA/cm³, is usually used to estimate the strength of bone tissue. At 6 weeks post-fracture, the SOC and MG groups exhibited a significantly lower mean value of TV than the OVXC group (*P* < 0.05). As to the vBMD, yet the SOC and MG groups showed a significantly higher mean value (*P* < 0.05). While the mean values of BMC showed no significant difference among the groups (*P* > 0.05) (**Figure 2**).

Biomechanical testing

Maximum load of bone represents the maximum strength of the sample can bear before refracture. Regarding the maximum load, the SOC and MG groups exhibited obviously higher values than the OVXC (*P* < 0.05). No significant differences were found between the SOC and MG groups (**Figure 3A**).

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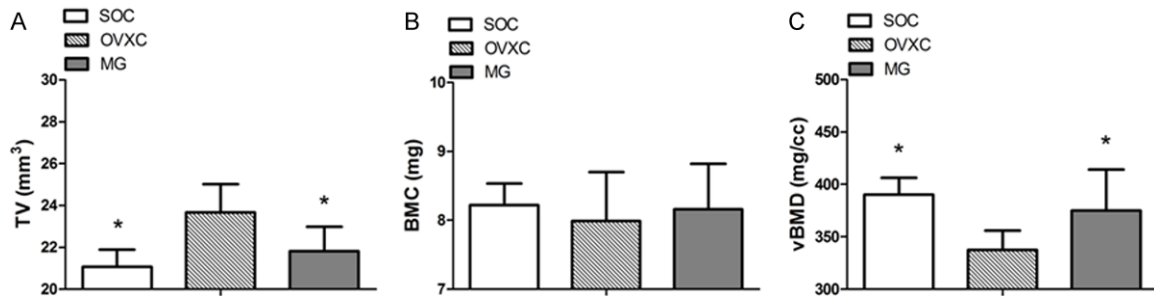


Figure 2. Micro-CT data of femoral callus compared at 6 weeks post-fracture. SOC: sham-operated control group. OVXC: OVX control group. MG: OVX+MG treatment group. Data represent mean value \pm standard deviation. * $P < 0.05$ versus OVXC.

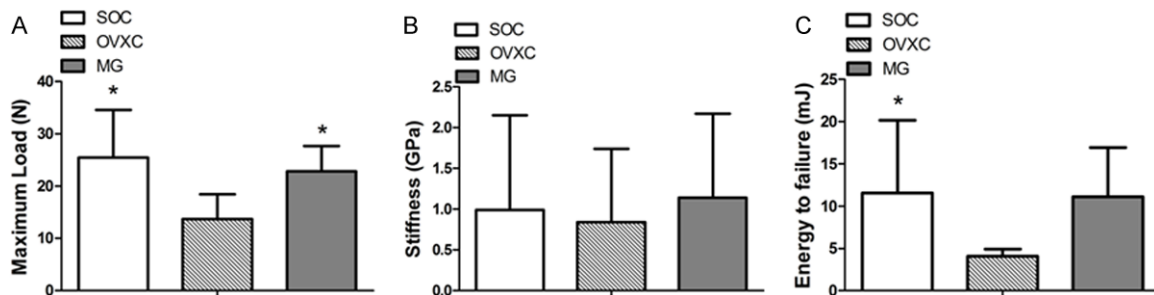


Figure 3. Biomechanical properties of fractured femur at 6 weeks post-fracture. SOC: sham-operated control group. OVXC: OVX control group. MG: OVX+MG treatment group. Data represent mean value \pm standard deviation. * $P < 0.05$ versus OVXC.

Stiffness of bone represents the extent to which resists deformation in response to an applied force. It is relevant to material Young's modulus, pattern of load and shape of sample. As to stiffness, no significant differences were detected among groups (**Figure 3B**).

Energy to failure was computed according to the area under the load-deformation curve. The SOC mice exhibited a significant higher energy to failure than the OVXC group ($P < 0.05$). No significant difference was detected in the energy to failure between the other groups (**Figure 3C**).

Discussion

Prolonged healing process and lower quality callus is common in the fracture healing process of osteoporotic patients. In the present study, we investigated the influence of post-menopausal osteoporosis on the bone healing process and the effects of MG on this process in osteoporotic animals. The healing quality was assessed by quantitative parameters of histomorphometry with micro-CT and biome-

chanical properties of anagenetic bone at the fracture zone. The measurement data of morphologic and biomechanical properties of the MG group were compared to two control groups. The OVXC and SOC groups had a role of control groups, and respectively represented osteoporotic and non-osteoporotic fracture. Our results showed that MG had a positive effect on vBMD of callus at the osteoporotic fracture site, and a positive effect on biomechanical properties of anagenetic bone.

As we all know, the main pathogenesis of primary osteoporosis Type1 is excessive bone resorption as a result of increased osteoclastic activity. It is well known that estrogen deficiency is a major contributory factor to primary osteoporosis Type1. Estrogen is one of the most important hormones which participate in regulating bone metabolism [30]. Estrogen deficiency due to menopause increases generation of osteoclast by activating precursor cells and offering a larger recruited pool. As a result of upregulation of generation and activation of osteoclast, resorption cavities was enlarged and deepened in trabecular surfaces [31]. The

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serial events result in augmented bone loss and increased bone fragility [32].

Many researchers have reported that fracture healing in postmenopausal osteoporotic bone appeared to be delayed with respect to bone callus remodelling and mechanical strength [33-37]. The study by Beil et al. [38] revealed that estrogen deficiency result in an delayed bone healing in animal model, with a postponed effect on the periosteal callus remodeling and reformation of marrow cavity, and provided evidence that the supplement of estrogen was a potential treatment of improving healing in osteoporosis. An other research by Stuermer et al. also revealed that application of estrogens could significantly enhance osteoporotic bone healing with regard to volume and mechanical strength of bone callus in experimental animal [39]. However, research on the side effects of estrogen therapy has hampered the use of this pharmaceutical agent [40]. The side effects of estrogen include increasing the risk of deep vein thrombosis, pulmonary embolus, endometrial cancer and breast cancer.

The effectiveness of drugs for osteoporosis on the osteoporotic fracture healing have been explored yet. Previous studies with efficacy of bisphosphonates on bone healing exhibited that it delayed bone tissue healing process owing to powerful inhibiting remodeling of bone callus [41]. Interestingly, these drugs did not hamper recovery of biomechanical strength of the traumatic bone.

The data from our study revealed that osteoporosis induced by ovariectomy impaired mineralization quality and mechanical strength of bone callus. This is consistent with the findings of previous studies. With respect to MG, treatment with MG increased vBMD and maximum load of callus. The higher vBMD, implying that the soft cartilaginous callus was well resorbed and replaced by the hard bony callus, indicated MG could promote mineral deposition. Biomechanical testing is universally regarded the golden standard of fracture healing evaluation. The higher maximum load, indicating that the callus will not easily fails, revealed MG could improve biomechanical properties of callus. Notebely, the callus volume was larger in OVXC groups, which indicated the delay of the remodelling process of callus in the OVX animals.

The herbs adopted to prepare MG have been widely applied for centuries in China, and the safety and efficacy of these herbs has been confirmed through long-term clinical application. The chemical composition of MG is quite diverse and is not completely detailed, and the interreaction among components also have not been clearly detailed. The main active components of these herbs have been identified, icariin in Yinyanghuo; stilbene glycoside in Heshouwu; calycosin in Huangqi; syringic acid in Shihu; echinacoside and cistanoside in Roucongong; naringin in Gushuibu; luteolin and apigenin in Juhua [42-47]. Previous studies indicated that MG had a positive role on enhancing bone mineral density and biomechanical property in osteoporotic animal models. Furthermore, clinical trials indicated that MG had an anti-osteoporosis action by improving bone mineral density in the vertebrae and femoral neck, with little adverse side effect [22, 23]. In molecular and cellular aspects, previous studies have revealed the water extract of BSF had an antiresorptive activity by encouraging expression of osteoprotegerin [48-50]. Furthermore, previous data also revealed the water extract of BSF had an anabolic activity by promoting release of IGF-I [51]. Anabolic activity may provide beneficial effect on bone regenerative process in the remodeling phase. Therefore, the further studies will be needed to clarify the underlying mechanisms of action of MG on bone healing.

The present study also has a few limitations. Firstly, the animal model used for experiment could be seen as a possible drawback of this study. In the real world, osteoporotic fracture usually occurs at the vertebration, distal radius and the neck of femur. However, to simplify the operation procedure, femoral shaft osteotomy were performed in this study. The major effect of estrogen deficiency on bone tissue is to promote cancellous bone resorption. A femoral shaft fracture model therefore may be not quite suitable for study on the effect of estrogen deficiency. Secondly, we did not detect estradiol blood concentrations of experimental animals in our study. However, it may be needed to verify accomplish of ovariectomy and estrogen deficiency.

In summary, the results shown here demonstrated that bone healing in a postmenopausal osteoporotic mouse model was significantly

improved via treatment with MG as revealed by micro-CT and biomechanical methods. Further studies are needed to clarify the underlying mechanism of action of MG on bone healing.

Acknowledgements

The current study was supported by grants from the National Natural Science Foundation of China (Grant no. 81674003, 81473703, 81403415, 81373665).

Disclosure of conflict of interest

None.

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