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Full Length Research Paper

Preventive and therapeutic effects of antler collagen on osteoporosis in ovariectomized rats

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The present study was aimed at evaluating the preventive and therapeutic effects of collagen extracted from Sika deer velvet on osteoporosis in ovariectomized rats. Histomorphometric indices, serum biochemical parameters and biomechanical properties were measured in ovariectomized rats treated with/without antler collagen and in sham-operated rats. Compared with the ovariectomized group, significant elevation in the levels of bone mineral density (BMD), nitrogen monoxide (NO), NO synthase, bone-Ca, bone-P, bone morphogenetic protein (BMP), trabecular bone volume (TBV), number of trabecular (N), mean trabecular plate thickness (MTPT), and biomechanical properties, while reduction in the level of serum alkaline phosphatase (ALP), and mean trabecular plate separation (MTPS) were observed in antler collagen-treated groups. The extracted collagen was found to play a role in the prevention and treatment of osteoporosis in ovariectomized rats.

Key words: Antler, collagen, osteoporosis, preventive, therapeutic.

INTRODUCTION

Osteoporosis is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to bone fracture. Reduction in the secretion of sex hormones is one of the important causes of osteoporosis. Hormone replacement therapy and bone resorption inhibitors such as selective estrogen receptor modulators, bisphosphonates, and calcitonin are widely used in the treatment and prophylaxis of post-menopausal osteoporosis. Sika deer velvet, an efficient traditional medicine for strengthening bones and tendons, has been used for thousands of years in China, but its substance foundations for use in curing diseases has not yet been elucidated. Sika deer velvet has a complex chemical composition (Kim, et al., 1999; Wang, et al., 2003; Feng, et al., 1997; Hemmings and Song, 2004; Banks and Newbrey, 1983; Chen, et al., 2004), such as amino, protein, vitamin, lipid, mucoitin, microelement, mineral and etc (Wang, 2006); crude protein accounts for more than 50% of the dry weight of plum velvet, and collagen is the major component of the crude protein. Collagen occurs ubiquitously in mammals in organs such as the skin, bone, joint cartilage, blood vessels, tendons, and even teeth. Due to factors such as low immunogenicity, absorption, and cell growth promotion ability, collagen has been widely used in the pharma-ceutical industry and medical research and has many clinical applications (Kawahara et al., 2007; Li et al., 2007; Ponsioen et al., 2008).

However, it has been approved that collagen gotten from different materials different in many aspects (Jiang, 2006). To investigate the chemical basis for the efficacy of collagen extracted from Sika deer velvet to strengthen bones and tendons, we evaluated the preventive and therapeutic effects of antler collagen on osteoporosis.

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Abbreviations: BMD, Bone mineral density; **NOS,** nitric monoxide synthase; **BMP,** bone morphogenetic protein; **TBV,** trabecular bone volume; **N,** number of trabecular; **MTPT,** mean trabecular plate thickness; **ALP,** alkaline phosphatase; **MTPS,** mean trabecular plate separation; **SHAM,** sham-operated group; **OVX,** ovariectomy group; **OVX + N,** ovariectomy with nilestriol treatment group; **OVX + L,** ovariectomy with low-dose antler collagen treatment group; **b-Ca,** bone calcium; **b-P,** bone phosphorous; **H and E,** hematoxylin and eosin stain.

MATERIALS AND METHODS

Chemicals

Nilestriol was purchased from Shanghai medicine group; pento

barbital sodium was obtained from Shanghai chemical reagent factory; bone morphogenetic protein (BMP) radioimmunoassay (RIA) kit, nitric monoxide synthase (NOS) kit, and Ca kit were obtained from Nanjing Jiancheng biological engineering research institute. All the chemicals were of analytical grade and used without further purification.

Instruments

GF-D800 Semi-automatic biochemical analyzer (Caihong Company, ShangDong), UVmini-1240 spectrophotometer (Japan), EG 1150H tissue embed machine (LEICA Germany), and dual-energy X-ray absorptiometer (DEXA, Italy) were used in the present study.

Preparation of collagen

After removing the skin and flesh, the antlers of fresh Sika deer velvet (500 g) provided by Dongfong medicine pharmaceutical factory (Jilin, China) were cut into 1-cm^3 pieces and washed with distilled water at 4℃; subsequently, the pieces were homogenized. The homogenate was incubated in 1000 ml of distilled water for 48 h at 60℃ and then centrifuged. The supernatant was dialyzed against distilled water for 24 h (molecular weight cut-off of 8 ~ 15 kDa). The dialysate was lyophilized and stored at -20℃ until further use.

Rat osteoporosis model

All the rats were anesthetized using 0.4% pentobarbital sodium at doses of 50 mg/kg body weight. Bilateral ovariectomies were performed in the rats via a dorsal approach (Xu et al., 2006). Sham operation was performed using the same procedure, but without removing the ovaries.

Experimental design

Fifty female wistar rats (weight, 220 ± 10 g; 3 months) obtained from the Changchun Gaoxin experimental animal center (Changchun, China) were randomly divided into the following 5 groups (n = 10, for each group): sham-operated group (SHAM), ovariectomy group (OVX), ovariectomy with nilestriol treatment group (OVX + N), ovariectomy with high-dose antler collagen treatment group (OVX + H), and ovariectomy with low-dose antler collagen treatment group (OVX + L). After 15 days of surgery, the OVX + N group was orally administered nilestriol at a dose of 0.36 $mg \cdot kg^{-1}$ every 2 weeks, and the OVX + H and OVX + L groups were orally administered antler collagen at doses of 0.5 $g \cdot kg^{-1} \cdot d^{-1}$ and 0.1 $g \cdot kg^{-1} \cdot d^{-1}$, respectively. The SHAM and the OVX groups received normal saline (0.9%) orally at the same volume. The treatment was continued for 90 days.

Preparation of the samples

Blood samples were collected from the abdominal aorta of the experimental rats, and the serum was immediately separated by centrifugation (4℃) at 1,500 g for 15 min and stored at -20℃. The right femurs and the third lumbar vertebrae were resected, the adhering connective tissue, cartilage, spinous processes, and articular processes were removed, and the bones were vacuum dried for 24 h. The third lumbar vertebrae were decalcified 3 times using trichloroacetic acid (24 h each time), and the decalcifying fluid was pooled for further use.

Measurement indicators

Bone mineral density (BMD) was determined by dual-energy X-ray absorptiometer. The levels of bone calcium (b-Ca), bone phosphorous (b-P) serum alkaline phosphatase (ALP), BMP, nitrogen monoxide (NO) and NO synthase were detected by using the corresponding kits. The mean trabecular plate thickness (MTPT, μm), mean trabecular plate separation (MTPS, μm), trabecular bone volume (TBV, %), and other histomorphometric parameters ratio were measured by using the BI-2000 system after the rats have been sacrificed, left femur taken, fixed in 10% formalin, decalcified in 10% formic acid, dehydrated using graded ethanol, vitrified by dimethylbenzene, paraffin section, hematoxylin and eosin (H and E) stained, at 100 times, under the metaphyseal cortical growth plate near 1 - 4 mm, to obtain vision range measurements. The left femur was used in calculating the bone weight coefficient and bone biomechanical testing, the maximum load, maximum deformation, bone stress and strain. All the statistical tests were performed using Microsoft Excel. Statistical significance of the difference between the groups was tested by unpaired Student's *t* tests. $P < 0.05$ was considered as statistically significant.

RESULTS

Amino acid analysis

As shown in Table 1, glycine (32.24%) was the major component in the amino acid hydrolysates of the extracted collagen. Proline and alanine accounted for 12.64 and 10.78% of the extracted collagen, respectively. The levels of histidine and tyrosine were low, that is, 0.57 and 0.21%, respectively. Cystine was not detected; hydroxyproline was also a component of the extracted hydrolysate, and the ratio of hydroxyproline to proline was 0.8, which was almost similar to that of type I collagen (0.7 ~ 0.8) (Jiang, 2006).

BMD and b-Ca

As shown in Table 2, compared to the SHAM group, the BMD value and the bone-Ca (b-Ca) level were lower ($P < 0.001$), and the b-P was lower ($P < 0.05$) in the OVX group. Compared to the OVX group, the BMD values of both the antler collagen-treated groups ($P < 0.01$ or $P < 0.05$) and the OVX + N group were higher ($P < 0.001$). The b-Ca was higher($P < 0.05$) than that of the OVX group in the OVX + H , OVX+L , and OVX + N groups .The b-P level was higher ($P < 0.05$) than that of the OVX group in the OVX + H , and OVX + N groups .

Biochemical markers

Table 3 shows that the levels of BMP, NO, and NO synthase were lower ($P < 0.01$), but the level of ALP was higher ($P < 0.05$) in the OVX group than in the SHAM group. Compared to the OVX group, the levels of NO and NO synthase were higher and that of ALP were lower in both the collagen-treated groups and the OVX + N group.

Table 1. Results of amino acid analyze of velvet antler collagen from sika deer.

Amino acid	Content (%)	Amino acid	Content (%)
Gly	32.24	Leu	2.59
Pro	12.64	Thr	2.33
Ala	10.78	Val	2.22
q-Pro	10.20	Phe	1.22
Glu	8.46	Ile	1.08
Asp	4.55	His	0.57
Arg	4.42	Tyr	0.21
Ser	3.56	Met	—
Lys	2.94	Cys	—

Table 2. BMD, b-Ca and b-P levels.

Target Group	g/kg	n	BMD (g/cm^2)	b-Ca (mmol/L)	b-P (mmol/L)
SHAM	—	8	0.185 ± 0.044	9.5 ± 1.1	62 ± 15
OVX	—	8	0.063 ± 0.014$^{\Delta\Delta\Delta}$	7.7 ± 0.4$^{\Delta\Delta\Delta}$	82 ± 14$^{\Delta}$
OVX + N	0.00036	8	0.127 ± 0.013***	8.7 ± 1.7*	80 ± 16*
OVX + H	0.5	8	0.084 ± 0.010**	8.9 ± 1.2*	79 ± 14*
OVX + L	0.1	8	0.080 ± 0.017*	8.8 ± 1.4*	72 ± 15

Values have been indicated as mean ± standard deviation; *P < 0.05 versus OVX ; **P < 0.01 versus OVX ; ***P < 0.001 versus OVX; Δ P < 0.05 versus SHAM; ΔΔΔ P < 0.001 versus SHAM.

Table 3. Biochemical parameters.

Group	g/kg	n	ALP	BMP (ng/mL)	NO (μmol/L)	NO synthase (μg/mL)
SHAM	—	8	200 ± 42	2.2± 0.32	54± 0.88**	58 ± 3.9**
OVX	—	8	303 ± 86$^{\Delta\Delta}$	1.6 ±0.22$^{\Delta\Delta}$	24± 4.9	38 ± 4.6
OVX + N	0.00036	8	205 ± 37*	2.1± 0.28**	30± 2.6*	41 ± 5.8*
OVX + H	0.5	8	228 ± 103*	1.9± 0.31*	39± 6.9**	49 ± 9.0**
OVX + L	0.1	8	218 ± 72*	1.7 ± 0.22	30± 3.8*	47 ± 3.3**

Values have been indicated as mean ± standard deviation; *P < 0.05 versus OVX; **P < 0.01 versus OVX; ΔΔP < 0.01 versus SHAM.

BMP level in the OVX + N group and the OVX + H group was higher than that in the OVX group. However, the BMP level in the OVX + L group did not differ significantly from that of the OVX group.

Bone tissue and morphogenetic indices

The values of histomorphometric indices in the OVX group were lower than those in the SHAM group. Compared to the OVX group, there was no marked difference in the MBCT and the MTPT in the collagen-treated groups and the OVX + N group, but MTPT in the OVX+N groups. On the other hand, MTPS, and number of trabecular values in the OVX + H, the OVX+L and OVX + N groups were higher than those in the OVX group. The TBV was higher in the OVX+N (P < 0.01) and OVX+H (P < 0.05) groups (Table 4 and Figure 1).

Biomechanical properties

The values of biomechanics indices in the OVX group were lower than those in the SHAM group. Compared to the OVX group, there was marked difference in maximum load (M-N), maximum deflection (M-D), bone stress and bone strain in the collagen-treated groups and the SHAM group (P < 0.05 or P < 0.01) (Table 5).

Table 4. Histomorphometric parameters.

Target Group	g/kg	n	MBCT (μm)	MTPS (μ)	TBV (%)	Number of bone trabecula	MTPT (μm)
SHAM	—	8	12.19±0.15*	9.5±1.0***	36.79 ± 3.2***	3.55 ±0.69***	8.33 ± 0.21**
OVX	—	8	12.00±0.20$^{\Delta}$	16.9 ±1.6$^{\Delta\Delta\Delta}$	19.43 ±4.6$^{\Delta\Delta\Delta}$	0.64 ±0.50	8.10 ±0.12$^{\Delta\Delta}$
OVX + N	0.00036	8	12.15±0.27	13.1±1.5***	26.20 ±4.5**	2.00±0.77***	8.23 ± 0.13*
OVX + H	0.5	8	12.06±0.27	14.1±2. 8**	24.82 ± 5.6*	1.69±0.63***	8.20 ± 0.16
OVX + L	0.1	8	12.01±0.28	14.3 ±3.0*	24.20 ± 7.8	1.55 ± 1.04*	8.17 ± 0.13

Values have been indicated as mean ± standard deviation; *P < 0.05 versus OVX; **P < 0.01 versus OVX; ΔP<0.05 versus SHAM; ΔΔP < 0.01 versus SHAM; ΔΔΔP < 0.001 versus SHAM.

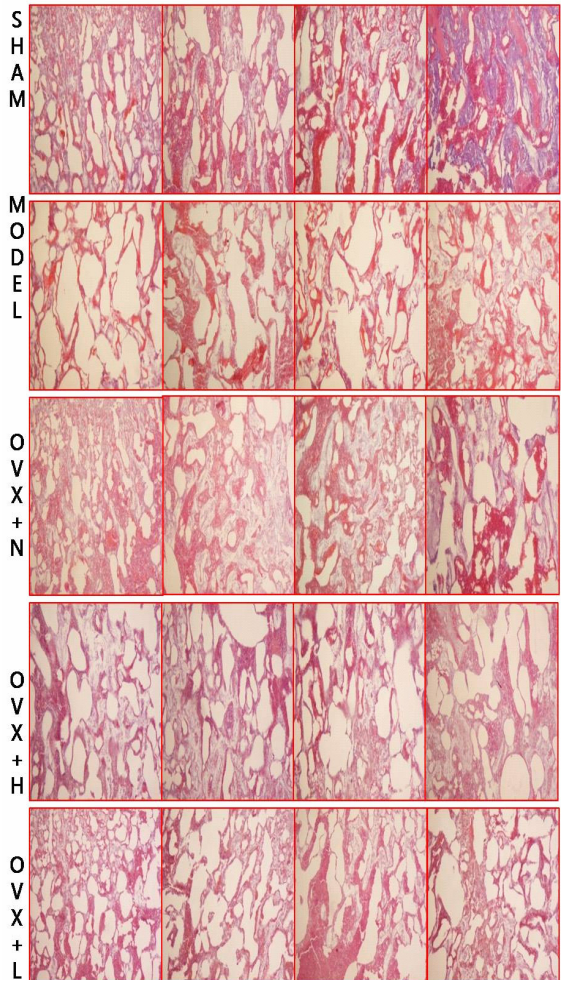

Figure 1. Effects of CSDV on static histomorphometric indexes of OP rats.

DISCUSSION

The osteoporosis model was established successfully in the present study. The values of BMD, bone-Ca, biochemical parameters, histomorphometric parameters and biomechanical properties were lowered in the OVX group than in the SHAM group. After 90 days of treatment, there was a marked increase in the BMD, bone-Ca, biochemical parameters, histomorphometric parameters and biomechanical properties in the collagen-treated groups compared with that in the OVX group. These results indicate that Sika deer velvet antlers play a role in the treatment and/or prevention of osteoporosis in OVX rats.

Human bones constituted of bone mineral and bone matrix; the ratio is about 2:1 (Jiang, 1987). The main component of bone mineral is Ca; the main component of bone matrix is collagen. Osteoporosis is mainly due to the loss of Ca and collagen degradation. As the result show, the level of BMD and the bone–Ca and P in collagen-treated rats increased (P < 0.01, P < 0.05), when the level of ALP and BMP was adjusted (P < 0.05; P < 0.01). Descriptions of these indicators show that the collagen increases the bone mineral level of OVX rats markedly. The above results show that rats treated with collagen were meliorated at corresponding indicators, and the bone matrix level in OVX rats, and collagen was well absorbed. The absorbing of collagen amends the indicators of bone biomechanics, as maximum load, maximum deflection and bone strain were higher and enhanced while brittleness was lowered in other to achieve the purpose of the prevention of osteoporosis.

The first step in the formation of new bone is collagen synthesis, which interlaced with each one another, while forming bone matrix net. Next, hydroxyl calcium phosphate deposits in the net. Hence, collagen is important for bone growth and bone development. It has been reported in literature that hydroxyproline, the characteristic collagen amino acid, was carrier absorption of calcium; it was thus concluded that hydroxyproline in collagen promote the absorption of calcium (Li et al., 2000). Through our experiments, collagen content of hydroxyproline to the total amino acid content was about 30% (Table 1), while collagen is the most abundant protein content above 50% in deer velvet. The results indicate that, deer antler and collagen may be used to prevent and treat osteoporosis and increase bone density through the use of hydroxyproline.

Furthermore, experiments have been carried out to show that collagen promote osteoblast in rat proliferation. The results showed that the collagen treated group proliferate more quikly than none treated group (Li et al.,

Table 5. Biomechanical properties.

Target Group	g/kg	n	M-N (N)	M-D (mm)	Bone-stress (N/mm^2)	Bone-strain (%)
SHAM	—	8	141.2±26.2**	0.88±0.27*	141.4±21.1**	4.60±1.24**
OVX	—	8	$105.1±23.3^{\Delta\Delta}$	$0.66±0.15^{\Delta}$	$112.7±25.2^{\Delta\Delta}$	$3.29±0.70^{\Delta\Delta}$
OVX + N	0.00036	8	128.1±21.7*	0.82±0.17*	146.9±29.1**	4.31±0.75**
OVX + H	0.5	8	127.5±26.0*	0.83±0.21*	139.4±27.1*	4.21±1.00**
OVX + L	0.1	8	122.9±17.9*	0.80±0.13*	135.5±19.1*	4.09±0.74*

Values have been indicated as mean ± standard deviation; *P < 0.05 versus OVX; **P < 0.01 versus OVX; ΔP < 0.05 versus SHAM; ΔΔP < 0.01 versus SHAM.

2009). These show that collagen act most likely through the promotion of osteoblast proliferation to prevent osteoporosis. The levels of NO and NO synthase, which are responsible for the activation of many cytokines, were also measured. The results showed that the levels of NO and NO synthase in the OVX group were significantly lower than those of the SHAM group, and those of the antler collagen-treated groups. Collagen treatment had a remarkable impact on the levels of NO and NO synthase. This suggests that the antiosteoporotic effect of antler collagen was mediated by the upregulation of NO and NO synthase that led to the stimulation of osteoblast proliferation and inhibition of osteoclast activity.

In summary, the Sika deer velvet collagen could significantly increase BMD and prevent osteoporosis induced by estrogen deficiency in ovariectomized rats. Antler collagen may thus have potential therapeutic applications in the treatment of osteoporosis.

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