Association of Bone Morphogenetic Protein (BMP)/Smad Signaling Pathway with Fracture Healing and Osteogenic Ability in Senile Osteoporotic Fracture in Humans and Rats

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

B 1 De-Bao Liu
B 1 Cong Sui
C 2 Ting-Ting Wu
D 2 Lian-Zhong Wu
E 2 You-Yu Zhu
A 2,3 Zhen-Hua Ren

1 Department of Orthopedics, First Affiliated Hospital of Anhui Medical University, Hefei, Anhui, P.R. China
2 Department of Anatomy, Anhui Medical University, Hefei, Anhui, P.R. China
3 Cell Therapy Center, Xuanwu Hospital, Capital Medical University, Beijing, P.R. China

Corresponding Author: Zhen-Hua Ren, e-mail: renzhenhua321123@126.com
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Background: To investigate the effect of the BMP/Smad signaling pathway on fracture healing and osteogenic ability in senile osteoporotic fracture on humans and rats.

Material/Methods: Sixty-two patients and well-matched normal controls were enrolled for clinical observation. A rat model of senile osteoporotic fracture was established. Serum BMP2 and Smad4 levels, as well as alkaline phosphatase (ALP) activity, were detected by ELISA. Fracture healing was observed by X-ray radiography and bone formation was analyzed by micro-CT.

Results: Serum BMP2 and Smad4 levels in patients with senile osteoporotic fracture were significantly lower than those in normal controls (all $P<0.01$). BMP2 was highly positively correlated with Smad4 in patients with senile osteoporotic fracture (r=0.738). Compared with patients with low serum BMP2 and Smad4 levels, visual analog scale scores decreased, bone mineral density (BMD) increased, and duration of fracture healing was shortened in patients with high levels (all $P<0.05$). Compared with the Model group, serum BMP2 and Smad4 levels increased, fracture healing was improved, BMD, trabecular bone volume (TBV), tissue volume (TV), bone volume fraction (BV/TV), mean trabecular thickness (Tb. Th), and mean number of trabecular bone (Tb. N) were increased, and ALP activity increased in the BMP2 overexpression group (all $P<0.05$), while each index in the NC group showed no statistical difference relative to rats in the Model group (all $P>0.05$).

Conclusions: BMP2 overexpression can promote fracture healing and osteogenic ability in senile osteoporotic fractures through activating the BMP/Smad signaling pathway.

MeSH Keywords: Ankle Fractures • Osteoporosis • Smad4 Protein

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Background

Osteoporosis is a widespread chronic metabolic disease of the bone [1], which is characterized by low bone mass, micro-architectural deterioration, and fragility fractures [2]; it has been suggested to influence populations with different ethnic backgrounds, and the elderly are a high-risk group [3]. As a common complication of osteoporosis, osteoporotic fracture results from decreased bone mass and micro-architectural deterioration of osteoporotic bone structure [4]. Osteoporotic fracture presents biomechanically impaired healing and seriously threatens human health as the global population ages [5]. It was reported that about 21 million males and 137 million females age 50 years and older around the world were at high risk of osteoporotic fracture in 2010 and these numbers are predicted to double by 2040 [6]. Moreover, the World Health Organization predicted that half of global osteoporotic hip fractures will occur in Asia by 2050 [7,8]. Therefore, determining reasonable and effective approaches for diagnosis and treatment for osteoporotic fracture is of great significance.

Bone morphogenetic proteins (BMPs), belonging to the superfamily of transforming growth factor β (TGF-β), have vital roles in bone development, postnatal bone growth, and fracture repair [9]. As an important member of the BMP family, BMP2 is located on the genome of 20p12 and participates in the early development of skeletal system and tissue construction [10]. In addition, BMP2 is reported to be involved in the induction of osteoblast differentiation and the enhancement of bone matrix production by osteoblastic cells [11]. Patients carrying *BMP2* gene mutations and BMP2 expression disorders were reported to be prone to bone diseases and fracture [12,13]. *Smad4* gene encodes a member of the Smad family of signal transduction proteins which are phosphorylated and activated by transmembrane serine-threonine receptor kinases responding to TGF-β signaling [14]. Smad4, which is the specific intracellular transducer of TGF-β, is thought to participate in bone metabolism by playing an important role in the development and function of osteoclasts and osteoblasts [15]. Research indicated that alleles and haplotypes in Smad4 promoter were significantly correlated with bone mineral density (BMD) at the lumbar spine and various proximal femur sites [16]. Although BMP/Smad signaling was commonly explored in bone disease, the interaction of BMP2 and Smad4 and its combined effect in osteoporotic fracture has seldom been reported. In addition, there are few studies focused on the investigation of the regulation of BMP/Smad signaling in senile osteoporotic fracture healing and osteogenic ability via both clinical observation and animal models. Therefore, this study aimed to investigate the effect of the BMP/Smad signaling pathway on fracture healing and osteogenic ability in patients with senile osteoporotic fracture, using a wider range of parameters and indexes, including visual analogue scale (VAS) score, BMD, BV, TV, BV/TV, Tb. Th, Tb. N, and ALP activity.

Material and Methods

Subjects

Between January 2014 and January 2016, 62 patients with senile osteoporosis treated in our hospital were selected. Inclusion criteria for the patients were as follows: 1) Patients meeting the diagnostic criteria of senile osteoporosis proposed by *Guiding Principle of Clinical Research on New Drugs of Traditional Chinese Medicine*; 2) T-score of bone mineral density (BMD) measured by dual energy X-ray absorptiometry $\leq-2.5SD$; 3) Patients ages 60~80 years; and 4) Non-violent fracture. Exclusion criteria included: 1) Patients with other bone metabolism-related diseases; 2) Violent fractures such as traffic accident and crashes; 3) Patients with bone tumors and other malignant tumors; and 4) History of administration of fracture-related drugs, such as corticosteroids, anticoagulants, and others. In addition, another 62 individuals with normal results in physical examination were selected from the same region as the control group. All patients with senile osteoporosis were treated with anti-osteoporosis drugs and 0.25 μg/d calcitriol (batch number: B4072, F. Hoffmann La Roche AG, Basel, Switzerland). After discharge, the patients were recommended to rest lying in plank bed and to perform functional exercise following doctor's advice. This study was approved and supervised by the Ethics Committee of our hospital. All subjects signed informed consent and had the right to know.

Observation of pain indexes

Clinical observation was performed at the first visit of fracture patients in the hospital, as well as the regular return visit at the 1st, 2nd, 3rd, and 4th week after fracture. Pain indexes were measured using the visual analogue scale to observe the efficacy of analgesics. The visual analogue scale used by marking the ruler with numbers 0–10. Afterwards, patients chose a corresponding mark according to their self-perceived degree of pain. Visual score was classified into 3 grades: 1–3 (mild pain), 4–7 (moderate pain), and 8–10 (severe pain).

Observation of fracture healing

Tenderness and percussion pain at fracture site and spinal motion were observed based on the criteria of fracture healing at the return visit in the 1st, 2nd, 3rd, and 6th month after fracture. In patients with the above symptoms, continuous bed rest and return visit at the 2nd month was advised. Clinical fracture healing was determined by no tenderness or percussion pain at the fracture site, no limitation in spinal motion, and high stability of compressed vertebral body in X-ray. Clinical duration of fracture healing was considered as the duration from the day of fracture to the day of clinical fracture healing. After fracture healing, BMD of the left femoral neck was

measured using Hologic QDR-200+ dual-energy X-ray absorptiometry (DEX; USA).

Establishment of a rat model of senile osteoporotic fracture

A total of 10 Sprague-Dawley (SD) rats (age, 12 months and 22 months) were randomly selected, which were purchased from Shanghai Silaike Experimental Animal Co., Ltd. NORLAND EXCELL dual-energy X-ray absorptiometry (NORLAND Corporation, USA) was used to measure BMD. QuikscanTM Technology RESEARCH software by the high-speed scanning mode was employed, with the energy spectrum preset at 46.8/80 keV, and each image count was 1.0 mm. Data between groups were compared to confirm the success of the establishment of the rat model of senile osteoporotic fracture. Rats were anesthetized with 30 mg/kg 3% pentobarbital sodium (Sigma, USA), then the rats were placed on the operating table in prone position. The hair of the left lateral thigh was shaved using an electric shaver. With the limbs fixed with rubber bands and disinfected with povidone iodine solution, an incision was longitudinally made on the left lateral thigh, followed by cutting open the skin, subcutaneous tissue and the deep fascia, separate from the lateral femoral muscle space, and exposing the middle shaft of the femur, protecting the surrounding soft tissue, and a transverse fracture of middle left femur was made by use of a fret saw. Immediately, retrograde intramedullary fixation from the distal medullary cavity was carried out using 1.5-mm Kirschner wire, which was then removed from the femoral condyle, and the Kirschner wire tail was in line with the fracture line. After the restoration of the fracture, we pulled the Kirschner wire into the proximal end of the fracture. Following the fixation, the excess Kirschner pins were cut at the femoral condyle, and the incision was closed successively. Postoperatively, 80×10^4 U penicillin (Shanghai Xianfeng Pharmaceutical Co., Ltd., China; batch number: S100824) was intramuscularly injected into the rats 2 times per day for 3 consecutive days. All rats were free to move and eat food.

Grouping and treatment of experimental animals

According to the establishment approach of the osteoporotic fracture model, 36 rats were selected for modeling. After the successful construction of the rat model, the 36 enrolled rats were divided into 3 groups, with 12 rats in each group: 1) model group (Model), 2) negative control group (NC), and 3) BMP2 overexpression group (BMP2). Rats in the model group were all established to have osteoporotic fracture; in the NV group, after the establishment of the rat model of osteoporotic fracture, the rats were intraperitoneally injected with negative plasmids with BMP2 overexpression (Shanghai GenePharma Biotechnology Co., Ltd., China); and in the BMP2 group, after the establishment of the osteoporotic fracture model, the rats were intraperitoneally injected with the same amount of plasmids with BMP2 overexpression (Shanghai GenePharma Biotechnology Co., Ltd., China). This experiment was approved by the Ethics Committee of our hospital, and animal experiments strictly followed the Declaration of Helsinki.

Observation of fracture healing in rats by X-ray radiography

At the 2nd, 4th, 6th, and 8th week after the establishment of the rat model of osteoporotic fracture, X-ray radiography (photographic machine: PHILIPS BUCKY TC, PHILIP, Netherlands; developing machine: KODAK CR 400, KODAK, US) was performed with the conditions of 50 kV, 50 mA, and 125 ms. Computer-aided image analysis was carried out by placing X-ray films in front of the light source, and applying low-magnification image analysis system (Axioplan 2, ZEISS, Germany) to collect X-ray images. The data and images were analyzed by a computer system.

Detection of fracture healing-related indexes in rats

At the 8th week after internal fixation, rats were killed. The left thigh bone was collected with the fixed Kirschner wire removed, and stored at –20°C. The tibia specimens were scanned along the long axis using Inveon micro-CT, with the angle of rotation and CT values manually corrected, planar pixel resolution set at 2048×2048, pixel size at 16×16 μm, and interlamellar spacing at 16 μm. The proximal fracture (thickness, 0.5 mm) was selected from scanning images for detection, with indexes including BMD, trabecular bone volume (TBV), tissue volume (TV), bone volume fraction (BV/TV), mean trabecular thickness (Tb. Th), and mean number of trabecular bone (Tb. N) detected. Among them, BMD was indirectly obtained from the quantification of voxel of CT scan images.

Detection of serum BMP2 and Smad4 levels and ALP activity by ELISA

The morning fasting peripheral venous blood (5 ml) was collected on the next day from all patients who were confirmed to have osteoporotic fracture on admission and centrifuged at 2000 rpm for 10 min, followed by serum separation and storage at –80°C. After experiments, the rats were anesthetized by 3% pentobarbital sodium, which was followed by transabdominal collection of arterial blood (4 ml), centrifugation at 4000 rpm for 10 min, serum separation, and storage at –20°C. Serum levels of BMP2 and Smad4 and the activity of alkaline phosphatase (ALP) in patients and rats were detected using the double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit (Blue Gene). These serum samples (100 μl) were collected, followed by reconstitution at room temperature, re-centrifugation, and collection of supernatant

Table 1. Comparison in general information between the case group and the control group.

Variables	Case group (n=628)	Control group (n=62)	t/χ^2	*P* value
Average age	69.1±8.3	67.4±7.9	0.93	0.351
Gender (male/female)	32/30	35/27	0.15	0.696
Smoking history (yes/no)	24/36	23/49	0.31	0.578
Drinking history (yes/no)	28/34	29/33	0.16	0.686
Bone mineral density (kg/cm²)	24.84±1.08	25.42±0.86	3.25	0.078
Educational degree			1.26	0.885
Junior middle school	17	18		
Junior and high school	32	33		
University and above	13	11		
Diet			0.91	0.340
Low calcium	23	18		
Normal	39	44		

for measurement. The optic density (OD) value was measured using a microplate reader at 450 nm.

Statistical analysis

Data analysis was conducted by using SPSS 21.0 (SPSS, Inc., Chicago, IL). Measurement data are expressed as mean ± standard deviation, and correlation between BMP2 and Smad4 was examined using Pearson correlation analysis. Comparison of measurement data following normal distribution between 2 groups was performed by the *t* test. Pairwise comparison was carried out using the least significant difference (LSD) test, and comparison among multiple groups by the single-factor analysis of variance (one-way ANOVA). *P*<0.05 was considered as statistically significant.

Results

General information of subjects

Sixty-two patients with senile osteoporotic fracture were included in the case group, including 32 males and 30 females (average age, 69.1±8.3 years). Among them, there were 30 cases of radius fractures, 18 cases of tibial fractures, 16 cases of proximal humeral fractures, 14 cases of hip fractures, and 2 cases of spinal fractures. Furthermore, there were 23 cases of hypertension, 12 cases of diabetes, 22 cases of bronchial diseases, 7 cases of other diseases, and 9 cases combined with at least 2 complications. The control group included 62 individuals (35 males and 27 females; average age, 67.4±7.9 years). Age, sex composition, smoking history, drinking history, BMI, educational level, and diet showed no statistically

significant differences between the 2 groups (all *P*>0.05), suggesting comparability (Table 1).

Serum BMP2 and Smad4 expressions in patients with senile osteoporotic fracture and normal controls

Serum BMP2 content was (70.31±5.48) ng/L in the patients with senile osteoporotic fracture and (86.92±5.86) ng/L in the controls, showing significantly lower serum BMP2 content in the patients with senile osteoporotic fracture than in the normal controls (*P*<0.01) (Figure 1A). Serum Smad4 content in the patients with senile osteoporotic fracture and controls was (4.98±0.51) ng/L and (9.27±0.65) ng/L, respectively. Statistical analysis demonstrated that serum Smad4 content was significantly lower in the patients with senile osteoporotic fracture as compared with the controls (*P*<0.01) (Figure 1B).

Correlation analysis of serum BMP2 and Smad4 levels in patients with senile osteoporotic fracture

Pearson correlation analysis showed that the correlation coefficient of serum BMP2 and Smad4 levels in patients with senile osteoporotic fracture was r=0.738 (*P*<0.01), suggesting a highly positive correlation between serum BMP2 and Smad4 levels in patients with senile osteoporotic fracture (Figure 2).

Correlation of serum BMP2 and Smad4 levels with fracture healing

Using mean serum BMP2 and Smad4 levels in the patients as boundaries, serum BMP2 and Smad4 levels in the patients were divided into high levels (higher than the mean levels) and low levels (lower than the mean levels). The correlations
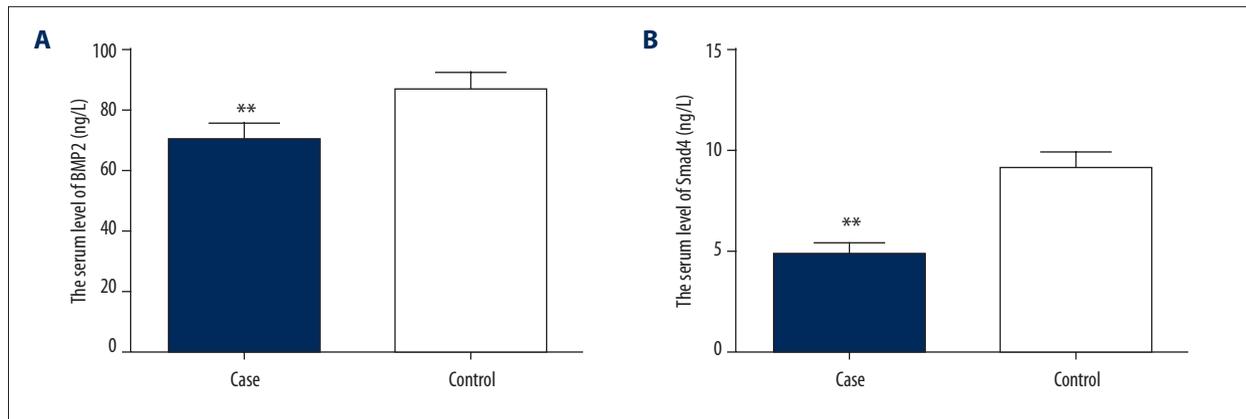
Figure 1. BMP2 and Smad4 levels in the case group and the control group. (**A**) BMP2 level in the case group and the control group; (**B**) Smad4 level in the case group and the control group. ** Compared with the control group, *P*<0.01; BMP2 – bone morphogenetic protein-2.
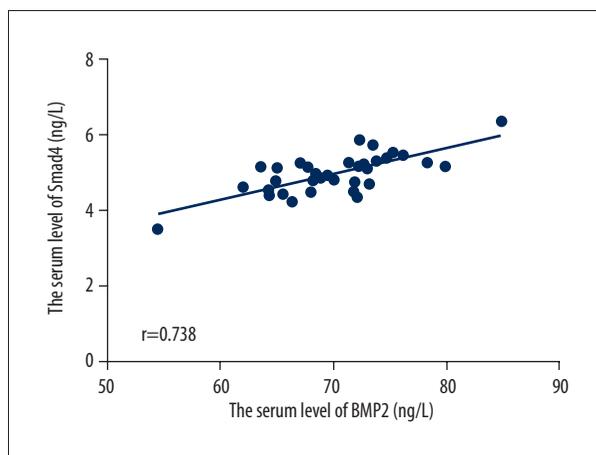
Figure 2. Correlation analysis of BMP2 and Smad4 levels in the case group. BMP2 – bone morphogenetic protein-2.

of serum BMP2 and Smad4 levels with fracture healing in the patients were analyzed, demonstrating that, as compared with the patients with low serum BMP2 level, VAS score was reduced significantly, BMD was increased significantly, and the duration of fracture healing was significantly shortened in the patients with high serum BMP2 level (all *P*<0.05). Moreover, compared with the patients with high serum Smad4 level, VAS score increased significantly, BMD decreased significantly, and

the duration of fracture healing was significantly prolonged in the patients with low serum Smad4 level (all *P*<0.05) (Table 2).

BMD measurement results in rats with osteoporotic fracture

After the establishment of senile osteoporotic fracture with rats ages 12 and 22 months, BMD was measured in the involved rats of different ages. The results showed that BMD in the whole body of the rats age 22 months with osteoporotic fracture was significantly lower than that in the rats in the age of 12 months (*P*<0.05), which suggested successful establishment of the rat model of senile osteoporotic fracture, as seen in Table 3.

Table 3. Bone mineral density measurement results in experimental rats.

Month age	Case	Bone mineral density (g/cm^2)
12 months old	10	0.189±0.0058
22 months old	10	0.165±0.0064*

* Compared with the rats in the age of 12 months, *P*<0.05.

Table 2. Correlation of serum BMP2 and Smad4 levels with fracture healing in patients.

Indicators	BMP2		Smad4	
	High level	Low level	High level	Low level
Pain score	1.04±0.15	3.63±0.36*	1.57±1.10	3.09±1.11*
Bone mineral density (kg/cm^2)	2.67±0.12	0.73±0.08*	2.26±0.82	1.14±0.82*
Duration of fracture healing (d)	43.07±3.18	49.86±4.72*	44.37±4.59	48.58±5.14*

* Compared with the same indicator at a high level, *P*<0.05; BMP2 – bone morphogenetic protein-2
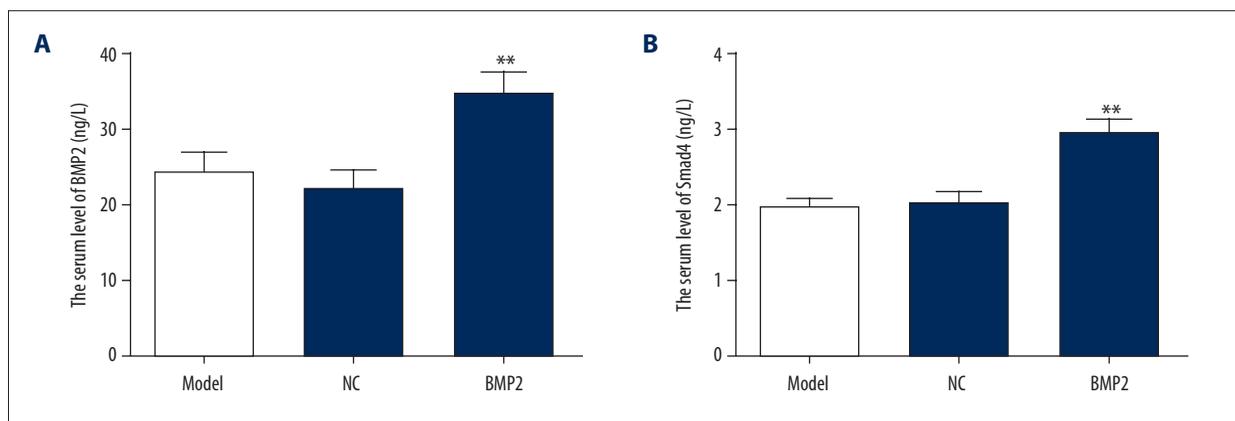
Figure 3. Serum (**A**) BMP2 and (**B**) Smad4 expressions in rats of each group. ** Compared with the Model group, $P<0.01$; BMP2 – bone morphogenetic protein-2.
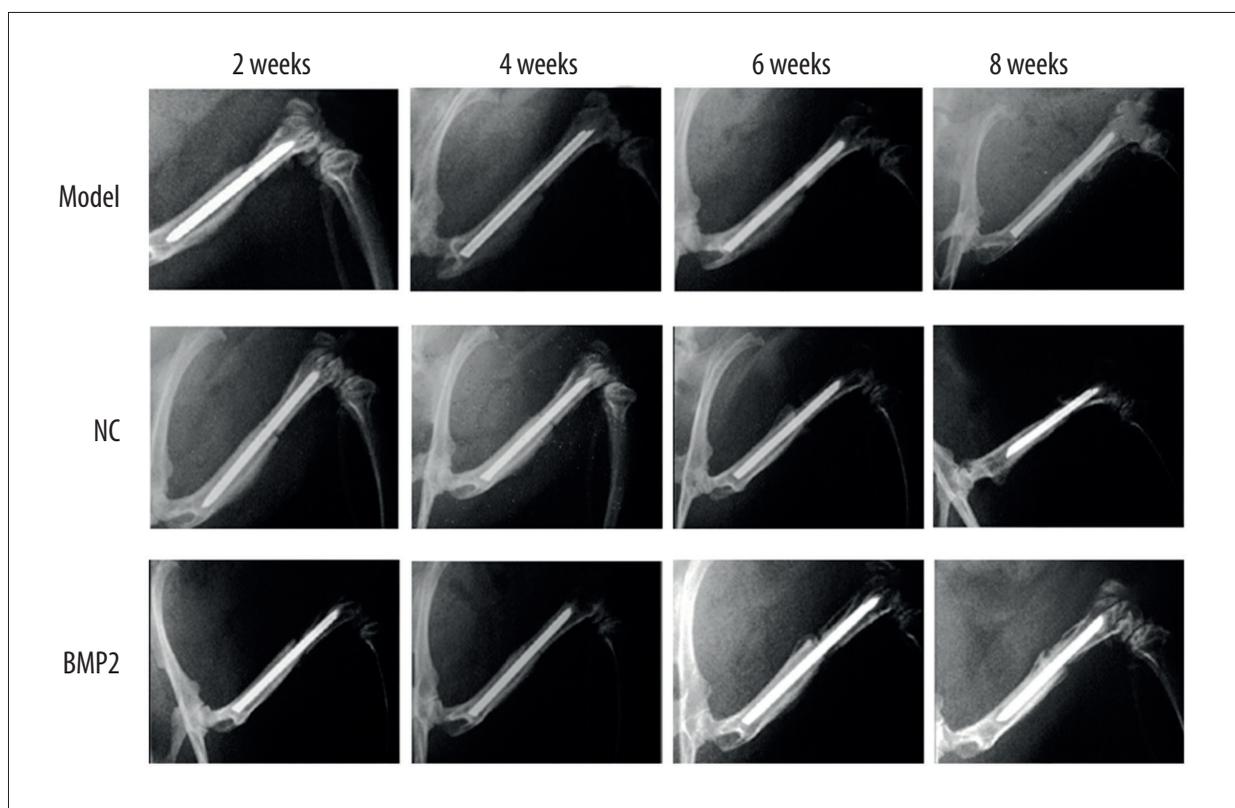
Figure 4. Observation of callus formation in rats of each group by X-ray radiography.

Serum BMP2 and Smad4 expressions in rats

Serum BMP2 and Smad4 expressions in rats were detected by ELISA. The results indicated that, as compared with the Model group, serum BMP2 and Smad4 expressions in the NC group showed no obvious differences (both $P>0.05$). After the over-expression of BMP2 in rats with senile osteoporotic fracture, serum BMP2 and Smad4 expressions increased significantly (both $P<0.05$), as shown in Figure 3.

Callus formation in rats

In the rats with osteoporotic fracture and the rats of the NC group, there was little callus tissue observed at the fracture end, callus density was heterogeneous, osteosclerosis was found at the fracture end, internal fixation was loosening, and the fracture line was still observable with slight force. As compared with the rats with osteoporotic fracture, there was more callus tissue at the fracture end in the BMP2 overexpression group, bone healing was better, there was continuous callus

Table 4. Changes in fracture healing-related indexes in rats of each group.

Indexes	Model	NC	BMP2
BMD (mg/mm)	427.31±28.75	425.72±27.96	469.39±30.48
TBV (mm³)	24.42±3.61	22.87±3.58	41.05±4.02
TV (mm³)	29.87±3.52	31.16±3.84	53.92±5.17
BV/TV (%)	58.09±5.65	57.74±5.83	71.58±6.41
Tb. Th (mm)	0.59±0.04	0.56±0.05	0.78±0.07
Tb. N (mm-1)	1.12±0.23	1.09±0.21	1.42±0.26

BMD – bone mineral density; TBV – trabecular bone volume; TV – tissue volume; BV/TV – bone volume fraction; Tb. Th – mean trabecular thickness; Tb. N – mean number of trabecular bone; NC – negative control; BMP2 – bone morphogenetic protein-2.
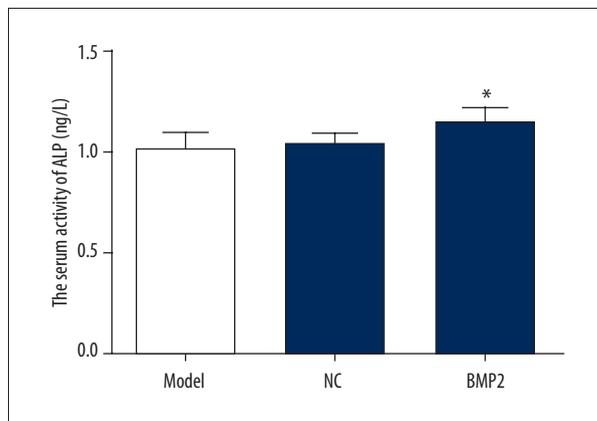
Figure 5. Serum ALP activity in rats of each group. * Compared with the Model group, $P<0.05$; ALP – alkaline phosphatase.

through the fracture end, the fracture line was fuzzy, internal fixation showed no displacement or loosening, and no malunion or abnormal angulation was found, as seen in Figure 4.

Changes in fracture healing-related indexes in rats

Micro-CT showed that after BMP2 overexpression, all BMD, BV, TV, BV/TV, Tb. Th, and Tb. N were significantly higher as compared with the Model group and the NC group (all $P<0.05$). However, no significant differences in each index were detected between the single Model group and the NC group (all $P>0.05$), suggesting that BMP2 overexpression promoted the restoration and reconstruction of bone microstructure during fracture healing in the rats with senile osteoporotic fracture, as seen in Table 4.

Serum ALP activity in rats

Serum ALP activity in rats of each group was detected using kits. We found that, as compared with the Model group, serum ALP activity in the rats with senile osteoporotic fracture

increased significantly in the NC group with BMP2 overexpression (all $P<0.05$). However, serum ALP activity in the NC group presented no obvious changes (all $P>0.05$), as seen in Figure 5.

Discussion

Osteoporosis risk increases with increased age, and the elderly are likely to suffer osteoporotic fracture frequently [17]. In this study, serum BMP2 and Smad4 contents were significantly lower in patients with senile osteoporotic fracture than in the normal controls, suggesting that BMP2 and Smad4 play crucial roles in senile osteoporotic fracture. As compared with patients with low serum BMP2 and Smad4 levels, VAS score was reduced, BMD was increased, and the duration of fracture healing was shortened in the patients with high serum BMP2 and Smad4 levels. Therefore, it is plausible that high BMP2 and Smad4 levels can promote fracture healing. Further, this study demonstrated the accelerated healing potential of BMP2 application in bone fractures in an osteoporotic rat model. As speculated, BMP2 can stimulate new bone growth and greater mineral density, and, perhaps most importantly, enhance the biomechanical strength of healed fractures as compared to the normal controls.

Furthermore, the role of BMP2 in the stimulation of osteogenesis and the promotion of fracture healing has been proved *in vivo* in a number of different models, including osteoporotic rats [18–20]. Furthermore, BMP2 stimulation of bone cell proliferation, osteogenic differentiation, and expression of early (ALP) and late (calcium mineralization) markers of bone formation have been reported *in vitro* in an osteoporotic environment [21,22]. BMD measurements and histological studies might provide a possible indication of new bone formation at the fracture site, showing accelerated new tissue formation and mineralization in the BMP2 overexpression group. There may be potential for BMP2 application to offer an alternative approach to osteoporotic fractures where bone fragility and

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instrumentation failure rates are high. During osteogenic differentiation, by regulating the Wnt signaling, BMP2 could promote bone development and differentiation [23]. After BMP2 activates the channel, there is a great increase in both specific activity of ALP and phosphorylation level of Smad proteins, which strengthens bone differentiation [24]. Therefore, the decreased expression of BMP2 will weaken the channel for bone differentiation, so as to cause bone mass loss, which will lead to a great elevation in the occurrence of osteoporotic fracture. In contrast to other studies which focused on understanding the mechanism by which BMPs transduce signaling and exert their functions in skeletal development and homeostasis [25,26], the present study focussed on the possible role of the BMP/Smad signaling pathway in fracture healing and osteogenic ability. Indeed, BMP2 was superior to other BMPs and growth factors in stimulating osteogenesis and enzyme function in bone tissue derived from elderly, osteoporotic fractures and induced mineralization in serum-free callus explant culture [21,22]. What remains unknown is whether BMP2 have the ability to reverse some of the adverse effects of oestrogen deficiency, such as osteoblast apoptosis and healing in the later stage, to accelerate bone repair. Certainly, BMP-7 has shown an anti-apoptotic effect in cartilaginous cells *in vitro* [27].

Moreover, there was a highly positive correlation between serum BMP2 and Smad4 levels in patients with senile osteoporotic fracture, indicating that BMP2 may have an up-regulating effect on Smad4 in senile osteoporotic fracture. In a study by Hsieh et al., BMP2 and Smad4 expressions were up-regulated in mice with osteoporotic fracture, and induction of BMP2 could regulate Smad4, which might contribute to the induction of osteoblasts proliferation and differentiation, resulting in bone formation [28]. Also, as a key signaling component in bone formation, BMP2 signals via Smad4, which is a nuclear transcription factor that regulates the activity of TGF-β ligands and plays an important role in bone formation [16].

Conclusions

In conclusion, serum BMP2 and Smad4 levels are lower in patients with senile osteoporotic fracture than those in the normal controls, and serum BMP2 shows a highly positive correlation with Smad4. Additionally, up-regulation of the BMP/Smad signaling pathway has a promoting effect on fracture healing and osteogenic ability in senile osteoporotic fracture, which provides an alternative way to treat senile osteoporotic fracture. Further studies are required to elucidate the mechanisms by which up-regulating the BMP/Smad signaling pathway protects against bone loss.

Conflicts of interests

None.

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