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Effects of an antler velvet-based natural compound on osteoporosis in a rodent model

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Abstract

Context. Velvet antlers (VA) are claimed to have the effects on osteoporosis in traditional Chinese medicine.

Aims. So as to scientifically confirm this claim, a VA-based compound (VAC, the mixture of upper part of VA, deer blood, and calcined oyster shell powder in a ratio of 4 : 1 : 1) was produced and administered to osteoporotic model rats, with osteoporosis being induced by retinoic acid via gavage.

Methods. In total, 48 rats were used and divided into six groups (8/group). Concentrations of alkaline phosphatase and osteocalcin in the rat serum were measured, and bone ash weight, concentrations of calcium and phosphorus in rat femur were measured. Bone biomechanical test was performed using a computer-controlled three-point bending tester. Ratio of trabecular bone area to tissue area in a given view field of the femoral tissue section was histologically examined and calculated. Bone histomorphometry was measured using micro-CT.

Key results. No significant difference was found between the VAC-treated groups and the positive control (alendronate sodium) on the basis of the following tested parameters: (1) levels of alkaline phosphatase and osteocalcin in rat serum; (2) maximum load value (N) of femur and maximum compression force of lumbar vertebra; (3) concentrations of calcium and phosphorus in femur; (4) ratio of trabecular bone area to tissue area; and (5) bone histomorphometry.

Conclusions. Effects of VAC used in the present study on osteoporosis in the model rats were comparable to the alendronate sodium (western medicine for treating osteoporosis) on the basis of our selected parameters.

Implications. This compound has the potential to be developed as an effective traditional Chinese medicine for clinic use to treat osteoporosis.

Additional keywords: calcined oyster shell, deer blood, osteoporosis, velvet antler.

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Introduction

Osteoporosis has major negative impacts on the quality of human life and on life expectancy (Szekanecz *et al*. 2019). This disease is a systemic metabolic disease and is characterised by decreased bone mass, density and strength, deteriorated bone microarchitecture, and predisposes the bones to an increased risk of fracture (Vestergaard *et al*. 2007; Anagnostis *et al*. 2019). Currently used drugs for treating osteoporosis in a clinical setting have been reported to have adverse effects (Papaioannou *et al*. 2007), such as aesophageal irritation (Watts and Diab 2010) and an increased risk of venous thromboembolism (Deeks and Dhillon 2010). Therefore, search for a more effective and safer alternative anti-osteoporosis medicine has never ceased (Zhang *et al*. 2009; Tseng *et al*. 2016).

Natural medicines is considered to be a healthier and safer alternative to the synthetic (chemical) ones (Brindha and Parvath 2003). For example, velvet antler (VA) has been found to have significant anti-osteoporotic effects in model rats (Duan *et al*. 2007; Yang *et al*. 2013; Gong and Li 2014; Wang *et al*. 2015; Qu and Gong 2019). Clinical application has also confirmed this finding, namely that VA can effectively ease osteoporotic symptoms (Zhao *et al*. 2003; Wang *et al*. 2004; Wu *et al*. 2015; Ma *et al*. 2019). Treatment with upper and middle parts of VA significantly increase strength of the femur and improve the microarchitecture of the trabecular bone (Tseng *et al*. 2014). There have been no reports of comparisons of efficacy between VA and Western medicine in treating osteoporosis; undeniably, this undesirable situation

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has hindered progress for the development of VA products for treating osteoporosis in clinics.

In the present study, a VA-based natural compound was produced, so as to investigate the effects of VA on osteoporosis in model rats, and the results showed that our VA compound was effective in treating osteoporosis of model rats, and even comparable to Western medicine, alendronate sodium, on the basis of our selected relevant parameters.

Materials and methods

The study was approved by the Animal Ethics Committee of Institute of Special Wild Economic Animals and Plants, Chinese Academy of Agricultural Sciences (Permit number: 2018-0024).

Manufacture of VA-based compound (VAC)

Two sticks of three-branched VAs (65 days after hard-button casting) were obtained from the farmed sika deer (*Cervus nippon*). VA removal was conducted under general anaesthesia (xylazine hydrochloride, 1.5–2.0 mL/100 kg bodyweight). Fresh VA was then divided into two parts vertically, namely, upper and the lower parts (Fig. 1). This division was based on the procedure described by the Agricultural Industry Standards of the People's Republic of China (NY/T 1162 2006). The upper part of VA was used for the subsequent experiments.

The VA was cut into 2-mm-thick slices with a manual VA slicer, and the slices were then ground into ultrafine powder (300 mesh) after being totally dried at 65°C. Deer blood was collected (in the growth period of VA) from the sika deer immediately after slaughter, and ground into ultrafine powder (300 mesh) after being totally dried at 65°C. Calcined oyster shells were purchased from Kangmei Pharmaceutical Co. Ltd (batch number: 170 901 611, Guangzhou, China), and also ground into ultrafine powder (300 mesh). VAC was then prepared by mixing powdered VA, deer blood and calcined oyster shells together at a ratio of 4 : 1 : 1.

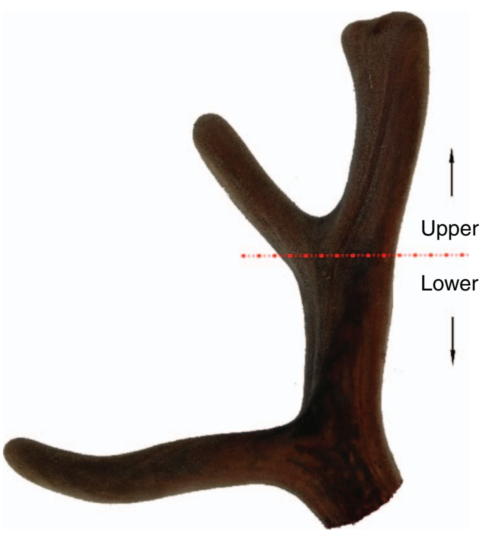

Fig. 1. Demarcation (dotted line) between the upper and the lower parts of a three-branched sika velvet antler. The dividing line is just below the second branch.

Establishment of osteoporotic rat model

Forty-eight 2-month-old female Wistar rats (bodyweight: 190 ± 20 g) were used and randomly allocated into six groups (8 rats/group; Table 1). The first five groups (Groups 1–5) were used to establish the osteoporotic model where the rats were treated with retinoic acid at a dosage of 85 mg/kg.day in 1 mL water (Sun *et al*. 2017; Fan *et al*. 2018) via gavage for 14 days. In Group 6, retinoic acid was substituted with 1 mL water, and this group was the overall intact control. Four weeks following the establishment of the osteoporotic model, each rat from Group 6 was treated with 1 mL of 0.5% carboxy methyl cellulose sodium (CMC–Na) via gavage daily. The negative control group (Group 5) of the osteoporotic model was also treated with 1 mL of 0.5% CMC–Na. The positive control group (Group 4) was treated with 5 mg/kg.day (equivalent to human dose × 6.2, assuming a bodyweight of ~60 kg for an adult person) of alendronate sodium (MSD Pharma Pte Ltd, Singapore; tablets were ground to powder before use). The three VAC-treated groups (Groups 1–3) were given VAC at 0.16 (low), 0.32 (middle) and 0.48 (high) g/kg.day. Dose judgement is a problem in traditional Chinese medicine (TCM) research, especially in TCM compounds, and the doses selected in the present study were based on the following rationale. The dose for traditional use of VA is ~1–2 g daily. Dose of VAC for rats was equivalent to the traditional human-dose regimen conversion where animal dose equals to human dose × 6.2, assuming that the bodyweight of an adult person is ~60 kg (Reagan-Shaw *et al*. 2008). Alendronate sodium and VAC were each dissolved in 1 mL 0.5% CMC–Na before gavage. Bodyweight of the rats was measured weekly. At the end of the experiment, all rats were killed (0.7 mL 10% chloral hydrate/rat), and their blood, organs and bones were kept for further analyses.

Biochemical analysis of rat blood

Blood for biochemical analysis was collected from rat heart immediately after the rat was euthanised, and it was centrifuged to obtain serum. Concentrations of serum alkaline

Table 1. Animal group allocation

Alendronate sodium and velvet antler-based compound (VAC) were dissolved in 1 mL 0.5% carboxy methyl cellulose sodium (CMC–Na) respectively before gavage

Group number	Group	Designation	Dosage
1	Osteoporotic model	VAC-low	0.16 g/kg.day VAC
2		VAC-middle	0.32 g/kg.day VAC
3		VAC-high	0.48 g/kg.day VAC
4		Positive control	5 mg/kg.day alendronate sodium
5		Negative control	1 mL 0.5% CMC–Na
6	Intact	Overall control	1 mL 0.5% CMC–Na

phosphatase and osteocalcin were analysed using ELISA kits (Shanghai Enzyme Biotechnology Co. Ltd, Shanghai, China).

Bone biomechanical testing

Femurs and the fourth vertebrae of the rats were collected and weighed immediately after sampling. Their biomechanical strength was measured by using a computer-controlled three-point bending tester (Jinan Times New Light Instrument Co. Ltd SDW-1, Jinan, China). The bone was placed horizontally on a two-point sample holder (20-mm span), and a load was administered to the centre of the bone at the rate of 2 mm/min until the bone broke. A compression test was performed to measure the maximum compression strength of lumbar vertebra. The unit for the bone-breaking load was Newton (N).

Measurement of bone ash weight, and concentrations of calcium and phosphorus

Femurs from the biomechanical test were reused for further measurement of bone ash, calcium and phosphorus concentrations. Bone samples were weighed, and then transferred to a porcelain crucible, which was then placed in a muffle furnace, and dried at 550°C for over 4 h until a constant weight was reached. The ash weight was determined, and concentrations of calcium and phosphorus in ash were determined according to the methods of the Association of Official Analytical Chemists (Horwitz 2005).

Histological examination

Femurs were collected immediately after the rats were euthanased, fixed in 10% buffered formalin for 2 days, decalcified in 10% formic acid, embedded in paraffin wax, sectioned at 5 μm, stained with haematoxylin and eosin, and examined under a light microscope. The area of femur tissue section was calculated by using Image Pro Plus 6.0 software (Media Cybernetics, Inc., Singapore). Four slices were selected from each group (slice/rat). In total, eight fields of view were randomly selected (2 fields of view/group) and examined under a microscope at a magnification ×40. The ratio of trabecular bone area (Tb.Ar) to tissue area (T.Ar) was calculated.

Micro-CT and bone histomorphometry

Bone morphometry of femoral head (proximal femur) was obtained through CT scanning with a mCT-40 imaging system (Scanco Medical, Bruttisellen, Switzerland), following the procedure described by Zheng *et al*. (2015) and Balbinot *et al*. (2019). A scan length of 12.3 mm extending from the top of the femoral head to the femoral shaft was imaged with a spatial resolution of 27 μm. Three-dimensional structures were then reconstructed. Bone volume ratio (BV : TV) and trabecular separation (Tb.Sp) were measured within the predetermined subchondral bone cylinder as a region of interest (0.5 mm diameter, 2.5 mm height).

Statistical analyses

The data were analysed using Graph Pad Prism 5 software (Graphpad Software, Inc., La Jolla, CA, USA). One-way ANOVA was used for statistical comparisons. Significant value was set at $P < 0.05$. The data are expressed as means ± standard deviation.

Results

Effect of VAC treatments on rat bodyweight and the ratio of organ weight to bodyweight

All of the rats showed increased bodyweight over the period of experiment and there was no significant difference among the different groups at the end of the experiment (Fig. 2). Two weeks later (the period of treatment with retinoic acid), growth rate of bodyweight in the osteoporotic-model groups (Groups 1–5) was higher than that in the overall control group (Group 6). There was no significant difference in the ratio of organ weight to bodyweight among the groups at the end of the treatment (Fig. 3). The results indicated that VAC treatments do not significantly affect rat bodyweight, and the ratio of organ weight to bodyweight.

Effect of VAC treatments on concentrations of alkaline phosphatase and osteocalcin

Alkaline phosphatase concentration (Fig. 4*a*) in serum in Group 5 was significantly higher than that in Group 6

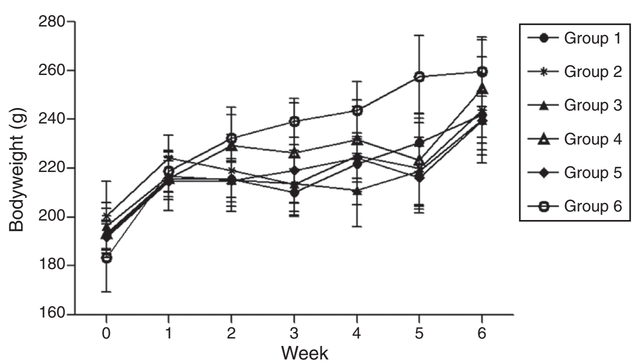

Fig. 2. Change in rat bodyweight over the experimental period. There was no significant difference among the groups at the end of the experiment. For explanation of Groups 1–6, refer to Table 1.

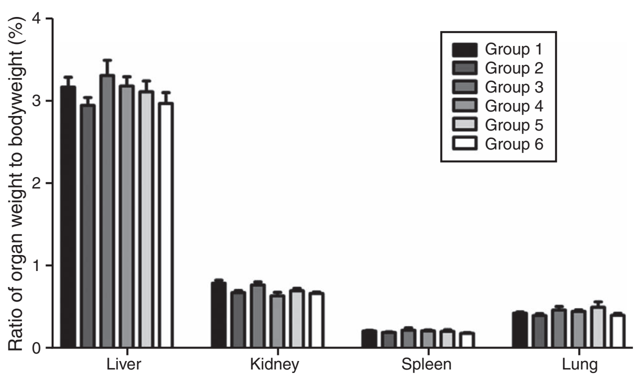

Fig. 3. Comparison of the weight ratios (organ to body) among all six groups. There was no significant difference in the ratio of organ weight to bodyweight, suggesting that treatments with velvet antler-based compound (VAC) do not affect rat growth. For explanation of Groups 1–6, refer to Fig. 2 and Table 1.

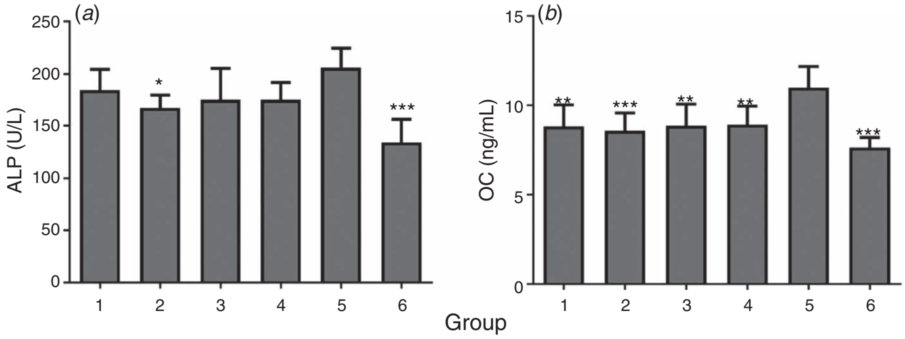

Fig. 4. Comparison of (*a*) alkaline phosphatase (ALP) and (*b*) osteocalcin (OC) concentrations among all six groups. Note that the concentrations of ALP and OC between Group 4 and the groups treated with velvet antler-based compound (VAC) were not significantly different. Serum ALP concentration in Group 5 was significantly ($P < 0.001$) higher than those in Group 6 and Group 2. For explanation of Groups 1–6, refer to Fig. 2 and Table 1. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

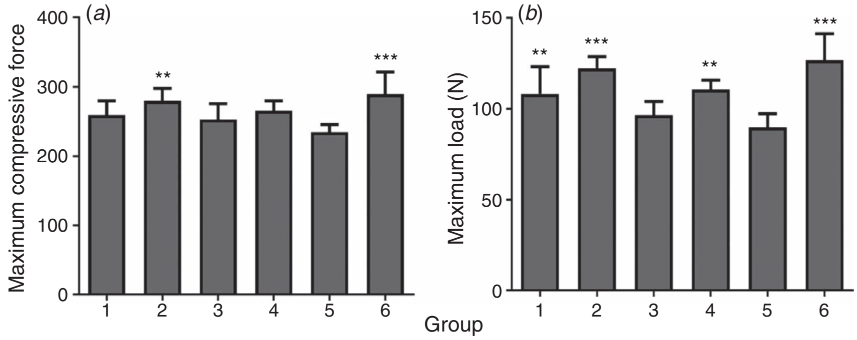

Fig. 5. Comparison of bone biomechanics among all six groups. Note that there was no significant difference in (*a*) maximum load (N) of femur and (*b*) maximum compression force of lumbar vertebra between Group 4 and the groups treated with velvet antler-based compound (VAC). Values of both maximum load and compression force in Group 2 were significantly higher than those in Groups 1 and 3. For explanation of Groups 1–6, refer to Fig. 2 and Table 1. **, $P < 0.01$; ***, $P < 0.001$.

($P < 0.001$) and Group 2 ($P < 0.05$). Osteocalcin concentration in the serum of Group 5 (Fig. 4*b*) was significantly ($P < 0.001$ or $P < 0.01$) higher than that in other groups. There was no significant difference in alkaline phosphatase and osteocalcin concentrations in serum between Group 4 and the VAC-reated groups (Fig. 4). The results indicated that VAC treatments can effectively decrease concentrations of alkaline phosphatase and osteocalcin, which are the markers for osteoporosis.

Effect of VAC treatments on rat-bone biomechanics

Maximum load value (N) of femur in Group 5 was significantly lower than those in Group 6 ($P < 0.001$), Group 4 ($P < 0.01$), Group 1 ($P < 0.01$) and Group 2 ($P < 0.001$; Fig. 5*a*). Maximum compression force (Fig. 5*b*) of lumbar vertebrae in Group 5 was significantly lower than those in Group 6 ($P < 0.001$) and Group 2 ($P < 0.01$) (Fig. 5*d*). There was no significant difference in maximum load of femur and maximum compression force of lumbar vertebrae between Group 4 and the VAC-treated groups. The results indicated that VAC treatments can effectively increase bone biomechanics in model rats.

Effect of VAC treatments on wet femur weight and ash content

Wet femur weight in Group 5 was significantly lower than those in Groups 6 ($P < 0.001$), 4 ($P < 0.05$), 2 ($P < 0.05$) and 3 ($P < 0.01$). Ash content of femur in Group 5 was significantly lower than that in Groups 6 ($P < 0.05$), 4 ($P < 0.01$), 1 ($P < 0.05$) and 2 ($P < 0.01$). There was no significant difference in wet femur weight and ash content between Group 4 and the VAC-treated groups (Fig. 6). The results indicated that VAC treatments can significantly increase wet femur weight and ash content.

Effect of VAC treatments on calcium and phosphorus concentrations of femur

Calcium concentration in femur in Group 5 was significantly lower than that in Groups 6 ($P < 0.001$), 4 ($P < 0.001$) and

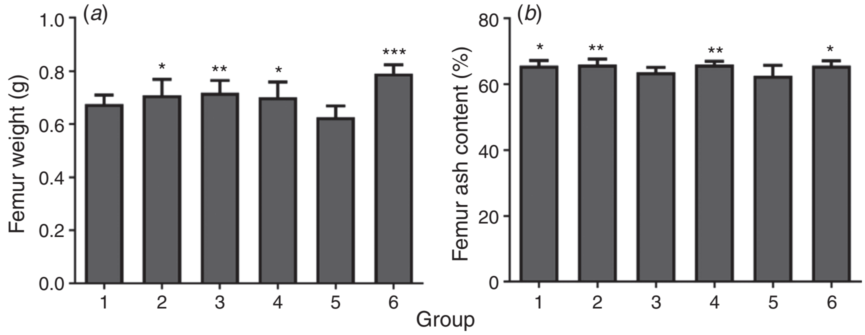

Fig. 6. Comparison of wet femur weight and femur ash weight among all six groups. There was no significant difference in wet femur weight and ash weight between Group 4 and the groups treated with velvet antler-based compound (VAC). For explanation of Groups 1–6, refer to Fig. 2 and Table 1. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

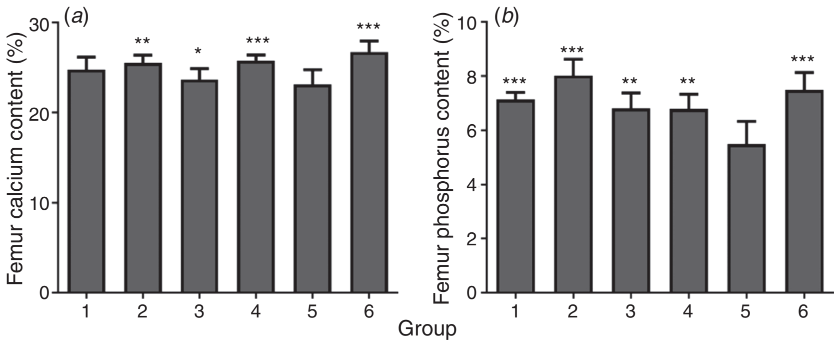

Fig. 7. Comparison of femur (*a*) calcium and (*b*) phosphorus concentrations in rat femur among all six groups. Note that the phosphorus concentration in Group 2 was significantly higher than that in Group 4. For explanation of Groups 1–6, refer to Fig. 2 and Table 1. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

2 ($P < 0.01$). There was no significant difference in calcium concentration in femur between Group 4 and the VAC-treated groups, except Group 3, which had a lower calcium concentration than did Group 4 ($P < 0.05$; Fig. 7*a*). Phosphorus concentration in femur in Group 5 was significantly ($P < 0.001$ or $P < 0.0001$) lower than that in the other groups. There was no significant difference in phosphorus concentration of femur between Group 4 and the VAC-treated groups, except Group 2, which had a higher concentration than did Group 4 ($P < 0.01$; Fig. 7*b*). The results indicated that VAC treatments can effectively increase calcium and phosphorus concentrations in femur.

Effects of VAC treatments on histological structure and the ratio of Tb.Ar to T.Ar

Unlike femurs of the VAC-treated groups (Fig. 8*a*, *b*, *c*), Group 4 (Fig. 8*d*) and Group 6 (Fig. 8*f*), femurs in Group 5 showed typical characteristics of osteoporosis (Fig. 8*e*); trabecular bone became sparse, space between trabecular bones became larger, trabecular bone became disintegrated, and ratio of Tb.Ar to T.Ar was significantly decreased ($P < 0.001$; Fig. 9). There was no significant ($P > 0.05$) difference in the ratio of Tb.Ar to T.Ar between Group 4 and Group 2, whereas the ratio in Groups 1 and 3 was significantly lower than that in Group 4 ($P < 0.001$). The results indicated that VAC treatments can effectively improve microstructure and increase the ratio of Tb.Ar to T.Ar.

Effect of VAC treatments on BV : TV and Tb.Sp in bone histomorphometry

Femoral head architecture was assessed using micro-CT (Fig. 10); in the Group 5 (Fig. 10*d*), spaces between trabecular bones were found to be bigger and there was less trabecular tissue. These phenomena were reversed by the administration of VAC (Fig. 10*a*) in Group 2 or alendronate sodium (Fig. 10*b*) in Group 4. In Groups 2 and 4, BV : TV (Fig. 11*a*) significantly ($P < 0.01$) increased, and Tb.Sp (Fig. 11*b*) significantly ($P < 0.05$) decreased compared with those in Group 5. There was no significant differences in BV : TV and Tb.Sp between Group 2 and Group 4. Increase of BV : TV and decrease of Tb.Sp in Group 2 and Group 4 indicated an increase in bone mass in the model rats.

Discussion

According to the theory of TCM, osteoporosis is caused by kidney deficiency and blood stasis (Li *et al*. 2009; Liu and Li

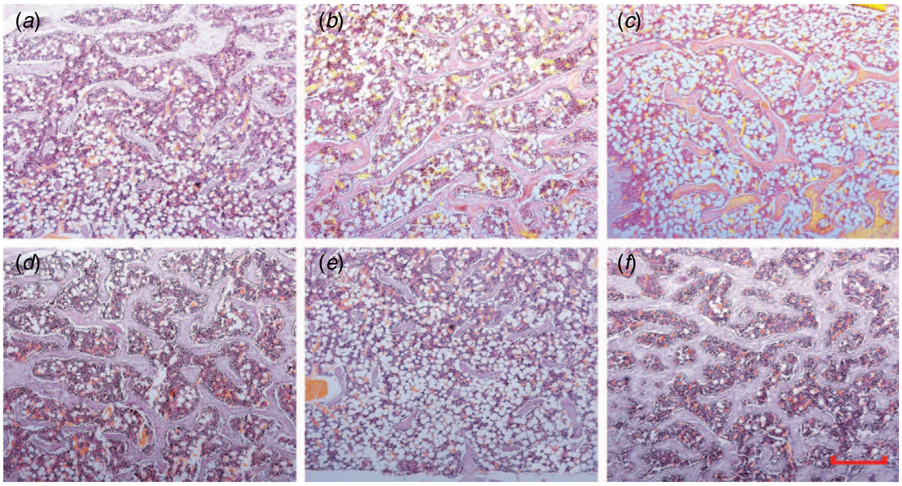

Fig. 8. Histological sections of the rat femur. Bone trabecular spaces in (*a*) Group 1 and (*c*) Group 3 were more sparse than those in (*b*) Group 2, which showed values similar to those in (*d*) Group 4. (*e*) Trabecular bone became more sparse, space between trabecular bones became wider, and trabecular bone became disintegrated, indicating typical characteristics of osteoporosis in Group 5. (*f*) Trabecular bone structure was integrated in Group 6. Scale bar = 1000 μm.

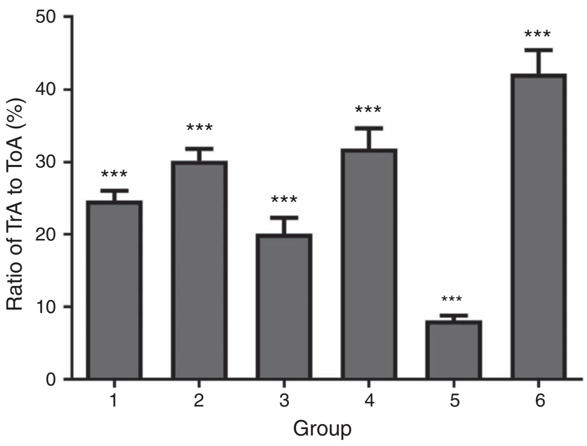

Fig. 9. Comparison of ratios (the ratio of trabecular bone area (Tb.Ar) to tissue area (T.Ar)) among all six groups. Note that ratios of Tb.Ar to T.Ar in Groups 1 and 3 were significantly lower than that in Group 4. However, there was no significant difference in the ratio between Group 2 and Group 4. For explanation of Groups 1–6, refer to Fig. 2 and Table 1. ***, $P < 0.001$.

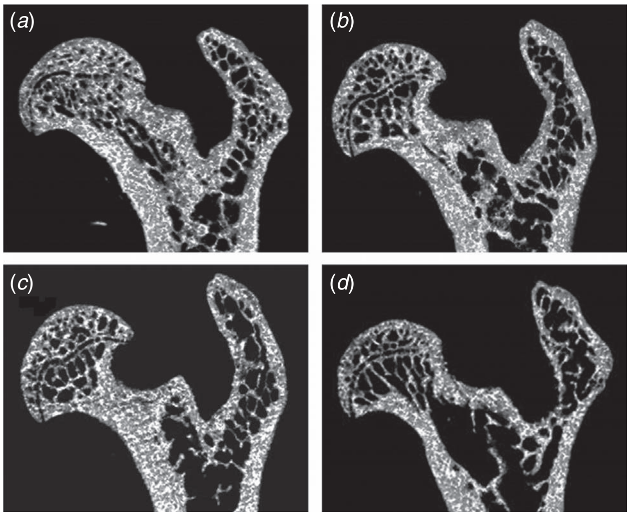

Fig. 10. Microarchitecture of the femoral head scanned with micro-CT. Note that treatments using (*a*) velvet antler-based compound (VAC) to rats in Group 2 or (*b*) alendronate sodium to rats in Group 4 restored the microarchitecture of bone similar to the extent of (*c*) Group 6, i.e. they reversed the osteoporosis symptoms induced by retinoic acid, compared with (*d*) the model group (Group 5).

2016). Implementation of symptomatic treatment, such as reinforcing kidney function and invigorating the circulation of blood, would have significant effects on osteoporosis (Liu and Li 2016). VA has been used in the eastern Asia for over 2000 years to reinforce kidney function and strengthen bones (Kawtikwar *et al*. 2010; Wu *et al*. 2013), and is used as an anti-osteoporotic component in many TCM prescriptions (Wang *et al*. 2004, 2015; Li *et al*. 2009; Meng *et al*. 2009; He *et al*. 2015; Sun *et al*. 2015; Liu and Li 2016).

It has been reported that VA can stimulate bone formation, increase bone mass and density, and, thus, achieve the purpose of easing symptom of osteoporosis (Duan *et al*. 2007; He *et al*. 2015; Qu and Gong 2019). Deer blood is reported to have the pharmacological activity of anti-blood stasis (Huang *et al*. 2017; Shi *et al*. 2017; Dong *et al*. 2018; Wang and Chen 2019). Calcined oyster shell is often being used in TCM as a complementary material for treatment of osteoporosis (Hu and Zheng 2009), possibly due to it being rich in $CaCO_3$ (Cao *et al*. 2018) and, so, providing an additional calcium source. In the present study, VA, deer blood and calcined oyster shell were selected and mixed together to make a VAC for the treatment of osteoporosis in the model rats. The overall results showed that the VAC treatments effectively restored

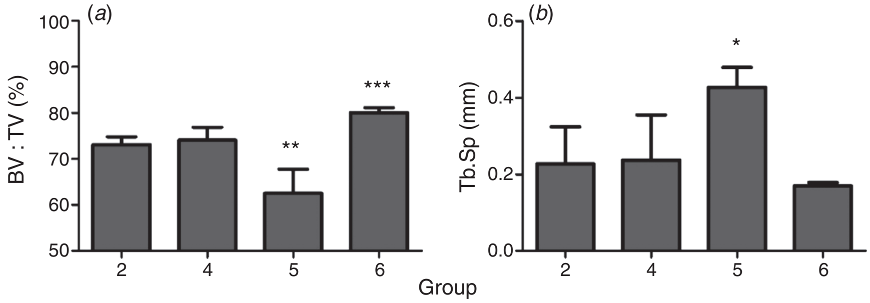

Fig. 11. Comparisons of Group 2 with Groups 4, 5, 6 in (*a*) bone volume ratio (BV : TV) and (*b*) trabecular separation (Tb.Sp). Note that Groups 2 and 4 increased in BV : TV ratio and decreased in Tb.Sp significantly compared with Group 5. There was no significant difference in ratios of BV : TV and Tb.Sp between Group 2 and Group 4. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 2. Concentration of chemical components in the upper part of a three-branched sika velvet antler (VA)

AA, total amino acid; SP, water-soluble protein; PL, total phospholipid; Ph, phospholipids; Nu, nucleoside; Po, polyamine; Ch, cholesterol; Ca, calcium; P, phosphorus; Mg, magnesium (Sun *et al*. 2018; He *et al*. 2019; Wang *et al*. 2019)

Concentration (%)									
AA	SP	PL	Ph	Nu	Po	Ch	Ca	P	Mg
56.3–73.9	0.55–0.94	0.14–0.59	0.12–0.26	0.07–0.11	0.14–0.93	0.07–0.18	0.24–9.94	0.89–5.74	0.06–0.23

the bone structure of osteoporotic rats, and the middle dose of VAC was found to be the best. On the basis of our selected parameters, effects of VAC on osteoporosis are comparable to those of alendronate sodium, a bisphosphonate drug that is being used for the treatment of osteoporosis in clinics. No obvious side effects (such as, for example, aesophageal irritation and increasing risk of venous thromboembolism) of using VAC were detected in the present study.

The efficacy of VAC on osteoporosis in the present study must be derived from its bioactive components and factors. Main chemical compositions (Table 2) of VA are reported to consist of proteins, steroids, carbohydrates and minerals, among others (Sun *et al*. 2018; He *et al*. 2019; Wang *et al*. 2019). Both polysaccharides and polypeptides have therapeutic effects on osteoporosis of rats induced by ovariectomy (Gong *et al*. 2019). A previous study showed that deer antler growth factors (extracted as a protein from deer growing-phase antlers) increased the number and viability of human alveolar osteoblasts significantly (Laky *et al*. 2009). Besides, multiple bioactive factors are also the integral part of VA, such as tropomyosins (Tpm1, 2 and 4), WD repeat-containing protein 1 (Wdr1), alpha-actinin-1 (Actn1), destrin (Dstn), alpha-2-macroglobulin (A2 m), serine protease inhibitor A3 N (Serpina3n) and apolipoproteins (Apoh and Apof). These factors interact with each other and ultimately achieve the final outcome of anti-osteoporosis (Yao *et al*. 2019).

Alkaline phosphatase concentration is high in patients with bone disorders such as Paget's disease or osteomalacia (Mukaiyama *et al*. 2015). Osteocalcin is released from the bone matrix into the blood stream during bone resorption, thereby osteocalcin is a marker of bone turnover (Neve *et al*. 2013). In the present study, retinoic acid treatment reduced bone biomechanics and increased concentrations of serum alkaline phosphatase and osteocalcin in the model rats, compared with the corresponding concentrations observed in control rats (Group 6). In contrast, VAC treatments reduced concentrations of serum alkaline phosphatase and osteocalcin, and increased bone biomechanics, compared with the respective values observed in Group 6. In addition, VAC treatments reduced the degree of osteoporosis by increasing wet weight, and ash, calcium and phosphorus concentrations, and the ratio of Tb.Ar to T.Ar in the rat femurs. On the basis of our results in the present study, VAC may exert its bone-protective effects through reducing bone resorption.

Conclusions

Velvet antler-based compound used in the present study is a mixture of upper VA, deer blood and calcined oyster shell powders at the ratio of 4 : 1 : 1, and was found to have significant effects on osteoporosis, which was induced by administration of retinoic acid to rats. The effects of VAC treatment on osteoporosis was found to be comparable to those of alendronate sodium (Western medicine for treating osteoporosis) on the basis of our selected parameters. The optimal dose for using VAC to treat osteoporosis of model rats was found to be 0.32 g/kg.day in the present study.

Conflicts of interest

The authors declare no conflicts of interest.

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