

11 β -hydroxysteroid dehydrogenases as targets in the treatment of steroid-associated femoral head necrosis using antler extract

RIBUSURONG PU¹ and HAO PENG²

¹Orthopaedics Department, The Affiliated Hospital of Inner Mongolia Medical University, Hohhot, Inner Mongolia 010050;

²Orthopaedics Department, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060, P.R. China

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Abstract. The aim of the present study was to investigate the therapeutic effect of deer antler extract on avascular necrosis of the femoral head (ANFH) induced by steroids, and to confirm that 11 β -hydroxysteroid dehydrogenases (11 β -HSD) are one of the targets of treatment with antler extract. A total of 30 rabbits were randomly divided into 5 groups (n=6): A control, ANFH, ANFH + antler (250 mg/kg), ANFH + antler (500 mg/kg) and ANFH + antler (1,000 mg/kg) group. Rabbits in the experimental groups were injected with methylprednisolone and horse serum to establish a steroid-induced ANFH (SANFH) model. Rabbits in the ANFH + antler (250 mg/kg), ANFH + antler (500 mg/kg) and ANFH + antler (1,000 mg/kg) groups were treated with intraperitoneal injection of 250, 500 or 1,000 mg/kg antler extract/day, respectively, for 60 days. Serum samples were then extracted to determine total cholesterol (CT) and triglyceride levels, treat osteoblasts, measure 11 β -HSD (11 β -HSD1) and 11 β -HSD2 and alkaline phosphatase (ALP) levels and cellular apoptosis, and determine the proportion of osteoblasts in each phase of the cell cycle. Serum CT and triglyceride levels in SANFH rabbits significantly decreased as the concentration of antler increased (P<0.05). 11 β -HSD1 levels in the femoral heads of SANFH rabbits and osteoblasts following treatment with antler-containing serum decreased as the concentration of antler used increased, whereas levels of 11 β -HSD1 increased significantly (P<0.05). The proliferation of osteoblasts and ALP levels in osteoblasts increased as the antler concentration increased, whereas the number of osteoblasts in the G0/G1 phase decreased significantly (P<0.05). The current study demonstrated that treatment with antler extract has a therapeutic effect on ANFH induced by steroids in rabbits and may regulate the expression

of 11 β -HSD in femoral heads and osteoblasts, as well as promoting the proliferation of osteoblasts.

Introduction

Osteonecrosis of the femoral head (ONFH) is caused by the interruption of the blood supply to the femoral head or the pathological process of osteoblast death (1,2). Steroid-induced avascular necrosis of the femoral head (SANFH) is a type of non-traumatic ANFH and the most common type of femoral head necrosis. It occurs following the long-term use of adrenocorticotrophic hormones (ACTH) or the use of high doses of ACTHs (3-5). The major clinical characteristics of ANFH include hip pain, limited mobility, lameness and a high disability rate, which negatively impact patient health and quality of life (6-8). It is estimated that ~50% of all ONFH cases are induced by ACTH (5,9,10). In China, there are 5-7.5 million patients with ONFH requiring treatment and the number of new ONFH cases was 150,000-200,000 in 1998, with the majority of cases diagnosed in young adults (11).

Corticosteroids are widely used to treat several diseases, including asthma (12), nephritic syndrome (13) and leukemia (14), as they suppress inflammation, allergic reactions and the immune response. However, in the process of producing marked improvements in the symptoms of these diseases, corticosteroids induce certain adverse effects, including growth retardation (15), obesity (16), osteoporosis (17), hyperglycemia (18), osteonecrosis (19), cataracts (20) and Cushing's syndrome (21), which limits their use clinically. Therefore, increasing attention has been given to identifying methods of treating corticosteroid-induced ANFH (22-25).

Currently, patients with advanced ANFH receive hip arthroplasty, whereas those at the pre-collapse stage receive salvage surgery; however, for patients with non-traumatic ANFH, as well as those for whom surgery would be high-risk, non-surgical treatment is more appropriate (26). Numerous studies have been conducted to investigate the potential of novel drugs, including statins (27) and alendronate (28) to treat ANFH without surgery; however, to date, their therapeutic effects have been unsatisfactory. For many years, Traditional Chinese Medicine (TCM) has been widely used to treat different diseases involving the bone, and deer antlers are often used in TCM (29,30). The outside of the antlers consist of skin covered with hair, while the inner antler is made up

Correspondence to: Professor Hao Peng, Orthopaedics Department, Renmin Hospital of Wuhan University, 99 Zhangzhidong Road, Wuchang, Wuhan, Hubei 430060, P.R. China
E-mail: penghaowuda@163.com

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of connective tissue, cartilaginous tissue, blood vessels and abundant nerves (31). It has been reported that antlers may have preventive and therapeutic effects on osteoporosis (32) and may also help with sperm production and strengthen the muscles and bones (33). However, there have been few papers investigating the therapeutic effect of deer antler extracts on femoral head necrosis.

The 11β -hydroxysteroid dehydrogenase (11β -HSD) family of enzymes, which includes 11β -HSD1 and 11β -HSD2, catalyze the interconversion of active glucocorticoids (34). Previous studies have demonstrated that 11β -HSD1 and 11β -HSD2 influence the function of adipocytes and endotheliocytes (35,36). Adipocyte and endotheliocyte dysfunction may cause blood flow in the terminal vessels to become abnormal and induce complications, including intravascular coagulation, microcirculation disturbance and vascular embolization, which in turn may lead to femoral head necrosis (37). The phenomenon indicates that 11β -HSD may be important in the development of SANFH.

The current study used antler extract to treat rabbit with SANFH and measured the expression of 11β -HSD to confirm that 11β -HSD was one of the targets in the treatment of SANFH using antler extract.

Materials and methods

Antler preparation. Mature deer antlers were obtained from Beijing Tong Ren Tang Chinese Medicine Co., Ltd., (Beijing, China). A total of 200 g antler was dried, milled and sliced, and then extracted 4 times by dissolving in 1 l distilled water and boiling for 5 h. Following filtration with 3 levels of sterile gauze (TZSB; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), the liquid containing the antler extract was concentrated at 20% by boiling for 5 h and stored at 4°C for 3 days.

Animals. A total of 30 male New Zealand rabbits, 4 months old and weighing 2.6–3.2 kg, were obtained from the Experimental Animal Center of the Affiliated Hospital of Inner Mongolia Medical University (Hohhot, China). Rabbits were housed in an animal chamber maintained at $22\pm 2^\circ\text{C}$ with a relative humidity of $50\pm 5\%$ and a 12/12 h light/dark cycle. Access to food and water was *ad libitum*. All animal experiments in the present study were approved by the Animal Care and Use Committee of the Affiliated Hospital of Inner Mongolia Medical University (Hohhot, China) and followed the ethical guidelines set by the European Community guidelines (38).

Establishment of SANFH rabbit model. SANFH rabbit models were established following a previously described method (39,40). The 30 healthy New Zealand rabbits were randomly divided into 5 groups ($n=6$): A control, ANFH, ANFH + antler (250 mg/kg), ANFH + antler (500 mg/kg) and ANFH + antler (1,000 mg/kg) group. All rabbits received an injection of 10 ml/kg horse serum (100%; Thermo Fisher Scientific, Inc., Waltham, MA, USA) via the ear vein for 2 weeks. Subsequently, 6 ml/kg/day horse serum was injected for 2 days.

Rabbits in the experimental groups received an intraperitoneal injection of 20 mg/kg methylprednisolone (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) twice

a week, over a 2-week period. Rabbits in the control group were intraperitoneally injected with the same volume of saline. Subsequently, 200,000 U penicillin was injected into the buttock of each rabbit. After 2 weeks, 1 rabbit in each group was randomly selected to confirm whether the SANFH model was successfully established by performing a femoral head histopathological examination. The femoral head was separated and fixed with 2% glutaraldehyde at 4°C overnight (Beijing Solarbio Science & Technology Co., Ltd.), decalcified with 5% nitric acid (Beijing Solarbio Science & Technology Co., Ltd.), dehydrated with a graded series of ethanol (70, 80, 90, 95 and 100%) embedded in paraffin and cut into 5 μm slices using a microtome (HM 355S; Thermo Fisher Scientific, Inc.). Sections were subsequently dewaxed with xylene, rehydrated with a graded series of ethanol (100, 95, 90, 80 and 70%) and stained with hematoxylin at 25°C for 10 min and eosin at 25°C for 1 min. Stained sections were observed under a light microscope (magnification, $\times 200$).

Following confirmation of successful establishment of the SANFH model, the antler extract was dissolved in distilled water to form final concentrations of 250 mg/kg, 500 mg/kg and 1,000 mg/kg. The different concentrations of antler extracts were administered to rabbits in the ANFH + antler (250 mg/kg), ANFH + antler (500 mg/kg) and ANFH + antler (1,000 mg/kg) groups, respectively, every day for 60 days by intraperitoneal injection. Rabbits in the control and ANFH groups were intraperitoneally injected with the same volume of saline.

CT and triglyceride determination. Following treatments, all rabbits were anesthetized with a combination of xylazine (6 mg/kg) and ketamine (40 mg/kg; both Seebio Biotech Co., Ltd., Shanghai, China) according to previous report and sacrificed by cervical dislocation (41). Blood was collected from the ear vein in EDTA tubes and centrifuged at 4°C and $5,000 \times g$ to separate serum. CT and triglyceride concentrations in the blood samples of all rabbits were measured using an automatic biochemistry analyzer (Siemens AG, Munich, Germany).

Histopathological examination. Femoral heads, including the metaphyses and thighbones, of all the rabbits were collected and cut into sections. Sections were fixed in 2% glutaraldehyde (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at 25°C for 24 h. Sections were then embedded in paraffin and cut into slices 5 μm thick. Following deparaffinization and rehydration with graded ethanol, sections were stained with hematoxylin and eosin (Thermo Fisher Scientific, Inc.). Histopathological changes of the femoral heads were observed under a light microscope (Olympus Corporation, Tokyo, Japan).

Osteoblast culture. A total of 2 male newborn New Zealand rabbits, 10 days old, weight 20.17 ± 1.03 kg, were obtained from the Experimental Animal Center of the Affiliated Hospital of Inner Mongolia Medical University and sacrificed as described above. Rabbits were housed in an animal chamber maintained at $22\pm 2^\circ\text{C}$ with a relative humidity of $50\pm 5\%$ and a 12 h light/dark cycle with access to food and water *ad libitum*. Primary osteoblasts were obtained from newborn rabbit calvarias. Tissues

Table I. Total cholesterol and triglycerides in the serum of rabbits with or without SANFH.

Groups	Total cholesterol (mmol/l)	Triglycerides (mmol/l)
Control	1.22±0.78	0.85±0.32
SANFH	2.58±0.90 ^a	1.27±0.60 ^a
SANFH + antler (250 mg/kg)	2.16±0.63 ^{a,b}	1.09±0.44 ^{a,b}
SANFH + antler (500 mg/kg)	1.72±0.59 ^{a,c}	0.97±0.58 ^{b,c}
SANFH + antler (1,000 mg/kg)	1.57±0.81 ^{b,c}	0.92±0.49 ^{b,c}

Data are presented as the mean ± standard deviation. n=25. ^aP<0.05 vs. control group; ^bP<0.05 vs. SANFH group, P<0.05; ^cP<0.05 vs. SANFH + antler (250 mg/kg) group. SANFH, steroid-associated femoral head necrosis.

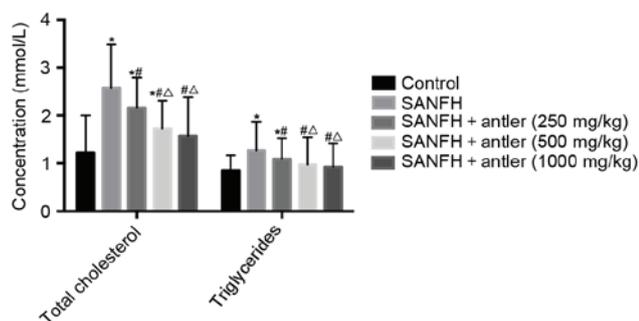


Figure 1. Total cholesterol and triglycerides in the serum of rabbits with or without SANFH following treatment with different concentrations of antler extract. *P<0.05 vs. control group; #P<0.05 vs. SANFH group; ΔP<0.05 vs. SANFH + antler (250 mg/kg) group. SANFH, steroid-induced avascular necrosis of the femoral head.

were digested with trypsin 3 times and incubated with type I collagenase and 0.25% trypsin-EDTA in Hank's buffer solution (all Thermo Fisher Scientific, Inc.). The tissues were then cut into pieces <0.5 mm³ and placed on the inner surface of the dish to release the cells. Cells were cultured with Dulbecco's Modified Eagle medium (DMEM; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 U/ml streptomycin (Sigma-Aldrich; Merck KGaA) at 37°C in 5% CO₂ for 24 h. The tissues were then washed with 1X PBS to discard unattached cells. The medium was replenished every 2-3 days and cells were passaged until 90% confluence was reached.

Antler serum treatment. Osteoblasts were seeded at 1x10⁶/ml and cultured in a 96-well plate. Cells from rabbits in the 5 groups including the control, ANFH, ANFH + antler (250 mg/kg), ANFH + antler (500 mg/kg) and ANFH + antler (1,000 mg/kg) groups and there were 3 replicates from each group. A total of 40 μl methylprednisolone solution (1 μmol/l) was added to the ANFH, ANFH + antler (250 mg/kg), ANFH + antler (500 mg/kg) and ANFH + antler (1,000 mg/kg) groups, while 40 μg/DMEM was added to the cells in the control group. Following incubation for 24 h, medium was replenished and

serum samples collected from the rabbits (1:40) were added to the corresponding group of cells for 24 h.

Western blotting. Total proteins from the femoral head tissue or the cells treated with antler serum were extracted using radio-immunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.) and measured using the BCA method (Thermo Fisher Scientific, Inc.). A total of 75 μg protein was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 10% separating gel and 5% stacking gel. Following SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane (Merck KGaA). The membrane was blocked with 5% skimmed milk at 25°C for 2 h. Rabbit anti-11β-HSD1 and rabbit anti-11β-HSD2 primary antibodies (1:1,000, Wuhan Sanyang Biotechnology, Wuhan, China) were incubated with the membrane at 4°C overnight. Membranes were washed with PBS 3 times for 7 min. Membranes were incubated with goat anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove PA, USA) at 25°C for 1 h. β-actin was used as an internal control. An electrochemiluminescence system (Thermo Fisher Scientific, Inc.) was used for exposure.

Alkaline phosphatase (ALP) detection. Following treatment with antler serum for 72 h, 20 μl 0.05% Triton X-100 was added to each well and incubated at 25°C for 5 min to induce cell lysis. An ALP assay kit (AP0100-1KT; Sigma-Aldrich; Merck KGaA) was used to detect ALP levels in the blood serum and osteoblasts. A total of 980 μl (blank) and 960 μl (test and control) of reaction buffer was pipetted into cuvettes and 20 μl of 0.67 M pNPP solution was added to each cuvette and equilibrated at 37°C. Subsequently, 20 μl of sample was added to each test cuvette, and 20 μl of diluted ALP solution was added to the enzyme control cuvette. Cuvettes were mixed by inversion and the A₄₀₅ nm was recorded (SpectraMax i3X plant reader; Molecular Devices, LLC, Sunnyvale, CA, USA). Obtained the maximum linear rate (ΔA₄₀₅ nm/minute).

Measurement of cell proliferation. Following treatment with antler serum for 72 h, 10 μl cell counting kit-8 (Thermo Fisher Scientific, Inc.) was added to each well and incubated at 37°C for 20 min. An automatic biochemistry analyzer was used to measure the optical density (OD) value at 450 nm.

Cell cycle analysis. Following treatment with antler serum for 72 h, cells were collected and fixed in pre-cooled 70% ethanol at 4°C overnight. A Cell Cycle and Apoptosis Analysis kit (C1052, Beyotime Institute of Biotechnology) was used to assess the cell cycle according to the manufacturer's protocol. A flow cytometer (Cytomics FC 500; Beckman Coulter, Inc., Brea, CA, USA) was used to detect and determine the proportion of cells in each phase of the cell cycle.

Statistical analysis. All data were analyzed using SPSS 19.0 software (IBM Corp, Armonk, NY, USA) and presented as the mean ± standard deviations. One-way ANOVA followed by Dunnett's test was used for comparison and analysis between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

CT and triglyceride concentration. CT and triglyceride levels in the blood serum of the rabbits with or without SANFH are presented in Table I and Fig. 1. The serum CT levels of the rabbits in the SANFH, SANFH + antler (250 mg/kg) and SANFH + antler (500 mg/kg) groups (2.58 ± 0.90 mmol/l, 2.16 ± 0.63 mmol/l and 1.72 ± 0.59 mmol/l) were significantly higher ($P < 0.05$) than that of the control (1.22 ± 0.78 mmol/l). Serum CT levels in the SANFH + antler (1,000 mg/kg) group (1.57 ± 0.81) mmol/l were higher than that of the control, but not significantly. Furthermore, serum CT levels in the SANFH + antler (250 mg/kg), SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups were all significantly lower than that of the SANFH group (all $P < 0.05$) and serum CT levels in the SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups were significantly lower than that of the SANFH + antler (250 mg/kg) group ($P < 0.05$). Serum CT levels did not differ significantly between the SANFH + antler (1,000 mg/kg) and SANFH + antler (500 mg/kg) groups (Fig. 1 and Table I).

Serum triglyceride levels of the rabbits in the SANFH and SANFH + antler (250 mg/kg) groups (1.27 ± 0.60 mmol/l and 1.09 ± 0.44 mmol/l, respectively) were significantly higher ($P < 0.05$) than that of the control (0.85 ± 0.32 mmol/l). However, serum triglyceride levels in the SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups (0.97 ± 0.58 mmol/l and 0.92 ± 0.49 mmol/l, respectively) did not differ significantly from those in the control. Serum triglyceride levels in the SANFH + antler (250 mg/kg), SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups were all significantly lower than those in the SANFH group ($P < 0.05$). Furthermore, serum triglyceride levels in the SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups were significantly lower than those in the SANFH + antler (250 mg/kg) group ($P < 0.05$). Levels of serum triglycerides did not differ significantly between the SANFH + antler (1,000 mg/kg) and SANFH + antler (500 mg/kg) group.

11 β -HSD expression. The expression of 11 β -HSD1 and 11 β -HSD2 in the rabbit tissues (Fig. 2) and osteoblasts (Fig. 3) are also presented in Table II.

In the rabbit tissues, relative 11 β -HSD ($P < 0.05$), SANFH + antler (250 mg/kg) and SANFH + antler (500 mg/kg) groups (0.49 ± 0.05 , 0.40 ± 0.07 and 0.33 ± 0.04 , respectively) were all significantly higher than that of the control (0.28 ± 0.08 ; $P < 0.05$). However, 11 β -HSD1 expression in the SANFH + antler (1,000 mg/kg) group (0.31 ± 0.07) did not differ significantly from that of the control. Furthermore, the expression of 11 β -HSD1 in the SANFH + antler (250 mg/kg), SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups were all significantly lower than that of the SANFH group ($P < 0.05$). Levels of 11 β -HSD1 in the SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups were also significantly lower than in the SANFH + antler (250 mg/kg) group ($P < 0.05$). There were no significant difference in the expression of 11 β -HSD1 in SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups (Fig. 2; Table II).

In the rabbit tissue, 11 β -HSD2 in both experimental groups was significantly lower than in the control group (0.29 ± 0.03 ;

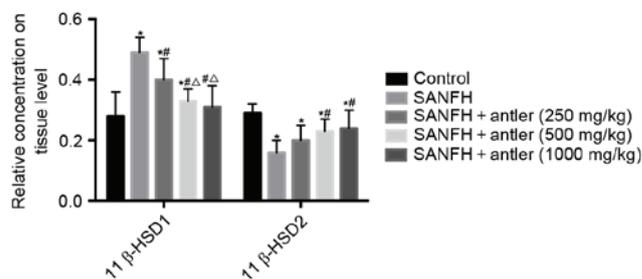


Figure 2. Relative expression of 11 β -HSD1 and 11 β -HSD2 in the rabbits' femoral head following different treatment with different concentrations of antler. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. SANFH group; $\Delta P < 0.05$ vs. SANFH + antler (250 mg/kg) group. 11 β -HSD, 11 β -hydroxysteroid dehydrogenases; SANFH, steroid-induced avascular necrosis of the femoral head.

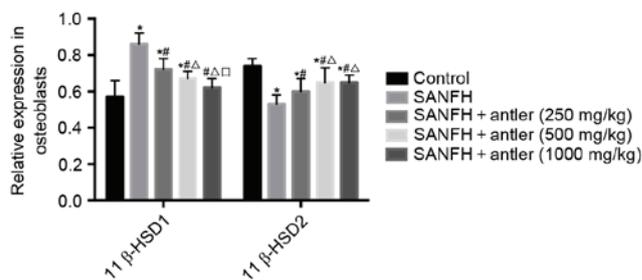


Figure 3. Relative expression of 11 β -HSD1 and 11 β -HSD2 in rabbit osteoblasts following treatment with different concentrations of antler extract. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. SANFH group; $\Delta P < 0.05$ vs. SANFH + antler (250 mg/kg) group; $\square P < 0.05$ vs. SANFH + antler (500 mg/kg) group. 11 β -HSD; 11 β -hydroxysteroid dehydrogenase; SANFH, steroid-induced avascular necrosis of the femoral head.

$P < 0.05$). Levels of 11 β -HSD2 in the SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups (0.23 ± 0.04 ; 0.24 ± 0.06 , respectively) were significantly higher than in the SANFH group (0.16 ± 0.04 ; $P < 0.05$). There were no significant differences in 11 β -HSD2 between the SANFH and SANFH + antler (250 mg/kg) groups, as well as between in SANFH + antler (500 mg/kg) and in SANFH + antler (1,000 mg/kg) groups (Fig. 2 and Table II).

In osteoblasts, 11 β -HSD1 levels in the SANFH, SANFH + antler (250 mg/kg) and SANFH + antler (500 mg/kg) groups (0.86 ± 0.06 , 0.72 ± 0.06 and 0.67 ± 0.04 , respectively) were all significantly higher than those of the control (0.57 ± 0.09 ; $P < 0.05$). However, 11 β -HSD1 levels in the SANFH + antler (250 mg/kg), SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups were all significantly lower than that of the SANFH group ($P < 0.05$). Furthermore, 11 β -HSD1 levels in the SANFH + antler (1,000 mg/kg) group were significantly lower than those of the SANFH + antler (500 mg/kg) group ($P < 0.05$; Fig. 3).

In osteoblasts, levels of 11 β -HSD2 in all experimental groups were significantly lower than that of the control (0.74 ± 0.04 ; $P < 0.05$). 11 β -HSD2 levels in the SANFH + antler (250 mg/kg), SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups (0.60 ± 0.07 , 0.65 ± 0.08 and 0.65 ± 0.04 , respectively) were all significantly higher than that of the SANFH (0.53 ± 0.05) group ($P < 0.05$). 11 β -HSD2 levels in the SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups were significantly higher than of the

Table II. Relative expression of 11 β -HSD1 and 11 β -HSD2 in the rabbit femoral heads and osteoblasts.

Groups	Tissues		Osteoblasts	
	11 β -HSD1	11 β -HSD2	11 β -HSD1	11 β -HSD2
Control	0.28 \pm 0.08	0.29 \pm 0.03	0.57 \pm 0.09	0.74 \pm 0.04
SANFH	0.49 \pm 0.05 ^a	0.16 \pm 0.04 ^a	0.86 \pm 0.06 ^a	0.53 \pm 0.05 ^a
SANFH + antler (250 mg/kg)	0.40 \pm 0.07 ^{a,b}	0.20 \pm 0.05 ^a	0.72 \pm 0.06 ^{a,b}	0.60 \pm 0.07 ^{a,b}
SANFH + antler (500 mg/kg)	0.33 \pm 0.04 ^{a-c}	0.23 \pm 0.04 ^{a,b}	0.67 \pm 0.04 ^{a-c}	0.65 \pm 0.08 ^{a-c}
SANFH + antler (1000 mg/kg)	0.31 \pm 0.07 ^{b,c}	0.24 \pm 0.06 ^{a,b}	0.62 \pm 0.05 ^{b-d}	0.65 \pm 0.04 ^{a-c}

Data are presented as the mean \pm standard deviation. ^aP<0.05 vs. control group; ^bP<0.05 vs. SANFH group; ^cP<0.05 vs. SANFH + antler (250 mg/kg) group; ^dP<0.05 vs. SANFH + antler (500 mg/kg); SANFH, steroid-associated femoral head necrosis. 11 β -HSD; 11 β -hydroxysteroid dehydrogenases.

Table III. Effect of antler-containing serum on ALP levels, proliferation and the cell cycle in osteoblasts.

Groups	Proliferation (%)	ALP (U/l)	Cell cycle (%)		
			G0/G1	S	G2/M
Control	0.53 \pm 0.07	0.64 \pm 0.12	63.39 \pm 4.51	28.04 \pm 4.50	8.57 \pm 0.95
SANFH	0.28 \pm 0.12 ^a	0.25 \pm 0.16 ^a	75.60 \pm 3.88 ^a	22.05 \pm 3.63	2.35 \pm 1.14
SANFH + antler (250 mg/kg)	0.33 \pm 0.07 ^{a,b}	0.43 \pm 0.09 ^{a,b}	70.25 \pm 4.06 ^{a,b}	25.37 \pm 3.84	4.38 \pm 0.87
SANFH + antler (500 mg/kg)	0.37 \pm 0.12 ^{a,b}	0.56 \pm 0.11 ^{a-c}	68.77 \pm 2.96 ^{a,b}	26.29 \pm 2.17	4.94 \pm 0.93
SANFH + antler (1,000 mg/kg)	0.40 \pm 0.17 ^{a-c}	0.58 \pm 0.13 ^{a-c}	67.12 \pm 3.58 ^b	26.96 \pm 4.11	5.92 \pm 1.06

Data are presented as the mean \pm standard deviation. ^aP<0.05 vs. control group, P<0.05; ^bP<0.05 vs. SANFH group; ^cP<0.05 vs. SANFH + antler (250 mg/kg) group; ALP, alkaline phosphatase; SANFH, steroid-associated femoral head necrosis.

SANFH + antler (250 mg/kg) group (P<0.05). However, 11 β -HSD2 levels did not differ significantly between the SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups (Fig. 3; Table II).

ALP levels and osteoblast proliferation. The ALP concentration (U/l) and osteoblast proliferation rate (%) are presented in Table III and Fig. 4. The proliferation rates of the osteoblasts in all experimental groups were significantly lower than that of the control group (0.53 \pm 0.07; P<0.05). The proliferation rate of the SANFH + antler (250 mg/kg), SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups (0.33 \pm 0.07, 0.37 \pm 0.12 and 0.40 \pm 0.17, respectively) were all significantly higher than that of the SANFH (0.28 \pm 0.12) group (P<0.05). The proliferation rate of the SANFH + antler (1,000 mg/kg) group was significantly higher than that of the SANFH + antler (250 mg/kg) group (P<0.05) but did not differ significantly from that of the SANFH + antler (500 mg/kg) group (Fig. 4; Table III).

ALP levels in all experimental groups were significantly lower than in the control (0.64 \pm 0.12; P<0.05). ALP in the SANFH + antler (250 mg/kg), SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups (0.43 \pm 0.09, 0.56 \pm 0.11 and 0.58 \pm 0.13, respectively) were all significantly higher than in the SANFH group (0.25 \pm 0.16; P<0.05). ALP in the SANFH + antler (500 mg/kg) and

SANFH + antler (1,000 mg/kg) groups were significantly higher than that of the SANFH + antler (250 mg/kg) group (P<0.05), however there were no significant differences in the ALP between the SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups.

Osteoblast cell cycle. As presented in Table III and Fig. 5, the proportion of osteoblasts in G0/G1 in the SANFH, SANFH + antler (250 mg/kg) and SANFH + antler (500 mg/kg) groups (75.60 \pm 3.88, 70.25 \pm 4.06 and 68.77 \pm 2.96, respectively) were significantly higher than that of the control (63.39 \pm 4.51; P<0.05), whereas the proportion of osteoblasts in G0/G1 in the SANFH + antler (1,000 mg/kg) group (67.12 \pm 3.58) did not differ significantly from that of the control. Furthermore, the proportion of osteoblasts in G0/G1 in the SANFH + antler (250 mg/kg), SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups were significantly lower than that of the SANFH group (P<0.05; Fig. 5).

Discussion

Steroids are clinically used to inhibit inflammation, allergic and immune responses in a number of diseases (42-44). For example, steroids were widely used to treat SARS during the 2003 outbreak (45,46). However, the chronic use of steroids

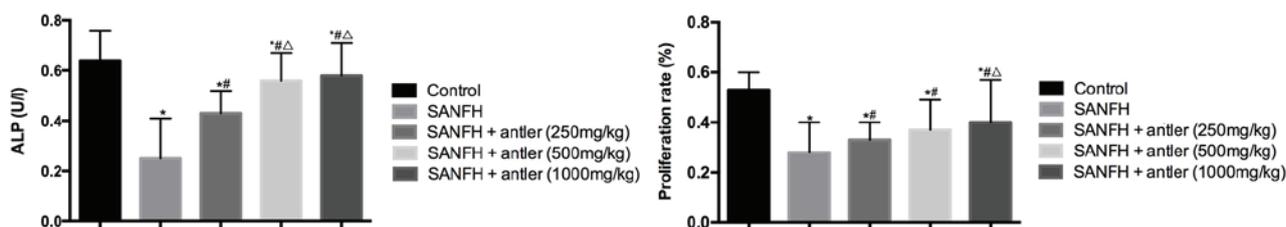


Figure 4. Effect of antler-containing serum on ALP (U/l) and proliferation (%) in osteoblasts. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. SANFH group; $\Delta P < 0.05$ vs. SANFH + antler (250 mg/kg) group. ALP, alkaline phosphatase; SANFH, steroid-induced avascular necrosis of the femoral head.

may induce severe side effects, including the onset of ANFH. Previous studies have demonstrated that the number of cases of ANFH that occur following the use of steroids is increasing (47-49). Without therapy or intervention, femoral head necrosis may become an irreversible process (50,51). Previously, the majority of treatments for hip arthroplasty (52), prosthesis and reconstructive surgery (53) were surgical. However, few cases could not undergo surgical treatment.

The current study used the steroid methylprednisolone to induce and establish a model of SANFH in rabbits. Following successful establishment of the model, different concentrations of antler extract were used to treat rabbits to determine the therapeutic effect of antler extract on SANFH. Subsequently, serum CT and triglyceride levels in the rabbits were measured and it was observed that CT and triglyceride levels increased following the establishment of SANFH. However, CT and triglyceride levels decreased in the serum following treatment with antler extract. Serum CT levels of the rabbits in the SANFH, SANFH + antler (250 mg/kg), SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups were all higher than that of the control, indicating the successful establishment of the SANFH model and the presence of metabolic disorder. Serum CT levels in the SANFH + antler (250 mg/kg), SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups were lower than those of the SANFH group. Furthermore, serum CT levels in the SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups were significantly lower than in the SANFH + antler (250 mg/kg) group ($P < 0.05$). Similar results were observed regarding triglyceride levels. This may be explained by the fact that SANFH induces fat embolism in the peripheral vessels, leading to intravascular coagulation (54-56). The results of the current study therefore indicate that antler extract induces a therapeutic effect on metabolic disorders in SANFH.

11 β -HSD1 are the critical enzymes that regulate glucocorticoids in bone tissue, which are expressed in osteoblasts and osteoclasts (57) and are able to participate in oxidation and reduction reactions (58). However, the mechanisms by which steroids induce ANFH remain unclear. The current study detected and compared 11 β -HSD1 and 11 β -HSD2 expression in femoral head tissue and osteoblasts with or without treatment with different concentrations of antler extract or antler-containing serum. The results demonstrated that in the femoral head tissues of SANFH model rabbits and osteoblasts treated with different concentrations of antler extract, 11 β -HSD1 levels significantly decreased, whereas levels of 11 β -HSD2 significantly increased ($P < 0.05$). In the femoral head tissue,

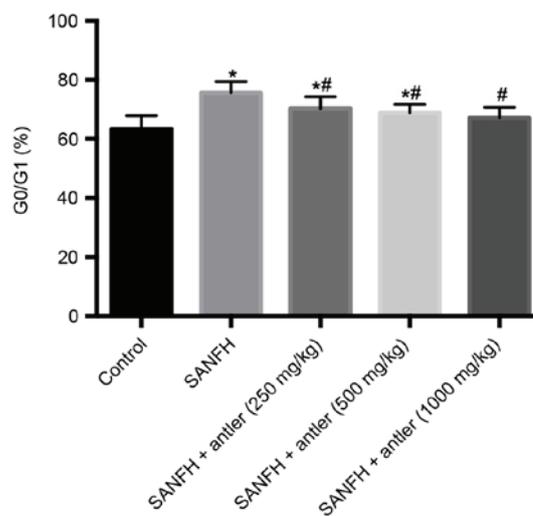


Figure 5. Effect of antler-containing serum on cell cycle in osteoblasts. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. SANFH group. SANFH, steroid-induced avascular necrosis of the femoral head.

11 β -HSD1 levels in the experimental groups, apart from in the SANFH + antler (1,000 mg/kg) group, were significantly higher than in the control ($P < 0.05$). Levels of 11 β -HSD1 in the SANFH + antler (250 mg/kg), SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups were all significantly lower than that in SANFH group ($P < 0.05$). 11 β -HSD2 levels in the experimental groups were lower compared with the control, significantly ($P < 0.05$). Levels of 11 β -HSD2 in the SANFH + antler (250 mg/kg), SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups were significantly higher than in the SANFH groups ($P < 0.05$). Similar results were found regarding in osteoblasts treated with different concentrations of antler extract, with decreasing 11 β -HSD1 and increasing 11 β -HSD2 levels. This phenomenon indicates that 11 β -HSD1 is upregulated, whereas 11 β -HSD2 is down-regulated in SANFH. Antler induced therapeutic effects in SANFH by downregulating 11 β -HSD1 and upregulating 11 β -HSD2. Following treatment with higher concentrations of antler extract, this therapeutic effect became more marked.

In order to evaluate the therapeutic effect of antler extract on SANFH, serum extracted from rabbits with SANFH that had or had not received treatment with different concentrations of antler, were used to treat osteoblasts extracted from rabbits. As well as the detection of 11 β -HSD expression, levels of ALP, which is an indicator of early osteoblastic differentiation (59), were measured and cell proliferation and the proportion of cells in each phase of the cell cycle were determined. The

proliferation rates of the osteoblasts in all experimental groups were all significantly lower than that of the control ($P < 0.05$), indicating that SANFH inhibits the proliferation of osteoblasts. The proliferation rate in the SANFH + antler (250 mg/kg), SANFH+antler(500 mg/kg) and SANFH + antler(1,000 mg/kg) groups were significantly higher than that of the SANFH group ($P < 0.05$) and increased as the concentration of antler extract increased, indicating that antler treatment may promote the proliferation of osteoblasts. ALP levels in all experimental groups were significantly lower than that of the control ($P < 0.05$), indicating that SANFH decreases ALP levels. However, ALP levels in the SANFH + antler (250 mg/kg), SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups were all significantly higher than that of the SANFH group ($P < 0.05$) and increased as the concentration of antler extract increased, indicating that treatment with antler extract may increase ALP levels following SANFH. The proportion of osteoblasts in the G0/G1 phase (%) in the experimental groups were higher than that in the control and the proportion of osteoblasts in the SANFH + antler (250 mg/kg), SANFH + antler (500 mg/kg) and SANFH + antler (1,000 mg/kg) groups were all significantly lower than that of the SANFH group ($P < 0.05$). This phenomenon demonstrates that SANFH may inhibit the proliferation of osteoblasts proliferation and attenuate the cell cycle at the G0/G1 phase. Antler extract, as a therapeutic drug, may promote the proliferation of osteoblasts and induce cellular differentiation.

In conclusion, the current study demonstrated that antler has a therapeutic effect on ANFH induced by steroids, which may promote biochemical metabolism, as well as promoting the proliferation and differentiation of osteoblasts. 11 β -HSDs may serve important roles in the development of SANFH and may be targeted by antler extract in order to prevent and treat SANFH.

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