As life expectancies increase worldwide and population ages, avascular necrosis of the femoral head (ANFH) has become a much more common condition (1, 2), and is listed as the third largest geriatric disease by the World Health Organization (WHO). As a result of its high rate of morbidity and disability, ANFH imposes significant financial burdens to patients, families, and healthcare systems. Current treatment of ANFH includes surgery and medication (3). Due to the serious trauma, long recovery period, high medical costs, and the risk of side effects of the surgery and medication associated with conventional ANFH treatment (4), new treatment options that effectively prevent, diminish,
or delay the development of ANFH, and that obviate
the need for invasive surgery, are of pivotal impor-
tance.

ANFH is a pathological process caused by
interruption of blood supply to the bone, resulting in
the eventual collapse of bone structure if not reme-
died in time (5, 6). Recently, enhancement of bone
repair and regeneration in the necrotic zone by bone-
marrow-derived mesenchymal stem cells (BMSCs)
has been highlighted for the treatment of
osteonecrosis prior to collapse of the head (7, 8).

Deer antler (Cervus elaphus Linn., antler) is a
highly valued traditional Chinese medicine, and has
long been considered to be beneficial in the preven-
tion and treatment of various diseases, such as acute
and chronic arthritis, osteoporosis, and fractures, as
evidenced in animal models and human clinical trials
(9). Each spring, the hard antler from the previous
season is cast off and the antler grows anew at
incredible speeds (10); this growth includes vascular,
connective tissues, and cartilage, which suggests that
the active compounds in antler can promote and sus-
tain bone formation and growth (11). Colla Cornus
Cervi is a refined extract from deer antlers obtained
by boiling the antler in water and concentrating the
extract. In the present study, BMSCs were isolated
and cultured, and BMSCs overexpressing human
bone morphogenetic protein 7 (BMP7) were generat-
ed using lentiviral transduction methods. We investi-
gated the combined effect of CCC and overexpres-
sion of BMP7 in BMSCs on osteogenic induction
and the treatment of ANFH in vitro and in vivo.

EXPERIMENTAL

Animals and reagents

Wistar rats weighting 180~220 g (License No.
scxk [Lu] 20130009) and 8 days old Sprague-
Dawley rats weighting 25~35 g (License No. scxk
(Lu) 20130009) were purchased from Shandong
University Laboratory Animal Center. All animal
experiments were approved by the Institutional
Animal Care and Use Committee of Shandong
Academy of Chinese Medicine. The experiments
were conducted according to the National Research
Council’s guidelines.

Deer antler tablets were purchased from
Shandong Baiweitang Chinese Herbal Medicine
Drinks Slice Company Limited. Quality manage-
ment of deer antler tablets was performed by
Department of Pharmacognosy of Shandong
Academy of Chinese Medicine. Quality control was
conducted in accordance with the regulations of the

The reagents used in experiments were as fol-
lows: Dulbecco’s modified Eagle’s medium
(DMEM-LG; Hyclone, USA), fetal bovine serum
(FBS; Hyclone, USA), polybrene (Sigma-Aldrich,
St. Louis, MO), phosphate buffered saline solution
(PBS; Solarbio, Beijing, China), TRIZol (Solarbio,
Beijing, China), fluorescence labeled mouse anti-rat
monoclonal antibodies (CD34-FITC, CD45-FITC,
and CD90-FITC, SinaSun, Beijing, China), primary
antibodies used in western blotting (Santa Cruz,
USA), secondary antibodies used in western blotting
(Beijing Chinese Fir Golden Bridge Biotechnology
Co. Ltd., China), Cell Counting Kit-8 (CCK-8, Boster,
Wuhan, China), colorimetric assay kit
(Nanjing Jian Cheng Bioengineering Institute,
Nanjing, China), enzyme-linked immunosorbent
assay (ELISA) kit (Nanjing Jian Cheng
Bioengineering Institute), ReverTra Ace qPCR RT
Kit (TOYOBA, Japan), and SYBR Green master
mix (Invitrogen, Carlsbad, California, USA).

CCC complex medium preparation

Deer antler tablets were pulverized and mixed
with 4-5 times (w/w) distilled water in a round-bot-
tom flask. The antler tablets were boiled and juices
were taken every 3 h. Supplemented distilled water
and boiled until antler tablets were soft and could be
pinched into powder. The juices were gathered and
concentrated in vacuo at 50°C until the water con-
tent was 11% (w/w%). The extracts were filtered
with double gauze. CCC was obtained after cooling
to room temperature.

CCC quality identification was conducted by
thin-layer chromatography (TLC) according to the
regulations of the Chinese Pharmacopoeia 2010
Edition. Briefly, 1 g CCC was added into 10 mL of
70% ethanol, and treated with ultrasonics for 15 min.
After filtration, filtrates were obtained as the experi-
mental sample. The control sample was 0.5 mg/mL
glycine standard substance (glycine dissolved in 70%
ethanol). The two samples were spotted on a same sil-
ica gel TLC plate, respectively, and then dipped in the
developing solvent (N-butyl alcohol, glacial acetic
acid and water, 3 : 1 : 1, v/v/v). The developing sol-
vent traveled up the plate until 1 cm from the top.
Then, the plate was taken out and dried in the air. The
spots on the plate were detected by ninhydrin solution.

The CCC were sterilized by γ-ray radiation for
2 h (accumulated 105 rad), and then prepared for
CCC complex medium. The CCC complex medium
was low glucose Dulbecco’s modified Eagle’s medi-
um (DMEM-LG) containing 0.25% CCC (w/v%),
20% FBS, 100 U/mL penicillin and 100 µg/mL
streptomycin.
Recombinant lentivirus construction and infection

Lentiviral vectors (LV) for overexpression of recombinant BMP7 (PLV-sfGFP(2A)-BMP7) and empty control vector (PLV-sfGFP(2A)-puro) were obtained from Inovogen Tech Company (Beijing, China).

LV transfection was performed by adding virus solution to BMSCs in the presence of 4 µg/mL polybrene. Forty-eight hours after infection, the cells were selected in complete culture medium containing 300 µg/mL puromycin. Puromycin-resistant cells were pooled and cultured for western blot detection of recombinant BMP7. Stable cell lines were cultured for further analysis.

BMSCs culture and characterization

Eight-days-old rats were sacrificed, and bone marrow was flushed out from femur and tibia with DMEM-LG under aseptic conditions. After digestion, cells were harvested through a cell strainer (100 micron mesh) and centrifuged (1200 rpm, 5 min). Cells were placed in a 25-cm² flask and cultured in DMEM supplemented with 20% FBS at 37°C in 5% CO₂ in a cell culture incubator (Gasaxy S, RS Biotech Co., UK).

The fourth generation of BMSCs were collected and washed three times with PBS. After digestion with 0.25% trypsin, single cell suspension was harvested, and then, respectively, incubated with monoclonal antibodies CD34-FITC, CD45-FITC, and CD90-FITC at room temperature for 30 min in the dark. After washing with PBS, cells were fixed with 20 g/L paraformaldehyde and analyzed using a flow cytometer (Beckman, USA).

The BMSCs were randomly divided into four groups: the normal control group, CCC group, BMP7 group, and CCC+BMP7 group. Briefly, cells were seeded in a 96-well plate at a density of 5 × 10⁴ cells per well. After incubation for 24 h, 48 h, and 72 h, 10 µL CCK-8 was added to each well and incubated for additional 4 h. The absorbance at 450 nm was measured with a microplate reader. Each experiment was performed in triplicate. The cell activity was calculated as follows: cell activity = (OD experimental group - OD blank control group) / (OD control group - OD blank control group) × 100.

Western blot assays

Cells were collected and lysed in RIPA lysis buffer, and the lysates were harvested by centrifugation (12,000 rpm) for 30 min. Then, 30 µg protein per sample was loaded into a 12% sodium dodecyl sulfate polyacrylamide gel for separation by electrophoresis, and were then transferred onto a polyvinylidene fluoride membrane. Membranes were blocked for 60 min with 5% non-fat milk, and incubated overnight at 4°C with the following primary antibodies: rabbit anti-rat monoclonal antibody against ACTIN, rabbit anti-rat monoclonal antibody against VEGF, and goat anti-rat polyclonal antibody against BMP7 (at a 1 : 200 dilution). After washing with TBST (Tris-buffered saline with 1% Tween 20) for 10 min, the membranes were probed with the following horseradish peroxidase (HRP)-conjugated secondary antibodies: goat anti-rabbit IgG antibody and mouse anti-goat IgG antibody at a 1 : 5000 dilution at 37°C for 1 h. After three washes with TBST, the membranes were developed by an enhanced chemiluminescence system (Cell Signaling Technology, Danvers, Massachusetts, USA).

Proliferation assay

The CCK-8 kit was used to evaluate the growth rate and relative activity of cells according to the manufacturer's protocol. The BMSCs were randomly divided into four groups: the normal control group, CCC group, BMP7 group, and CCC+BMP7 group. Briefly, cells were seeded in a 96-well plate at a density of 5 × 10⁴ cells per well. After incubation for 24 h, 48 h, and 72 h, 10 µL CCK-8 was added to each well and incubated for additional 4 h. The absorbance at 450 nm was measured with a microplate reader. Each experiment was performed in triplicate. The cell activity was calculated as follows: cell activity = (OD experimental group - OD blank control group) / (OD control group - OD blank control group) × 100.

Analysis of alkaline phosphatase (ALP) activity

ALP activity was measured using a colorimetric assay kit following the manufacturer's instructions. The BMSCs were randomly divided into four groups: the normal control group, CCC group, BMP7 group, and CCC+BMP7 group. Briefly, after incubation for 48 h, the medium in the 12-well plate was removed and the cells were washed three times with PBS. Cell lysis was accomplished by adding 20 µL 0.05% Triton X-100 to each well and incubating for 5 min at room temperature. The absorbance at 520 nm, indicative of ALP activity, was measured with a microplate reader. The total protein concentration was measured with a BCA kit (Nanjing Jian Cheng Bioengineering Institute) according to the manufacturer's protocol. The ALP activity was calculated as follows: ALP activity (King unit/gram protein (gprot)) = (ODsample - ODblank control)/ (ODstandard - ODblank control) × phenol standard concentration (0.02 mg/mL) - total protein concentration (gprot/mL).

Determination of osteocalcin levels

Osteocalcin determination was performed by an ELISA kit following the manufacturer's instruc-
tions. BMSC media were collected every 3 days and frozen at -20°C for further analysis. The standard curve was protracted according to the absorbance of standard samples measured at 450 nm with a microplate reader. Osteocalcin content was calculated according to the standard curve.

**Staining of calcified nodules**

CCC+BMP7 BMSCs were seeded in a 12-well plate at a density of 5 × 10^3 cells per well. After incubation for 21 days, the medium in the wells was removed and the cells were washed twice with PBS. The cells were fixed with 95% ethanol at 4°C for 10 min. After three washes with double distilled water, 0.1% alizarin red-Tris HCl (pH 8.3) was added for staining, and samples were incubated in a 37°C water bath for 60 min. Stained cells were then observed under an inverted phase contrast microscope and calcified nodules were counted under low power magnification. Quantification of nodules was performed on five random fields per well.

**Real time quantitative PCR (RT-qPCR)**

Total RNA was extracted from BMSCs using TRIzol according to the manufacturer's instructions. RNA concentration and quantity were assessed by measuring absorbance at 260 nm using a Nanodrop spectrophotometer (ND-1000, Thermo Scientific).

### Table 1. Primer sequences and PCR products size.

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<thead>
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<th>Gene</th>
<th>Primer</th>
<th>PCR products size</th>
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<tr>
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<tr>
<td>Integrin α2</td>
<td>forward 5'-CAGTGAAGCCAGAAGAAA-3' reverse 5'-AACCATAGCAGACAA-3'</td>
<td>665 bp</td>
</tr>
<tr>
<td>Integrin α5</td>
<td>forward 5'-CTTCGGTTCACTGCTCCTC-3' reverse 5'-TGCCCTTCGAGCATT-3'</td>
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</tr>
<tr>
<td>Integrin β1</td>
<td>forward 5'-ACAGAAGTAGAGGTGTC-3' reverse 5'-GAGGTTGAAATGGGAGC-3'</td>
<td>660 bp</td>
</tr>
<tr>
<td>β-Actin</td>
<td>forward 5'-GCTTACATGTGTCGATCC-3' reverse 5'-CTCGCGCTACTCTCTTTG-3'</td>
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### Table 2. General observation table.

<table>
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<th>Specific performance</th>
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<td>Behavior function</td>
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<td></td>
<td></td>
<td>Mild</td>
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<td></td>
<td>Moderate</td>
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<td>Serious</td>
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<td>Flexion</td>
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<td></td>
<td></td>
<td>&lt; 30°</td>
<td>0</td>
</tr>
<tr>
<td>Joint range of motion</td>
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<td>&gt; 30°</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt; 15°</td>
<td>2</td>
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<td></td>
<td>&gt; 5°</td>
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<tr>
<td></td>
<td></td>
<td>&lt; 5°</td>
<td>0</td>
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<td></td>
<td>Pronation</td>
<td>&gt; 15°</td>
<td>2</td>
</tr>
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<td></td>
<td></td>
<td>&gt; 5°</td>
<td>1</td>
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<td>1</td>
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<tr>
<td></td>
<td></td>
<td>&lt; 5°</td>
<td>0</td>
</tr>
</tbody>
</table>
Effect of Colla Cornus Cervi combined with LV-mediated BMP7 transfected... 1525

USA). First-strand cDNA synthesis was performed using a ReverTra Ace qPCR RT Kit according to the manufacturer’s recommendations. RT-qPCR was employed to evaluate the relative mRNA expression of COLI, integrin α2, integrin α5, integrin β1, and β-actin (as an internal control). The primers and PCR products size are shown in Table 1. Gene-specific amplification was performed using an ABI 7900HT real-time PCR system (Bio-Rad, American) with a 15-µL PCR mix containing 0.5 µL of cDNA, 7.5 µL of 2 × SYBR Green master mix, and 200 nM of the appropriate oligonucleotide primers. The cycling parameters are as follows: 95°C for 15 min, followed by 45 cycles of 95°C for 30 s and 60°C for 1 min. The resolution curve was measured at 95°C for 15 s, 60°C for 15 s and 95°C for 15 s. Ct values were calculated from the threshold cycles with the instrument's software (SDS 2.3), and the relative expression of mRNA was normalized against the results for β-actin. Data were analyzed using the comparative threshold cycle (2⁻∆∆CT) method.

Transplantation of BMSCs

After 1 week of acclimatization, the 30 Wistar rats weighting 180~220 g were randomly divided into two groups: the control group and the ANFH model group. The rats of ANFH model group were given 70 mg/kg retinoic acid by oral gavage once per day for 6 weeks. After 6 weeks, two rats were randomly selected from the ANFH group to confirm whether the treatment with retinoic acid successfully induced ANFH. PLV-sfGFP(2A)-BMP7 transfected BMSCs cultured in CCC complex medium were resuspended at 6 × 10⁶ cells/mL in normal saline, and injected into the necrotic femoral head of the left lower extremity. As a model control, the necrotic femoral head of right lower extremity did not receive any treatment in the ANFH group. After 4, 12, and 24 weeks, general observation, femoral head gross observation, X-ray observation, and pathological examination of the femoral head were performed. The general observation and score were conducted according to Table 2.

Statistical analysis

All experiments were performed in triplicate. All statistical analyses were performed using the Statistical Package for the Social Sciences, version 16.0 (SPSS Inc., Chicago, IL, USA). The comparison of multiple means was analyzed using the F test. The comparison of the rate was analyzed using the
σ^2 test. The results were presented as the mean ± standard deviation (SD). A two-sided p-value less than 0.05 was considered to be statistically significant.

RESULTS

BMSCs identification
Prior to their use in other experiments, cultured BMSCs were characterized. Results of Giemsa staining showed triangular or fusiform cells with full cytoplasm and clearly visible nucleoli stained uniformly (Fig. 1A). The expression of CD34, CD45 and CD90 was detected in BMSC populations by flow cytometry. Results showed that more than 90% of BMSCs were CD90+, while fewer than 5% of BMCs were CD34+ or CD45+. The percentages of positively stained BMSCs were as follows: CD34+ cells, 2.65 ± 0.56%; CD29+ cells, 96.87 ± 2.54%; CD90+ cells, 90.37 ± 3.55%; and CD45+ cells, 1.62 ± 0.16%.

BMP7 expression analyzed by western blotting
BMSCs were transfected with recombinant PLV-sfGFP(2A)-BMP7 and empty control vector PLV-sfGFP(2A)-puro. Following selection of stable transductants, BMP7 expression was detected using western blotting. The results showed a BMP7 band at the expected size and the amount of BMP7 protein present was further measured by densitometry (statistical analysis of significance showed p < 0.05). BMP7 protein expression was markedly increased in the PLV-sfGFP(2A)-BMP7 transfected cells compared with control vector PLV-sfGFP(2A)-puro transfected cells (Fig. 1B).

Characteristics of Colla Cornus Cervi
The CCC product was yellowish-brown, translucence, crisp and fragile, with a smooth section profile (Fig. 1C). The moisture content of CCC was 11%, which was determined according to Chinese Pharmacopoeia 2010 Edition (Appendix IX H, the first method). Quality identification showed that the CCC displayed the same spots as glycine standard at the corresponding position of TLC plate (Fig. 1D).

The role of CCC and BMP7 in BMSC proliferation
The CCK-8 proliferation assay revealed that proliferation rate of the CCC, BMP7, and CCC+BMP7 BMSC groups was significantly greater than the control BMSCs (Fig. 2A). Additionally, the proliferation rate of CCC+BMP7 group was significantly higher than either the CCC or BMP7 groups. These results suggested that CCC combined with BMP7 can enhance BMSC proliferation.

Alkaline phosphatase activity
The activity of ALP was significantly increased in CCC+BMP7 BMSCs compared with the CCC, BMP7, and control BMSC groups (p < 0.05, Fig. 2B). There was no significant difference in ALP activity between CCC group and BMP7 group. These results indicated that CCC can enhance the production of ALP in BMP7-overexpressing BMSCs.

Osteocalcin determination
After osteogenic induction of BMSCs, the osteocalcin content increased over time. The osteocalcin content in the CCC, BMP7, and CCC+BMP7 BMSC groups was significantly higher than the control BMSC group (Fig. 2C). The osteocalcin content in CCC+BMP7 group was significantly higher than

<table>
<thead>
<tr>
<th>Group</th>
<th>COL I</th>
<th>Integrin α2</th>
<th>Integrin α5</th>
<th>Integrin β1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.9 x 10^-3 ± 9.9 x 10^-4</td>
<td>6.1 x 10^-3 ± 3.5 x 10^-4</td>
<td>6.4 x 10^-3 ± 1.2 x 10^-3</td>
<td>1.2 x 10^-3 ± 6.0 x 10^-4</td>
</tr>
<tr>
<td>CCC</td>
<td>1.5 x 10^-3 ± 2.5 x 10^-4</td>
<td>1.1 x 10^-3 ± 0.5 x 10^-4</td>
<td>3.2 x 10^-3 ± 7.5 x 10^-4</td>
<td>2.5 x 10^-3 ± 0.9 x 10^-4</td>
</tr>
<tr>
<td>BMP7</td>
<td>1.9 x 10^-3 ± 1.7 x 10^-4</td>
<td>1.3 x 10^-3 ± 6.5 x 10^-4</td>
<td>1.4 x 10^-3 ± 3.8 x 10^-4</td>
<td>3.6 x 10^-3 ± 0.6 x 10^-4</td>
</tr>
<tr>
<td>CCC+BMP7</td>
<td>2.6 x 10^-3 ± 5.6 x 10^-4</td>
<td>1.5 x 10^-3 ± 4.0 x 10^-4</td>
<td>2.4 x 10^-3 ± 0.6 x 10^-4</td>
<td>2.2 x 10^-3 ± 1.1 x 10^-3</td>
</tr>
</tbody>
</table>

*p < 0.01, *p < 0.05, compared with control group; p < 0.05, compared with CCC or BMP7 group.

Table 4. Comprehensive score of general observation.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank control</td>
<td>10</td>
<td>22.20 ± 1.32</td>
</tr>
<tr>
<td>Model control</td>
<td>10</td>
<td>4.60 ± 1.35</td>
</tr>
<tr>
<td>CCC+BMP7</td>
<td>10</td>
<td>13.60 ± 1.90*</td>
</tr>
</tbody>
</table>

n: numbers of cases in each group. ## p < 0.01, compared with model group.
that in other groups (p < 0.05), which suggested that CCC can enhance osteoblastic differentiation of BMP7-overexpressing BMSCs.

**Calcified nodules staining**

Results of alizarin red staining showed that there were no calcified nodules in blank control group (Fig. 2D). The number of calcified nodules in CCC group and BMP7 group was significantly less than that in CCC+BMP7 group (Fig. 2D); the staining intensity in CCC+BMP7 group was also stronger than that in the other groups.

**mRNA expression of osteoblast collagen type I (COL1), integrin α2, integrin α5 and integrin β1**

At the seventh day after infection with lentiviral vectors, the mRNA expression of the osteogenesis related genes COL1, integrin α2, α5 and β1 were determined by RT-qPCR. Expression of COL1, integrin α2, α5 and β1 were significantly higher in CCC+BMP7 group compared with other groups (Table 3, p < 0.05). The expression of these genes in CCC group and BMP7 group were significantly higher than in the control BMSC group (Table 3, p < 0.05).

**The function of CCC and BMP7 on repair of ANFH**

Results of general observation were recorded and scored in Table 4. Results showed that score of CCC+BMP7 group was significantly higher than the model group (Table 4, p < 0.01).

**X-ray observation**

The necrotic femoral head of the left lower extremity in ANFH model group showed cystic degeneration and osteosclerosis, and some animals displayed segmental femoral head flattening or subchondral collapse (Fig. 3Ab). X-ray film showed smooth articular surface, and restoring necrotic and cystic area (Fig. 3Aa). Significant alleviation of femoral head necrosis was observed in the rats treated with CCC+BMP7 BMSCs (Fig. 3A).

**Microscopic observation**

Femoral head samples obtained from control rats showed normal ultrastructure (Fig. 3B). In the model group, femoral heads exhibited osteocyte degeneration and necrosis, cell surface collapse, marrow cavity bleeding, and synovial necrosis (Fig. 3B).

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**Figure 2. The effect of CCC and BMP7 on BMSCs.**

A: Proliferation of BMSCs. * p < 0.05, # p < 0.01, compared with normal control group. ° p < 0.05, compared with CCC or BMP7 group. B: ALP activity. * p < 0.05, ** p < 0.01, compared with normal control group. # p < 0.05, compared with CCC or BMP7 group. C: Osteocalcin content. * p < 0.01, # p < 0.05, compared with normal control group. ° p < 0.05, compared with CCC or BMP7 group. D: Results of calcified nodules staining in BMSCs (200× magnification)
In the CCC+BMP7 group, the femoral head cells were gradually recovered. Samples showed smooth fibrous cartilage and articular cartilage, and little cartilage cell destruction (Fig. 3B). Our results suggest that CCC and BMP7 play important functions in the ability of BMSCs to repair ANFH in rats.

DISCUSSION AND CONCLUSION

As world’s population aging, ANFH has become a widespread disease worldwide. Due to high rate of morbidity and disability, ANFH bring huge financial burdens to patients and family. Presently, less-invasive methods replacing arthroplasty are becoming a new trend in the treatment of ANFH. Stem cell therapies provide a new avenue for the treatment of ANFH (7, 12). Deer antler is highly valued in traditional Chinese medicine, and has been used for over 2000 years (13). CCC, the refined extract from deer antler, is thought to have the function of warming and tonifying the liver and kidney, replenishing vital essence and blood, and has been commonly used in the prevention and treatment of osteoporosis, to increase bone mineral content and density (10, 15). Modern chemical research indicates that CCC contains 82.49% protein and more trace elements, which are the material bases of replenishing vital essence and blood (9). Recently, CCC is used in the treatment of osteoporosis and osteonecrosis of the femoral head. In support of this, our study found that the proliferation and osteogenic differentiation of BMSCs treated with CCC were significantly enhanced compared to control BMSCs.

BMP7, a member of the transforming growth factor β family, has many biological functions, including regulating cell growth, proliferation, dif-

Figure 3. The combined effect of CCC and BMP7 BMSCs on the treatment of ANFH in rats. A: X-ray observation of rat femoral head. a - CCC+BMP7; b - ANFH model control. B: Pathological observation of the femoral head (200× magnification)
Effect of Colla Cornus Cervi combined with LV-mediated BMP7 transfected... 1529

ferentiation, apoptosis, and in inducing bone forma-
A study by Jin Dan et al. revealed that overex-
expression of BMP7 significantly promoted the
osteogenic differentiation of BMSCs (16). Kang
Yan et al. found that BMP7 enhances osteogenic
derivation of BMSCs transfected with a BMP7-
combinant-vector (17). Adeno-associated virus
mediated VEGF and BMP7 gene transfer stimulates
angiogenesis and bone regeneration, which may be
a new therapeutic technique for the treatment of
ANFH (18). These studies prompted us to consider
BMP-7 overexpressing BMSCs as ideal seed cells
for engineering bone tissue remodeling. Consistent
with these findings, our research revealed that over-
expression of BMP7 through lentiviral transduction
of PLV-sfGFP(2A)-BMP7 vector promoted the pro-
liferation and osteogenic differentiation of BMSCs
in rats, indicating that BMP7 can induce undifferen-
tiated mesenchymal cells to differentiate into chon-
drocytes or osteoblasts.

In the current study, cell proliferation assays
showed that the growth rate of CCC+BMP7 BMSC
group was significantly higher than CCC or BMP7
groups. Compared with the CCC, BMP7, control
groups, the activity of ALP was significantly
increased in CCC+BMP7 BMSCs. Additionally, the
content of osteocalcin in CCC+BMP7 group was
significantly higher than in other groups. The
mRNA expression of osteogenesis related genes
COLI, integrin α2, α5 and β1 was significantly
higher in CCC+BMP7 group compared with other
groups. These results suggested that CCC can sig-
ificantly promote the proliferation and differentia-
tion of BMP7-overexpressing BMSCs in vitro.

Additionally, we used a rat model of ANFH
model to investigate the function of CCC combined
with BMP7 on the ability of BMSCs to induce repair
of ANFH in rats. Results of general observation
were recorded and scored, and indicated that the
score of ANFH rats treated with CCC+BMP7
BMSCs was significantly higher than the control
group. Moreover, the number of calcified nodules in
CCC+BMP7 group was significantly higher than that
in other groups. X-ray and microscopy revealed that
the femoral head necrosis was significantly
relieved and the femoral head cells were gradually
recovered in the CCC+BMP7 BMSCs treated
ANFH rats compared with the model group.

In conclusion, we have demonstrated that CCC
and BMP7 can significantly promote the prolifera-
tion and osteogenic differentiation of BMSCs.
Furthermore, our results demonstrate that CCC and
BMP7 play important functions on the ability of
BMSCs to facilitate repair of ANFH in rats, and
indicate that this stem cell therapy can favorably
enhance the repair of avascular necrosis of the
femoral head. Our research suggests that CCC com-
bined with BMP7 may provide a new therapeutic
strategy in the use of BMSC and stem cell therapy
for the treatment of ANFH.

Conflict of interests

All authors have read and approved submission
of the manuscript. The authors declare that they
have no competing interests.

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