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Effects of herbaceous plant preparation of Cistanche on *in vitro* cytokine expression by rat osteoblasts

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ABSTRACT

Osteoblasts (OB) play an important role in bone remodeling through the release of cytokines such as osteoprotegerin **(**OPG), a receptor activator of nuclear factor kappa-B ligand (RANKL) and osteopontin (OPN). The objective of our study was to evaluate the effects of Chinese herb extracts of Cistanche *(Cistanche deserticola*) on the *in vitro* expression of these cytokines in rat OB cell culture. The primary OBs were isolated from the rat skull by trypsin and collagenase digestion. The cellular phenotype was determined by the Swiss dyeing method, alkaline phosphatase staining, Von Kossa staining, and Alizarin red staining. The gene expression of OPG, RANKL and OPN were analyzed by quantitative real-time PCR. All treatments with herbaceous plant extracts of Cistanche increased the gene expression of OPG and RANKL at 72 hours of culturing, especially at concentrations of 5×10^{-2} mg/mL, which were significantly different compared to the control cultures. Treatment significantly reduced the expression of the OPG/RANKL ratio at 48 hours and the expression of OPN mRNA at 72 hours of culturing. The *in vitro* exposure of OB to the plant preparation of Cistanche promoted the gene expression of OPG and RANKL and reduced the gene expression of OPG/RANKL and OPN, and thus, could maintain the balance of bone formation and resorption during bone metabolism.

Key words: Cistanche, osteoblasts, osteoprotegerin, RANKL, osteopontin, rat

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Introduction

Bone strength is determined by structure and composition. Bone tissue adapts to accommodate to a variety of loads by modeling during the growth phase and remodeling throughout life. The bone remodeling decreases with aging because of a reduction in the volume of bone formed in the basic multicellular unit, sex hormone deficiency and reduced periosteal bone formation (SEEMAN, 2008). Imbalance in bone remodeling can cause bone-related diseases, such as osteoporosis, and increased fracture risk. Osteoporosis, causing fractures, pain and disorders in locomotion, affects millions of individuals in the United States (NAYAK et al., 2012; SILVERMAN, 2010). The key process in prevention of the incidence of bone-related diseases is to maintain bone remodeling capacity.

Bone remodeling is the dynamic balance of bone formation and resorption (SEEMAN and DELMAS, 2006). These processes are mainly controlled by osteoblasts (OBs) and osteoclasts in adult bone, and by expression of cytokines, growth factors and hormones (PARFITT, 2000). OBs, which are derived from primordial mesenchymal cells, are found on the bone surface. OBs are responsible for bone formation, and indirectly impact bone resorption (MACKIE, 2003). The cytokines expressed by OBs, such as osteoprotegerin (OPG), receptor activator of nuclear factor kappa-B ligand (RANKL), and osteopontin (OPN), may promote fracture healing and increase bone formation in osteoporosis (LINKHART et al., 1996). OB development is a sequential process which includes osteoprogenitor proliferation, matrix maturation and cell mineralization (GIUSTINA et al., 2008). This process is regulated by multiple OB-associated genes and highly associated with the development of bone formation (HUNG et al., 2010). The progression of osteogenic mineralization stimulates the bone formation during bone fracture healing. Thus, research into the expression of osteogenic cytokines and their effects on bone mineralization is essential in searching for an innovative and effective therapeutic agent of natural origin for prevention of bone-related diseases.

Traditional Chinese Medicine has been used for thousands of years in the treatment of bone-related diseases. The herbaceous plant Cistanche, which is widely used for invigorating the kidneys and in the therapy of bone diseases, belongs to a genus of the *Orobanchaceae* family. It has been reported that the constituents of the Cistanche species are: phenylethanoid glycosides (PhGs), iridoids, lignans, alditols and oligosaccharides (JIANG and TU, 2009; LI et al., 2010). Cistanche plants are perennial parasite herbs, which mainly grow in the desert or on barren salt licks, mainly distributed in the arid and warm deserts in northwest China. In Traditional Chinese Medicine Cistanche is considered an extraordinary tonic, and is named the "Ginseng of the desert". A study performed in ovariectomized rats (LIANG et al., 2011) showed that Cistanche extracts administered orally significantly enhanced bone mineral density and bone mineral content in a dose dependent manner. However, the detailed mechanisms of the action of Cistanche on bone

formation are still unclear. Therefore, the aims of this study were to assess the effects of Cistanche on the *in vitro* expression of genes for OPG, RANKL and OPN cytokines by rat OB cell cultures, and to evaluate the mechanisms by which Cistanche promotes bone healing or prevents/controls bone-related diseases.

Materials and methods

Preparation of rat osteoblasts. Rat OBs were prepared from neonatal rat calvarias (<24 hours) and processed by the trypsin (Sigma, USA) and collagenase II（Sigma, USA）digestion methods. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, GIBCO, USA) supplemented with 15 % inactivated fetal calf serum, penicillin (100 IU/mL), streptomycin (100 IU/mL) and L-glutamine in 6 well culture plates, and incubated at 37 ° C with 5 % CO_2 in the air. The cellular phenotype was determined by the Swiss dyeing method, alkaline phosphatase staining, and Von Kossa and Alizarin red dye staining.

Preparation of water extracts of Cistanche in serial dilutions. The Cistanche used in this study was provided from Tongrentang Pharmacy in China. Dry pieces of Cistanche were ground into a powder. The Cistanche liquid extracts were prepared by decocting 50 g of herbal powder for 1 hour. Next, the preparation was concentrated to 100 mL using a rotary evaporator, and filtered through 0.22 mm filters. The filtrate was stored at 4 °C in dark. The liquid extract of Cistanche was diluted to serial concentrations ranging from 5×10^{-1} to 5×10^{-4} mg/mL with the DMEM fluid.

Isolation of RNA and reverse transcription PCR. The third generation of cultured OBs was treated with different concentrations of Cistanche extracts for 24, 48 and 72 hours. The OB cell culture treatments were performed in triplicate. The cells were washed with Phosphate buffered saline (PBS) twice and RNA was isolated from the cells with TransZol reagent (Beijing TransGen Biotech Co., Ltd.; Beijing, China) according to the manufacturer's instructions, and extracted with chloroform, precipitated with isopropyl alcohol, washed with 75 % ethyl alcohol and then dissolved in water. Reverse transcription of RNA (5 μL) was performed using the TransScript First-Strand cDNA Synthesis Supermix (Trans, Beijing, China).

Confirmation of real-time PCR products. Following electrophoresis on 1.5 % agarose gel, DNA sequencing was performed in order to prove that PCR products were the encoding gene segments of OPG, RANKL and OPN in rat OB.

The establishment of plasmid containing the target gene. The DNA sequence in 2 % agarose gel was purified and recovered by E.Z.N.A. gel extraction (Kangweishiji, China) and transferred to a pEASY-T3 cloning kit (Beijing TransGen Biotech Co., Ltd.; Beijing, China). The cell transformation was achieved via a constructed plasmid containing the target gene. Positive colonies were screened by the Blue-white spot screening test and

subsequently sequenced. The plasmids extracted from the positive colonies, containing the target gene, were proportionally diluted with deionized water to achieve a standard curve.

Quantitative real-time PCR (RTQ PCR). Specific primers were constructed according to the published gene sequences (NCBI, Nucleotide Database) and synthesized by Liuhe-huada Gene (Beijing, China) as shown in Table 1.

Table 1. Primer sequences used for the RTQ PCR to quantify the target genes that encode the expression of cytokines in rat OB cultures

mRNA of the target genes	Forward primer	Reverse primer	Product size (bp)
OPG	5'CGTCACCCACAGTCTGAGGAA3'	5'TCAACTGCCATTTCAAGAGCC3'	216
RANKL	5'CGTACCTGCGGA CTATCTTCA3'	5'GTTGGACACCTGGACGCTAA3'	196
OPN	5' ACAGCAACGGGAAGACCAGC3'	5' GCTTTGGAACTCGCCTGACTG3'	208
β-actin	5'TCCTAGCACCATGAAGATC3'	5'AAACGCAGCTCAGTATAACAG3'	190

The expression of mRNAs in the samples was quantified using the specific plasmid standard curves described above. The products were compared to the house-keeping gene β-actin. The quantity of target gene was quantified by RTQ PCR, using the SYBR Green RealMaster Mix (TIANGEN, China) according to the manufacturer's protocol. The PCR conditions were as follows: 94 °C for 1 minute, 58 °C for 30 seconds, 68 °C for 1 minute and were repeated for 40 cycles. The data were calculated using the 7500 Real Time PCR System (Applied Biosystems; Waltham, MA, USA).

Statistical analyses. The data were processed by one-way ANOVA, using commercial statistical software (SPSS 17.0, IBM Corporation; Armonk, NY, USA). The results were expressed as the mean ± standard deviations of the target genes and β-actin ratio. Differences were considered significant at $P<0.05$ values.

Results

The identification of rat OBs. The OBs were identified by specific cell staining methods and were phenotypically demonstrated as shown in the Figure 1. (A, B, C and D)

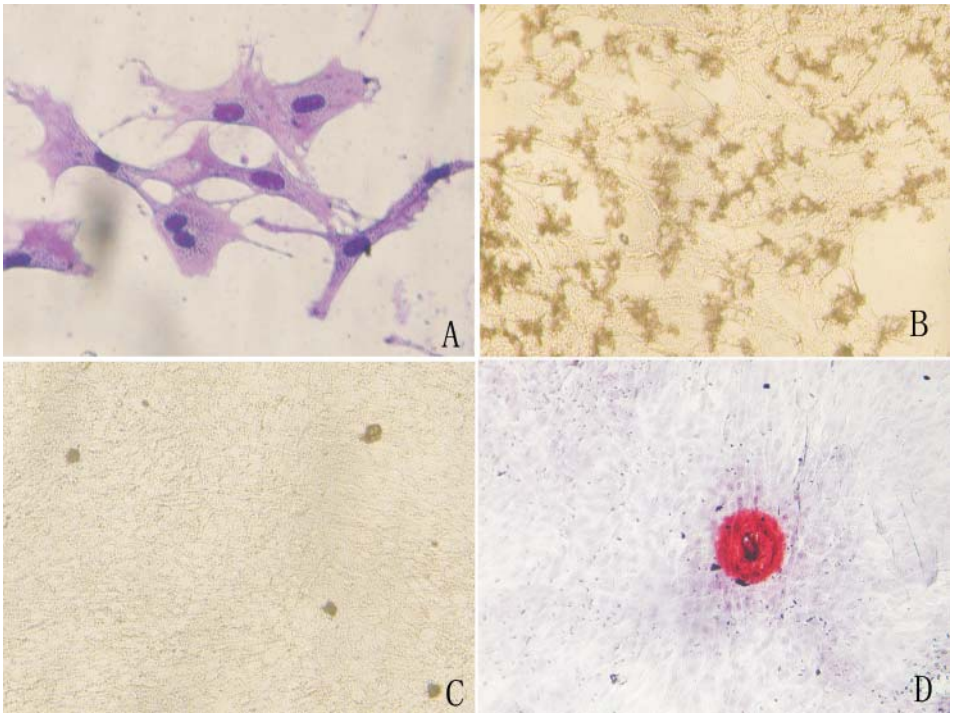

Fig. 1. The phenotype of rat OBs as demonstrated by: Swiss dyeing method (A) ×400, alkaline phosphatase staining (B) ×200, Von Kossa staining (C) ×200 and Alizarin red dye staining (D); ×200.

Confirmation of the target genes. The band sizes of the target genes were analyzed in 2 % agarose gel and were found to be consistent with the expected size (Fig. 2).

The results obtained for OPG, RANKL, OPN and β-actin gene segments that contained the target gene plasmid sequences were compared with the corresponding gene sequences previously published in the GENBANK by BLAST-N analysis, and were found to be: 100 %, 100 %, 100 % and 99 % identical, respectively.

Effect of liquid extracts of Cistanche on OPG, RANKL and OPN expression. Levels of the OPG mRNA were down-regulated when the OBs were exposed to all the applied concentrations of Cistanche for 24 h and 48 h (Table 2).

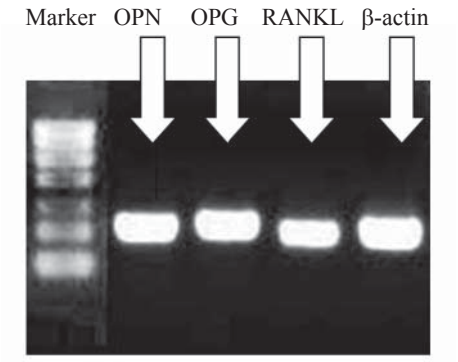

Fig. 2. The electropherograms of the target genes (OPG, RANKL and OPN) encoding the expression of cytokines in rat OB.

Table 2. Effect of different concentrations of water extracst of Cistanche on OPG expression, by rat OB cell culture.

Concentrations of water extract of Cistanche (mg/mL)		The OPG expression (Mean ± SD) by rat OB after culturing for:		
		24 hours	48 hours	72 hours
Treated cultures	5×10^{-1}	0.714 ± 0.043**	2.460 ± 0.569	1.519 ± 0.198
	5×10^{-2}	1.931 ± 0.345 *	2.308 ± 0.873**	18.280 ± 0.276**
	5×10^{-3}	2.537 ± 0.421	3.559 ± 0.774 *	20.453 ± 3.536**
	5×10^{-4}	1.770 ± 0.178**	3.412 ± 0.325	8.688 ± 6.171
Control culture	None	3.676 ± 0.348	4.775 ± 0.623	1.086 ± 0.537

The values marked with an asterisk were significantly different either at * $P<0.05$ or ** $P<0.01$ from those obtained for control non-treated cultures.

Significant differences were observed at concentrations of 5×10^{-1}, 5×10^{-2} and 5×10^{-4} mg/mL after 24 hours, and at concentrations of 5×10^{-1} and 5×10^{-2} mg/mL after 48 hours of culturing ($P<0.05$). However, when compared with control cultures, the expression of OPG was significantly increased after treatment with Cistanche for 72 hours, Cistanche concentrations of 5×10^{-2} mg/mL and 5×10^{-3} mg/mL had particularly increased OPG expression ($P<0.01$). In contrast, the RANKL mRNA expression was down-regulated after 24 hours with the concentration of 5×10^{-1} mg/mL and 5×10^{-2} mg/mL Cistanche extracts compared to the controls. However, RANKL mRNA was up-regulated after culturing with Cistanche extracts for 48 hours and 72 hours, especially the Cistanche

extract concentrations of 5×10^{-2} mg/mL, 5×10^{-3} mg/mL and 5×10^{-4} mg/mL (Table 3). The RANKL content continued to increase significantly after 72 hours of culturing.

Table 3. Effect of different concentrations of water extract of Cistanche on RANKL expression, by rat OB cell culture

Concentrations of water extract of Cistanche (mg/mL)		The RANKL expression (Mean ± SD) by rat OBs after culturing for:		
		24 hours	48 hours	72 hours
Treated cultures	5×10^{-1}	0.840 ± 0.030	2.331 ± 0.390*	1.042 ± 0.695
	5×10^{-2}	0.904 ± 0.024	1.992 ± 0.790	16.478 ± 0.718**
	5×10^{-3}	1.508 ± 0.305	1.846 ± 0.599	9.709 ± 2.067*
	5×10^{-4}	1.455 ± 0.590	1.683 ± 0.717	6.335 ± 1.875*
Control culture	None	1.282 ± 0.693	0.856 ± 0.188	0.855 ± 0.097

The values marked with asterisk were significantly different either at *P<0.05 or ** P<0.01 from those obtained for control non-treated cultures.

The ratio of OPG/RANKL was derived from the data presented in Table 2 and Table 3. The level of OPG/RANKL was significantly down-regulated at concentrations of 5×10^{-1} mg/mL after 24 hours and 5×10^{-1} mg/mL, 5×10^{-2} mg/mL and 5×10^{-3} mg/mL after the cells were cultured for 48 hours (Table 4). There were no significantly different changes in the gene expressions of OPG/RANKL as compared to the control cultures after 72 hours of culturing.

Table 4. Effect of different concentrations of water extract of Cistanche on OPG/RANKL expression, by rat OB cell culture

Concentrations of water extract of Cistanche (mg/mL)		The OPG/RANKL expression (Mean ± SD) by rat OBs after culturing for:		
		24 hours	48 hours	72 hours
Treated cultures	5×10^{-1}	0.849 ± 0.026*	1.049 ± 0.104*	2.048 ± 1.423
	5×10^{-2}	2.132 ± 0.343	1.410 ± 0.950**	1.111 ± 0.031
	5×10^{-3}	1.698 ± 0.191	2.094 ± 0.810*	2.194 ± 0.660
	5×10^{-4}	1.358 ± 0.531	2.271 ± 0.874	1.253 ± 0.641
Control culture	None	3.956 ± 3.028	5.706 ± 1.171	1.276 ± 0.656

The values marked with asterisk were significantly different either at * P<0.05 or ** P<0.01 from those obtained for control non-treated cultures.

Table 5 shows that the concentrations of the water extract of Cistanche significantly down-regulated the *in vitro* expressions of OPN mRNA by rat OBs, especially at the concentrations of 5×10^{-1} mg/mL and 5×10^{-2} mg/mL after 72 hours of culturing.

Table 5. Effect of different concentrations of water extract of Cistanche on OPN expression by rat OB cell culture

Concentrations of water extract of Cistanche (mg/mL)		The OPN expression (Mean ± SD) by rat OBs after culturing for:		
		24 hours	48 hours	72 hours
Treated cultures	5×10^{-1}	0.650 ± 0.271	1.338 ± 0.408	0.376 ± 0.257**
	5×10^{-2}	0.683 ± 0.273	1.788 ± 0.837	0.433 ± 0.145*
	5×10^{-3}	0.625 ± 0.126	1.236 ± 0.296	2.235 ± 1.190
	5×10^{-4}	0.942 ± 0.105	1.135 ± 0.230	2.648 ± 0.098
Control culture	None	1.261 ± 0.227	2.757 ± 0.823	2.021 ± 0.353

The values marked with asterisk were significantly different either at * $P<0.05$ or ** $P<0.01$ from those obtained for control cultures.

Discussion

Ostoblasts (OBs) not only play an essential role in creating bone architecture but also in calcium homeostasis, by regulating osteoclastic activity (MACKIE, 2003). There are certain bone diseases associated with OB function or dysfunction, such as osteoporosis. The phenotypic markers of OB appearance are primarily their proliferation and differentiation, and, secondarily, mineralization of bone nodules (PAN et al., 2005). OPG and RANKL are members of the tumor necrosis factor (TNF) family of cytokines, and they are the OB-derived proteins crucial for the regulation and maintenance of bone mass (HUMPHREY et al., 2006; REINHOLZ et al., 2002). RANKL regulates osteoclast differentiation, activation, and survival. RANKL may also increase bone resorption by interacting with its receptor on the osteoclastic membrane (SODEK et al., 2000). OPG is the decoy receptor for the RANKL and prevents RANKL from binding to its receptor (RANK). The RANK/RANKL/OPG relationship controls the balance between bone formation and resorption, by regulating OB and osteoclast activities (SODEK et al., 2000; HUMPHREY et al., 2006; MAZZIOTI et al., 2012). Accordingly, it could be concluded that a decreased OPG/RANKL ratio may induce bone resorption by increasing osteoclast activation.

In this study, all treatments with herbaceous preparations of Cistanche increased the gene expressions of OPG and RANKL at 72 hours, especially at the concentrations of 5×10^{-2} mg/mL and 5×10^{-3} mg/mL, which were significantly different compared to the control cultures.

In contrast, OPG expression was significantly down-regulated after 24 and 48 hours of culturing with Cistanche extracts.

The expression of the OPG/RANKL ratio was significantly down-regulated when the OBs were treated with Cistanche for 24 hours and 48 hours. Also, the Cistanche extract had no significant effect on OB cultures after 72 hours of treatment. We hypothesize that the liquid extracts of Cistanche may improve osteoclast activation after 24 hours and 48 hours through regulated OPG/RANKL expression, but may not have any effect on osteoclast activation after 72 hours.

OPN has several functional diversities in bone formation and remodeling relating to the fundamental roles of OPN in host defense and tissue repair. The primary role of OPN appears to be regulation of negative feedback at the systemic level, promotion of OB cell adhesion, induction of early calcification events, and also control of the size and shape of the mineral crystals (SODEK et al., 2000). Moreover, it has been confirmed that OPN may represent a bridge protein, increasing arterial stiffness during inflammation, and may consequently cause joint damage and increase cardiovascular risk in patients with rheumatoid arthritis (BAZZICHI et al., 2009).

In our study we have shown that a liquid extract of Cistanche down-regulated the expression of OPN. The trend of decreasing OPN concentrations in the OB cultures compared to the control cultures observed at 24 hours and 48 hours of culturing, and significantly lower levels recorded at 72 hours, suggests that Cistanche may improve bone formation, as demonstrated by the *in vitro* experiment.

The *in vitro* exposure of OB cultures to Cistanche significantly promoted the gene expressions of OPG and RANKL after 72 hours, reduced the gene expression of OPG/RANKL after 24 hours and 48 hours, and decreased the expression of OPN after 72 hours of culturing.

The Cistanche herb is always used as a prescription consisting of multiple Chinese herbs, therefore the clinical effectiveness of Cistanche used in this study and the components in the liquid extract are complex and not yet clear. The results of our study demonstrate that Cistanche extract affects OPG and RANKL expression when applied to rat OB in cell culture, but the level of expression varies, depending on the concentration of Cistanche extracts and the duration of culturing. The appropriate concentration of the liquid extracts of *Cistanche deserticola,* in combination with the appropriate duration of exposure to rat OBs, may maintain the balance of bone formation and resorption during bone metabolism.

Further research is needed in order to improve our knowledge on the bioactive ingredients of Cistanche and their concentrations in preparations, for them to be validated as cytokine modulators for bone restoration and repair.

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SAŽETAK

Osteoblasti (OB) imaju važnu ulogu u remodeliranju kostiju tako što izlučuju citokine, kao što su osteoprotegerin (OPG), ligand receptora aktivacije jezgrenog činitelja kappa-B (engl. receptor activator of nuclear factor kappa-B ligand; RANKL) i osteopontin (OPN). Cilj našeg istraživanja bio je vrednovanje učinaka ekstrakata kineske biljke *Cistanche deserticola* na *in vitro* ekspresiju tih citokina u OB štakora u staničnoj kulturi. Primarni OB bili su izdvojeni iz lubanje štakora digestijom pomoću tripsina i kolagenaze, a njihov je fenotip bio određen uporabom Swiss metode bojenja, bojenjem alkalnom fosfatazom, te bojenjem crvenom bojom po Von Kossi i alizarinom. Ekspresije gena za OPG; RANKL i OPN analizarne su pomoću kvantitativne RT-PCR metode. Svi su tretmani s biljnim iscrpcima biljke Cistanche povećali ekspresiju gena za OPG i RANKL nakon 72 sata kultiviranja, posebice u koncentraciji od 5×10^{-2} mg/mL, kad su one bile značajno različite u usporedbi s vrijednostima dobivenima za kontrolne kulture, dok je većina tretmana značajno umanjila ekspresiju OPG/RANKL nakon 48 sati te ekspresiju OPN mRNA nakon 72 sata kultiviranja. *In vitro* izlaganje OB biljnom pripravku Cistanche u primijenjenim koncentracijama potaknulo je ekspresiju gena za OPG i RANKL te umanjilo ekspresiju gena za OPG/RANKL i OPN, pa bi stoga moglo uspostaviti ravnotežu pri obnavljanju i resorbiranju kostiju tijekom koštanog metabolizma.

Ključne riječi: *Cistanche deserticola*, osteoblasti, osteoprotegerin, osteopontin, RANKL