Dynamic expression of matrix metalloproteinases 2, 9 and 13 in ovariectomy-induced osteoporosis rats

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Received May 10, 2017; Accepted December 1, 2017

DOI: 10.3892/etm.2018.6356

Abstract. The aim of the present study was to examine the dynamic expression of matrix metalloproteinase (MMP)-2, MMP-9 and MMP-13 in an ovariectomy (OVX)-induced osteoporosis rat model. A total of 80 Sprague-Dawley female rats (age, 3 months) were randomly divided into the OVX and sham groups, with 40 rats in each group. Rats in the sham group received sham surgery, while the remaining rats were ovariectomized. After 12, 16, 20 and 24 weeks, 10 rats from each group were randomly sacrificed, respectively. It was observed that the bone mineral density (BMD) and the trabecular bone area in the OVX group were significantly lower as compared with those in the sham group ($P<0.01$). The expression levels of MMP-2 and MMP-9 were negatively correlated with the BMD, while MMP-13 was positively correlated with the BMD. The expression levels of MMP-2 and MMP-9 increased more abruptly and were significant higher in the OVX group in comparison with those in the sham group between 12 and 24 weeks after surgery ($P<0.01$). More specifically, the MMP-9 mRNA expression level in the OVX group increased abruptly between 12 and 24 weeks after surgery. By contrast, in the sham group, the MMP-9 mRNA level was undetectable between 12 and 16 weeks, and increased steadily between 16 and 24 weeks. Furthermore, the mRNA and protein expression levels of MMP-13 initially increased and then decreased in the OVX group ($P<0.01$ vs. the sham group), whereas they continuously increased in the sham group between 12 and 24 weeks after surgery. In conclusion, MMP-2, MMP-9 and MMP-13 regulated the development of osteoporosis, and MMP-9 may be used as an important marker in the early diagnosis of osteoporosis.

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Abbreviations: OVX, ovariectomy; BMD, bone mineral density; MMP, matrix metalloproteinase

Key words: osteoporosis, bone resorption, MMP-2, MMP-9, MMP-13

Introduction

Osteoporosis is a systemic disease, characterized by reduced bone mass and a T-score [bone mineral density (BMD) score] of ≤-2.5 (1). This disease is frequently associated with an increased risk of fractures and is considered a public health issue, threatening a large part of the population aged >50 years (2,3). Osteoclasts, which are essential in bone homeostasis, serve a key role in the development of osteoporosis. Therefore, the study of osteoclast function has become important in the investigation of osteoporosis.

The regulation effect of matrix metalloproteinases (MMPs) on the osteoclastic bone resorption has received increasing attention (4). MMPs are a group of proteolytic enzymes that dependent on Zn^{2+} hydrolysis. To date, the MMP family has been found to only degrade fibrillar collagen (5). As three members of the MMP family, MMP-2, MMP-9 and MMP-13 serve an important role in the activation of osteoclasts (6,7). MMP-2 has been reported to degrade type I collagen barrier bone cells and, subsequently, active osteoclasts (8). MMP-9 is able to specifically degrade non-mineralized cartilage and release extracellular matrix proteins in combination with vascular endothelial growth factor (9). In addition, the serum MMP-9 concentration was observed to be negatively correlated with BMD and is considered as a biochemical marker of bone resorption (10). Furthermore, MMP-13 enhances the degradation of collagen fragments, leading to an increase of specific integrins and collagen fragments in osteoclasts, and an increase in bone remodeling and bone resorption (11). A previous study demonstrated that decreased expression of MMP-13 reduced the osteoclastic bone resorption and was negatively correlated with the percentage of trabecular bone area (Tb.Ar) (12).

At present, although the contributions of MMP-2, MMP-9 and MMP-13 in osteoporosis are increasingly investigated, the dynamic alterations in in osteoporosis remain unclear. Therefore, in the present study, it was hypothesized that the MMP-2, MMP-9 and MMP-13 expression levels are

continuously increased in osteoporosis. To test this hypothesis, the study attempted to demonstrate the dynamic expression of MMP-2, MMP-9 and MMP-13 in ovariectomy (OVX)-induced osteoporosis model in rats.

Materials and methods

Animals. A total of 80 Sprague-Dawley specific-pathogen-free female rats (3-month-old; weight, 284.72±20.32 g) obtained from the Shanghai Animal Experiment Center [Shanghai, China; certification no. SCXK(Hu)2012-0002] were used in the present study. The rats were housed in the Experimental Animal Center of Fujian University of Traditional Chinese Medicine (Fuzhou, China) under controlled environmental conditions (12-h light/dark cycle; temperature, ~24˚C; humidity, 40-70%) and were provided with standard food for laboratory animals and water *ad libitum*. The present study was approved by the Ethics Committee of Fujian University of Traditional Chinese Medicine (project no. 20140120), and followed the international guidelines for the Care and Use of Laboratory Animals provided by the National Institutes of Health (Bethesda, MD, USA).

OVX-induced osteoporosis rat model. The rats were randomly divided into the OVX and sham groups, with 40 rats in each group. Rats in the OVX group were bilaterally ovariectomized following anesthesia with 2% pentobarbital sodium. The ovaries were exposed and removed along with the surrounding fat, oviduct and a small portion of the uterus. Rats in the sham group underwent sham surgeries, during which the ovary was exposed but left intact.

Sample collection. In total, 10 rats from each group were randomly sacrificed at the 12, 16, 20 and 24 weeks after surgery. Following anesthesia, the left tibia was removed, fixed in 10% neutral buffered formalin and stored at room temperature for histological examination. The right femur was also collected and stored in -80˚C prior to BMD analysis by dual-energy X-ray absorptiometry. The first and second lumbar vertebrae were collected and stored in a -80˚C refrigerator for subsequent reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting, respectively.

Histological analysis. The left tibia were removed and immediately fixed in 10% neutral buffered formalin. Next, the sample was cleaned of soft tissue, placed in decalcifying solution (10% EDTA/PBS; pH 7.4) for ~21 days at room temperature, dehydrated in 95% (v/v) ethanol and then embedded in paraffin. Subsequently, three 5-mm paraffin-embedded horizontal bone sections were cut from the upper part of the left tibia, stained with hematoxylin-eosin and examined by light microscopy. The quality of the bone and trabecular density was assessed, according to the scores shown in Table I. The percentage of the Tb.Ar was calculated as follows: Tb.Ar (%) = Tb.Ar/T.Ar (bone tissue area) x100%.

BMD assay. The BMD of the right femur was measured using dual-energy X-ray absorptiometry (Lunar-DPX-IQ; GE Healthcare, Chicago, IL, USA) equipped with Hologic APEX software (Hologic, Inc., Marlborough, MA, USA), which assessed bone density in small laboratory animals. A measured value of ±1.5% was considered as an acceptable measurement. Whenever two points obtained in succession were outside the limits of the quality control curve, the procedure was repeated. Furthermore, the accuracy of the final BMD measurements was determined by duplicate scans of the femur.

RT-qPCR analysis. Total RNA was extracted from the first lumbar vertebra using TRIzol reagent according to the manufacturer's protocol (R0016; Beyotime Institute of Biotechnology, Haimen, China). The RNA concentration was measured by NanoDrop™ 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA was reverse transcribed into cDNA in each sample using PrimeScript™ RT reagent kit (RR047A; Takara Bio, Inc., Otsu, Japan), which was then used in PCR amplification. The qPCR primers were as follows: MMP-2 (143 bp), 5'-TCCCGAGATCTGCAAGCAAG-3' (forward) and 5'-AGAATGTGGCCACCAGCAAG-3' (reverse); MMP-9 (173 bp), 5'-AGCCGGGAACGTATCTGGA-3' (forward) and 5'-TGGAAACTCACACGCCAGAAG-3' (reverse); MMP-13 (131 bp), 5'-CCCAGATGATGACGTTCAAGGA-3' (forward) and 5'-CTCGGAGACTAGTAATGGCATCAAG-3' (reverse); and β-actin (131 bp) 5'-GGAGATTACTGCCCTGGCTCCTA-3' (forward) and 5'-GACTCATCGTACTCCTGCTTGCTG-3' (reverse). β-actin was used as the internal control. The conditions of qPCR conducted on a 7,500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) were the following: One cycle at 95˚C for 30 sec, 40 cycles at 95˚C for 5 sec and 60˚C for 34 sec. The amplification curve and Ct values were produced (13). To obtain the dissolution curve, one cycle at 95˚C for 30 sec, 40 cycles at 95˚C for 3 sec and 60˚C for 30 sec were conducted. The expression levels of MMP-2, MMP-9 and MMP-13 were quantified and normalized to β-actin expression.

Western blotting. Protein was extracted from the second lumbar vertebral samples in each group, and the protein concentrations were determined by the BCA assay (P0012S; Beyotime Institute of Biotechnology). Next, 50 μg samples were loaded and separated by 10% SDS-PAGE. Following electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes in a Tris-glycine transfer buffer (containing 20% v/v methanol, 3.03 g Tris and 14.4 g glycine) using a wet transfer system. Subsequently, the target proteins in the samples were detected with primary antibodies against MMP-2 (1:1,000; cat. no. sc-10736), MMP-9 (1:1,000; cat. no. sc-10737) and MMP-13 (1:1,000; cat. no. sc-30073; all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA), as well as an anti-β-actin antibody (1:1,000; cat. no. 4970; Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4˚C. The samples were then incubated with ImmunoPure® goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature (1:3,000; cat. no. LP31460; Xiamen Lulong Biotech Development Co., Ltd.). Proteins were developed with BeyoECL Plus (P0018; Beyotime Institute of Biotechnology). The optical density value of each band was read and recorded using Image Laboratory software (version 5.0; Bio-Rad Laboratories, Inc., Hercules, CA, USA), and normalized to the band intensity of β-actin.

Table I. Percentage of Tb.Ar of the two group (mean ± standard deviation).

	12 weeks		16 weeks		20 weeks		24 weeks	
Group	n	Tb.Ar (%)	n	Tb.Ar (%)	n	Tb.Ar (%)	n	Tb.Ar (%)
Sham	10	0.527±0.026	10	0.501±0.009	10	0.476±0.183	10	0.446±0.161
OVX	10	0.368±0.006[a]	10	0.318±0.067[a]	10	0.253±0.333[a]	10	0.236±0.211[a]

[a]P<0.01 vs. sham group. Tb.Ar, trabecular bone area; OVX, ovariectomy.

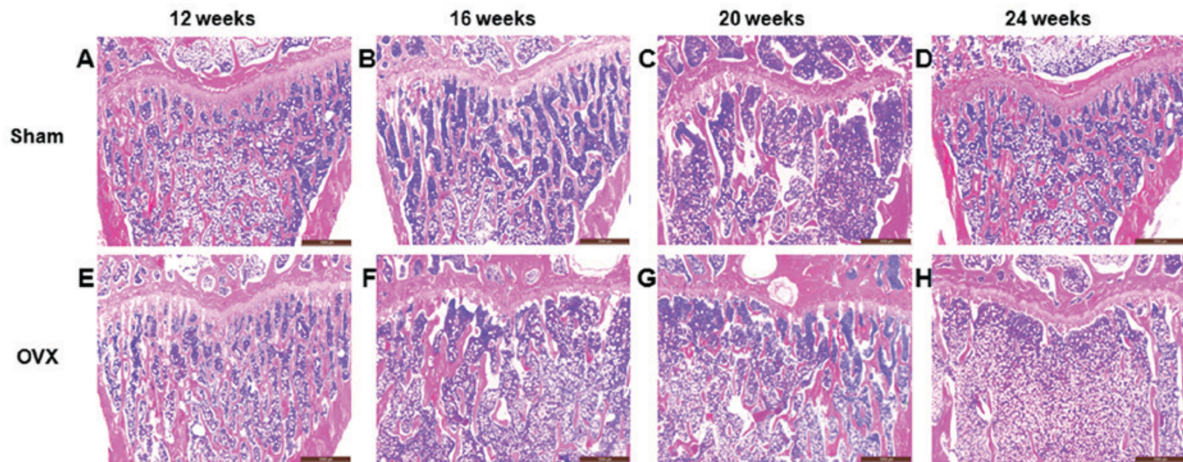

Figure 1. Trabecular bone morphology examined by hematoxylin and eosin staining. Sham group at (A) 12, (B) 16, (C) 20 and (D) 24 weeks after surgery. OVX group at (E) 12, (F) 16, (G) 20 and (H) 24 weeks after surgery. Magnification, x200. OVX, ovariectomy.

Statistical analysis. All experiments were repeated at least three times, and representative experiment results are shown. Correlation coefficient data were determined by Spearman's rank correlation analysis. Other data are reported as the mean ± standard deviation. Significance of differences between the mean of each group were determined by one-way analysis of variance and Student-Newman-Keuls test. P-values of <0.05 were considered to indicate differences that were statistically significant.

Results

Morphology of the rat tibia. In the sham group, the surface of the trabecular bone was smooth, and the trabecular plate remained intact (Fig. 1A-D). However, the structure of the trabecular plate in the OVX group was absorbed and became progressively thinner due to the increased osteoclast function. The surface of the trabecular bone was rough, while the trabecular plate underwent severe deterioration and was even completely removed (Fig. 1E-H). Compared with the sham group, the percentage of Tb.Ar in the OVX group at 12, 16, 20 and 24 weeks after surgery was significant lower (P<0.01; Table I).

BMD of the right femur. The BMD of the right femur in the sham group was significantly higher in comparison with that in the OVX group, except at 12 weeks (P<0.01; Fig. 2). The BMD in the sham group was initially increased abruptly between 12 and 16 weeks, and then remained stable between 16 and 24 weeks after surgery. However, the BMD in the OVX group demonstrated a gradual and continuous downward trend (Fig. 2).

MMP-2, MMP-9 and MMP-13 mRNA expression levels. The mRNA expression levels of MMP-2, MMP-9 and MMP-13 were detected by RT-qPCR. As shown in Fig. 3A and B, the levels of MMP-2 and MMP-9 expression were significantly higher in the OVX group as compared with those in the sham group at 12, 16, 20 and 24 weeks after surgery (P<0.01). The MMP-9 mRNA expression in the OVX group increased abruptly between 12 and 24 weeks after surgery. By contrast, MMP-9 expression was extremely low in the sham group between 12 and 16 weeks, while it increased steadily between 16 and 24 weeks after surgery. In addition, the MMP-13 expression level in the OVX group was increased until 20 weeks and then decreased, whereas a continuous increase was detected in the sham group between 12 and 24 weeks after surgery, with a significant difference observed between the two groups at all time-points (all P<0.01 except at 24 weeks, P<0.05; Fig. 3C).

MMP-2, MMP-9 and MMP-13 protein expression levels. The protein expression levels of MMP-2, MMP-9 and MMP-13 were detected by western blotting. The MMP-2 protein expression in the two groups increased between 12 and 24 weeks after surgery. However, MMP-2 protein expression increased more sharply in the OVX group as compared with that in the sham group, with a significant difference observed (P<0.01; Fig. 4). A similar trend was also detected for MMP-9 protein

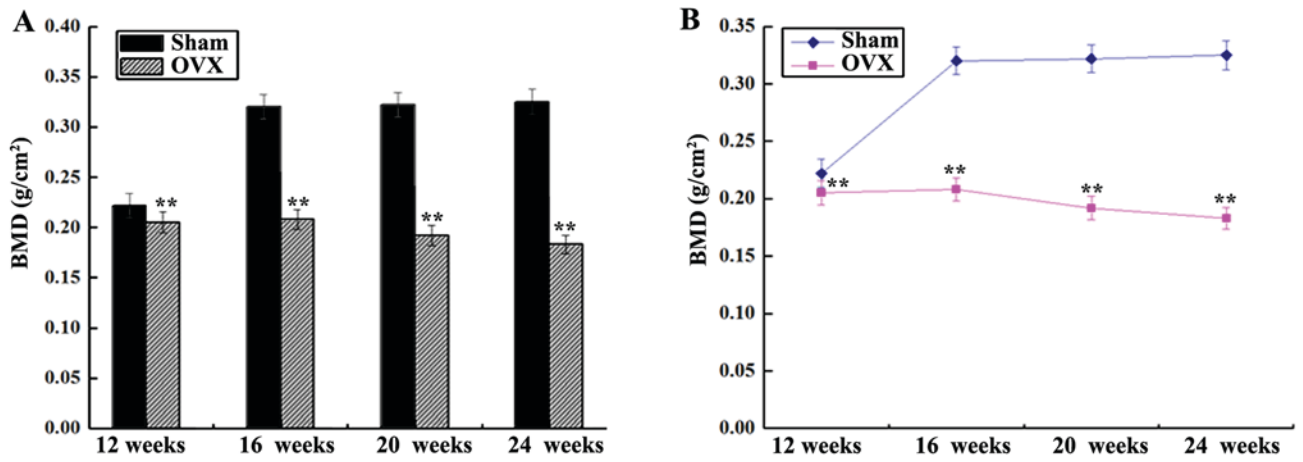

Figure 2. BMD in the right femur of the sham and OVX groups, examined by dual-energy X-ray absorptiometry. (A) Bar graphs and (B) line chart with error bars representing the mean ± standard deviation. **P<0.01 vs. sham group. OVX, ovariectomy; BMD, bone mineral density.

Figure 3. Relative mRNA expression levels of (A) MMP-2, (B) MMP-9 and (C) MMP-13 in the sham and OVX groups were investigated by reverse transcription-quantitative polymerase chain reaction. Bar graphs and line charts are shown, with error bars representing the mean ± standard deviation. *P<0.05, **P<0.01 vs. sham group. MMP, matrix metalloproteinase; OVX, ovariectomy.

Table II. Correlation coefficient (r) value indicating the correlation of BMD with the protein expression levels of MMP-2, MMP-9 and MMP-13 in the OVX group.

Parameter	12 weeks	16 weeks	20 weeks	24 weeks
MMP-2	-0.645[a]	-0.795[a]	-0.733[a]	-0.753[a]
MMP-9	-0.738[a]	-0.687[a]	-0.618[a]	-0.684[a]
MMP-13	-0.602[a]	-0.679[a]	-0.588[a]	-0.745[a]

[a]P<0.01 vs. sham group. MMP, matrix metalloproteinase.

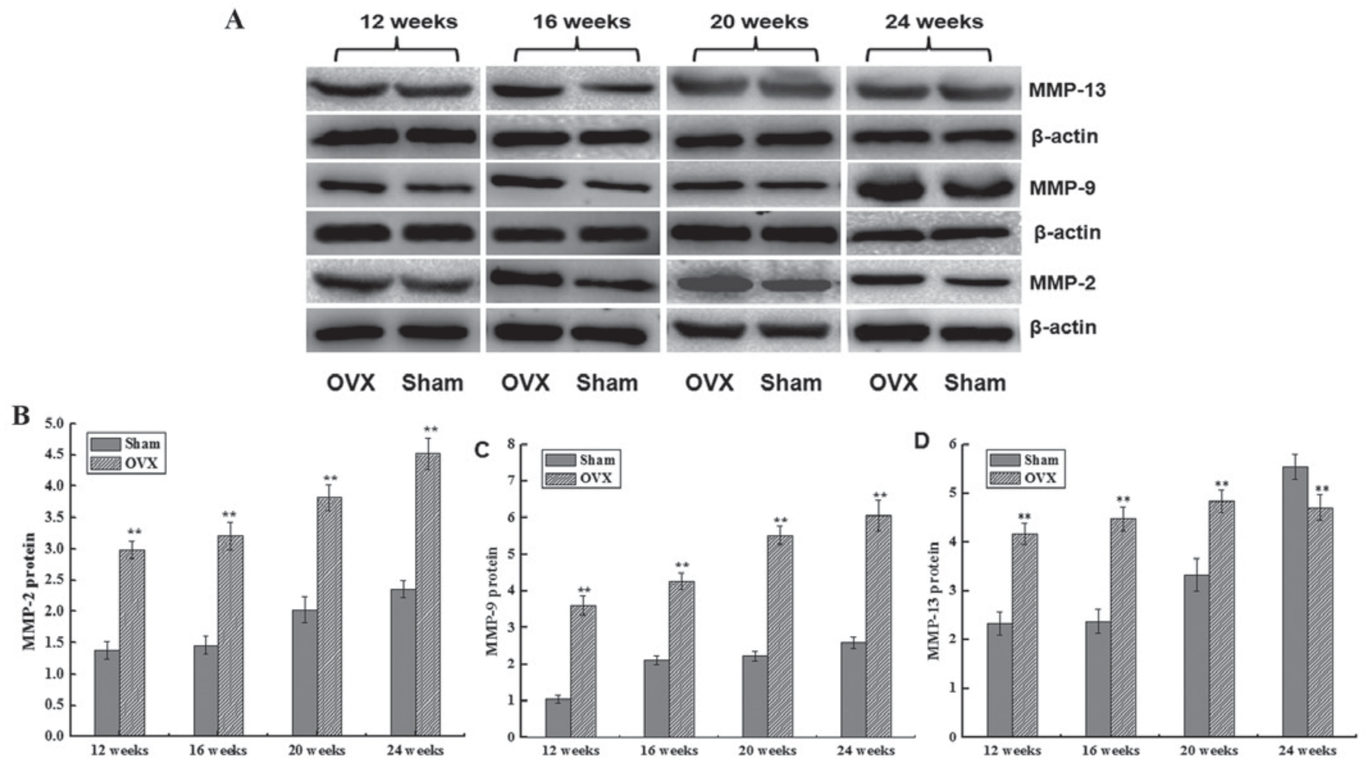

Figure 4. Relative protein expression levels of MMP-2, MMP-9 and MMP-13 in the two groups were investigated by western blotting. (A) Representative protein bands of the MMP proteins are shown. (B) MMP-2, (C) MMP-9 and (D) MMP-13 protein levels demonstrated as bar graphs, with error bars representing the mean ± standard deviation. **P<0.01 vs. sham group. MMP, matrix metalloproteinase; OVX, ovariectomy.

expression, which was significantly higher in comparison with the sham group (P<0.01; Fig. 4). In addition, the MMP-13 expression level initially increased and then slightly decreased in the OVX group, while it was continuously increased in the sham group between 12 and 24 weeks after surgery. A significant difference was detected in MMP-13 expression between the two groups at all time points (P<0.01; Fig. 4). Furthermore, as shown in Table II, a significantly negative correlation was identified between BMD and the expression levels of MMP-2 and MMP-9, while MMP-13 expression was positively correlated with the BMD.

Discussion

Osteoporosis is a bone disease mainly linked to postmenopausal complications, which is characterized by low BMD and micro-damage of the bone structure. The use of ovariectomized rat or mice models is a well-established and reproducible method of simulating the postmenopausal conditions, and has been observed to effectively cause postmenopausal cancellous bone loss over a relatively short period of time (14). In the present study, the ovaries of rats in the OVX group were removed, and this resulted in thinner trabecular bone in the OVX group as compared with that in the sham group (Fig. 1). In addition, the Tb.Ar in the OVX group was significant lower in comparison with that in the sham group (P<0.01; Table I). The BMD of the OVX group was significantly lower compared with that of the sham group between 12 and 24 weeks after the surgery (P<0.01; Fig. 2A), and it was decreased with time (Fig. 2B). A low BMD and destructive trabecular morphology indicated that the osteoporosis model was successfully established in the OVX group.

MMPs are enzymes responsible for the degradation of collagen fibrils. Accumulated evidence indicated that MMPs serve a critical role in osteoclastic bone resorption and facilitate the migration of osteoclasts to bone surfaces via the

extracellular matrix (15). To date, 18 different mammalian MMPs have been identified, including MMP-2, MMP-9 and MMP-13. Decrease of MMP-2, MMP-9 and MMP-13 expression levels is known to reduce the bone resorption (4). However, little is known regarding the dynamic expression of MMP-2, MMP-9 and MMP-13 in the bone during the development of osteoporosis.

MMP-2 is mainly expressed in osteoblasts and osteoclasts, which serve an important role in bone absorption (7). The MMP-2 protein activates bone absorption through enhancing the osteoclast MMP-2 activity and promoting the degradation of the bone matrix (16), or through the degradation of type I collagen barrier under the osteoblast bone cell and activation of osteoclast functions (17). MMP-2 deficiency and the extracellular matrix breakdown defect create an imbalance between bone synthesis and resorption (18). It was thus hypothesized that MMP-2 expression will continue increasing in the OVX group, whereas it will remain low in the sham group. However, the present study results clearly demonstrated that the expression of MMP-2 mRNA and protein increased with time in the two groups. An interesting finding in the present study was that the expression levels of MMP-2 mRNA and protein in the OVX group increased more rapidly as compared with those in the sham group between 20 and 24 weeks after surgery, while they increased at the same rate between 12 and 20 weeks. The possible reason underlying this effect may be that the estrogen level decreased rapidly in osteoporosis, weakening its inhibition effect on MMP-2 activity, and consequently accelerating BMD decrease. As for the sham group, the natural decline of estrogen expression may have led to slow rise of the MMP-2 level with the increase in the age of the rats. With the bone turnover acceleration at the late stage of osteoporosis, the expression of MMP-2 increased sharply between 20 and 24 weeks in the present study, and was observed to be significantly negatively correlated with BMD. Therefore, it is suggested that MMP-2 may serve an important role at the late stage of osteoporosis.

MMP-9 is one of two known gelatinases in the MMP family, along with MMP-2, and shares extensive structural similarities with the MMP-2 gene (19). MMP-9 mainly degrades collagen, integrin protein denaturation and protein polysaccharide. A high expression of MMP-9 in osteoclasts has previously been detected (7). The present study demonstrated that, the levels of MMP-9 mRNA and protein expression in the OVX group were significantly higher in comparison with those in the sham group, which is consistent with the conclusions of a previous study (20). Similar trends were observed for MMP-9 expression as those detected for MMP-2 expression in the OVX group between 12 and 24 weeks after surgery. Notably, an evidently low level of MMP-9 mRNA expression and a relatively high level of MMP-9 protein expression were observed in the sham group between 12 and 16 weeks, which indicated that the expression levels of MMP-9 mRNA and protein were not exactly associated. It has been reported that an adequate expression of MMP-9 is necessary for osteoblast differentiation in the initial phase of osteogenesis (21). In the present study between 16 and 24 weeks after surgery, the level of MMP-9 mRNA expression in the OVX group increased abruptly, while it remained steady in the sham group. The high level of MMP-9 gene expression in the OVX group and very low level in the sham group between 12 and 16 weeks after surgery indicated that MMP-9 may serve as an important marker for the early diagnosis of osteoporosis. In addition, it was observed that MMP-9 expression was negatively correlated with the BMD.

Another notable finding in the current study is that the expression levels of MMP-13 gene and protein in the OVX group were initially increased and then decreased. MMP-13 is one of the most significantly expressed genes in patients with postmenopausal osteoporosis (22). MMP-13 promotes osteoclast precursor cells to differentiate into osteoclasts, and indirectly promotes bone resorption (23). It is known that estrogen is able to regulate the expression of MMP-13; thus, in theory, the decrease of estrogen levels will lead to the significant increase of the expression levels of MMP-13 mRNA and protein with the occurrence of postmenopausal osteoporosis (24). However, according to the findings of the present study, the expression levels of MMP-13 gene and protein continued to increase between 12 and 20 weeks after surgery, but gradually decreased at 24 weeks. By contrast, in the sham group, the expression levels of MMP-13 gene and protein increased sharply, particularly at 24 weeks. These observations suggest that the levels of estrogen decreased, while MMP-13 expression gradually increased at the late phase of postmenopausal osteoporosis. Therefore, it is considered that MMP-13 may serve an important role in the primary bone loss at the early stage of postmenopausal osteoporosis. Due to the reduction of regulatory factors, such as estrogen, during the late stage, MMP-13 and BMD are positively correlated. Notably, the present study demonstrated that MMP-13 expression decreased in the OVX group and was significant lower in comparison with that in the sham group at 24 weeks after surgery ($P<0.05$). However, the mechanisms responsible for this trend in MMP-13 expression remain unclear. It is hypothesized that high or low levels of estrogen may exert a two-way regulation on MMP-13 expression, or other types of hormones and cytokines may be associated with the negative regulation on MMP-13. The mechanisms underlying this effect need to be elucidated through long-term, multi-sample and multi-center studies in the future.

In conclusion, MMP-2, MMP-9 and MMP-13 were demonstrated to regulate the occurrence and development of osteoporosis, and may serve as potent markers in the development of novel prediction and diagnostic methods for postmenopausal osteoporosis. Furthermore MMP-9 may be used as an important marker for the early diagnosis of osteoporosis.

Acknowledgements

Not applicable.

Funding

The current study was supported by grants from the National Natural Science Foundation of China (grant nos. 81574003 and 81674054), the 2015 Strategic Emerging Industries Project of Fujian Province (grant no. 2015Y0069), the Fujian Fuzhou Science and Technology Project (grant no. 2016-S-125-3) and the Science and Technology Project in Fujian Province Department of Education (grant no. JK2015021).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

XZ participated in the design of the study, all experiment procedures and manuscript editing. YZ mainly developed the postmenopausal osteoporosis model and performed the hematoxylin and eosin staining. SG performed the bone mineral density of lumbar vertebra analysis. WZ and JW performed the western blot analysis. YL conceived the study, analyzed the data and prepared the manuscript. All authors read, discussed and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Fujian University of Traditional Chinese Medicine (project no. 20140120), and followed the international guidelines for the Care and Use of Laboratory Animals provided by the National Institutes of Health (Bethesda, MD, USA).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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