

Effect of advanced oxidation protein products on the proliferation and osteogenic differentiation of rat mesenchymal stem cells

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Abstract. Advanced oxidation protein products (AOPPs) as a novel marker of oxidative stress, are involved in a variety of diseases, including osteoporosis. Although a number of studies have shown the possible functions of AOPPs in biological processes, little is known about the role of AOPPs in the pathogenesis of osteoporosis. In this study, we aimed to investigate the effect of AOPPs on the proliferation and osteogenic differentiation of rat mesenchymal stem cells (MSCs). MSCs, isolated from bone marrow, were cultured in the absence or presence of AOPPs (50, 100, 200 and 400 mg/ml). MTT assay was used to determine the proliferative ability of the cells. Alkaline phosphatase (ALP) activity, the mRNA expression of ALP and collagen I and bone nodule formation were detected to assess osteogenic differentiation. Reactive oxygen species (ROS) generation was analyzed with the probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). The expression of receptor of advanced glycation end-products (RAGE) at the mRNA and protein level was detected by real-time PCR and western blot analysis, respectively. Compared with the control group, AOPPs inhibited MSC proliferation in a dose- and time-dependent manner. Moreover, AOPPs induced a significant reduction in ALP activity, as well as a decrease in ALP and collagen I mRNA levels in the MSCs; bone nodule formation was also inhibited. Furthermore, AOPPs increased ROS generation in the MSCs, and upregulated the expression of RAGE at

the mRNA and protein level. These results suggest that AOPPs inhibit the proliferation and osteogenic differentiation of MSCs, possibly through the AOPPs-RAGE-ROS pathway; this may be an important mechanism in the development of osteoporosis.

Introduction

Osteoporosis is a skeletal disorder characterized by an imbalance between bone resorption and bone formation, culminating in fragility fractures, pain and disability (1). Although the mechanisms involved are unclear, increasing evidence indicates that oxidative stress plays a role in the pathogenesis of osteoporosis (2,3). Oxidative stress is an imbalance between the free radicals and antioxidant mechanisms in biological systems and damages cellular macromolecules and functions. It can lead to an accumulation of reactive oxygen species (ROS) in the body which has adverse effects on the skeleton (4).

Advanced oxidation protein products (AOPPs) are defined as dityrosine-containing cross-linked protein products formed during oxidative stress by the action of chlorinated oxidants (5). Thus far, AOPPs have received increasing attention as novel markers of oxidative stress. There is a close correlation between AOPPs and monocyte activation markers *in vivo*, such as neopterin and interleukin-1 receptor (IL-1R) antagonist. AOPPs trigger the oxidative burst of human monocytes in culture (6). Evidence indicates that AOPPs increase ROS levels in human umbilical vein endothelial cells by activating nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, extracellular-signal-regulated kinase (ERK)1/2, p38 and nuclear factor (NF)- κ B (7).

AOPPs accumulate in the body and increase with age (8). Elevated levels of AOPPs are detected in many types of diseases, including osteoporosis (9-14). Certain studies have demonstrated that oxidative stress leads to increased levels of AOPPs in osteoblastic MC3T3-E1 cells (15,16); AOPPs have been shown to inhibit the proliferation and differentiation of rat osteoblastic cells through NF- κ B (17); these data suggest that AOPPs are associated with the progression of osteoporosis. However, to our knowledge, there are no studies to date on the effects of AOPPs on mesenchymal stem cells (MSCs).

Since impaired bone formation is an important mechanism involved in the pathological process of osteoporosis, the inhibition of osteoblast proliferation and the inability to form bone

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may result in osteoporosis (18). Bone cells purportedly originate from MSCs that commit to the osteogenic cell lineage, becoming osteoprogenitor cells, pre-osteoblasts, osteoblasts and osteocytes (19,20). MSCs in patients with osteoporosis also have abnormal gene expression (21,22). Thus, the negative effects on MSC osteogenic differentiation may aggravate the development of osteoporosis. Therefore, the aim of this study was to investigate the role of AOPPs in modulating the proliferation and osteogenic differentiation of rat bone marrow-derived MSCs, as well as the possible mechanisms involved.

Materials and methods

Materials. Trypsin-EDTA, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), the amebocyte lysate assay kit, dexamethasone, ascorbic acid, β -glycerophosphate, alizarin red, p-nitrophenyl phosphate and 3-(4,5-dimethylthiazozyl)-2,5-diphenyl tetrazolium bromide (MTT) were all obtained from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) was from Roche Diagnostics GmbH (Mannheim, Germany); Oil Red O was purchased from Amresco, Inc. (Solon, OH, USA); the BCA assay kit was from CoWin Biotech (Beijing, China); dimethyl sulfoxide (DMSO) and TRIzol reagent were obtained from Invitrogen (Buenos Aires, Argentina); adipogenic differentiation medium, fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were all from HyClone (Logan, UT, USA); the PrimeScript[®] one step RT-PCR kit and SYBR[®] Premix Ex Taq[™] II were from Takara Biotechnology (Dalian, China); RIPA lysis buffer was from Beyotime Biotech (Shanghai, China); mouse anti-receptor for advanced glycation end-products (RAGE) antibody and phycoerythrin (PE)-conjugated antibody against CD34 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); mouse anti-GAPDH antibody was from Abcam (Cambridge, UK); PE-conjugated antibody against CD29 was from BioLegend Inc. (San Diego, CA, USA); the Detoxi-Gel column was from Pierce (Rockford, IL, USA); the ECL kit was from Applygen Technologies Inc. (Beijing, China). All other chemicals and reagents were purchased from commercial sources and were of analytical grade.

MSC isolation and culture conditions. All animal procedures were approved by institutionally approved protocols and guidelines. After the rats (Sprague-Dawley, 4 weeks of age, male) were sacrificed by the controlled inhalation of CO₂, the hind legs were removed, the soft tissue was removed and bone marrow from the femurs was flushed out with serum-free DMEM. The cells were then cultured in H-DMEM with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. The medium was first changed after 2 days and subsequently every 2 to 3 days. MSCs were purified by plastic adherence and expanded in culture flasks. Third or fourth passage cells were used for all experiments.

AOPP preparation. AOPPs-BSA was prepared as described previously (17). Briefly, BSA (100 mg/ml) was incubated with 200 mM hypochlorous acid (HOCl) for 30 min at 37°C and dialyzed overnight against PBS to remove free HOCl. AOPPs-BSA was passed through a Detoxi-Gel column to remove contaminated endotoxin. Endotoxin levels in the prepa-

ration were determined using the amebocyte lysate assay kit and were found to be <0.25 EU/ml. AOPP content in the preparation was then determined. Briefly, 200 μ l of sample or chloramine-T were placed in a 96-well plate, followed by 20 μ l of acetic acid. The absorbance at 340 nm was determined immediately in a microplate reader. AOPP content was 57.6 \pm 4.9 nmol/mg protein in the AOPPs-BSA and 0.24 \pm 0.09 nmol/mg protein in the native BSA preparation.

Flow cytometry. The MSCs were analyzed by flow cytometry to determine the pluripotent cell characteristics. Following trypsinization, the cells were resuspended in PBS reaching a concentration of 3.0 \times 10⁶/ml. Cell suspension per sample (100 μ l) was then incubated with PE-conjugated antibodies against CD34, and CD29. The cells were incubated in the dark for 30 min at 4°C. The control samples were incubated with PE-conjugated isotype-matched antibodies. Following incubation, the cells were washed with PBS and centrifuged to remove unbound antibody. The cells were resuspended in 400 μ l PBS and evaluated by flow cytometry (Beckman-Coulter, Brea, CA, USA).

Adipogenic differentiation assay. MSCs were seeded at a density of 5 \times 10⁵ cells/well in 6-well plates and cultured in commercially available adipogenic differentiation medium for 3 weeks. For Oil Red O staining, the cells were fixed with 4% paraformaldehyde, washed in water and stained with a 0.6% (w/v) Oil Red O solution for 1 h at room temperature, followed by washing with water to remove unbound dye. Oil Red O staining of lipid droplets was analyzed under a phase-contrast inverted microscope (Olympus, Japan).

Osteogenic differentiation assay. MSCs were seeded at 5 \times 10⁵ cells/well in 6-well plates and were stimulated with osteogenic differentiation medium as previously described (23) for 3 weeks. To evaluate the effects of AOPPs on the osteogenic differentiation of MSCs, the cells were cultured in the induction medium for 2 weeks, followed by stimulation in 10% FBS-DMEM with the addition of BSA (200 μ g/ml) or AOPPs (200 μ g/ml) for 1 week. For the detection of osteogenic differentiation, bone nodule formation was examined by alizarin red staining. Briefly, the cells were fixed with 95% ethanol for 10 min and washed 3 times with PBS. Alizarin red solution (1%) was added to the cells for 30 min at 37°C, and then washed with PBS. Bone nodule formation was observed under a phase-contrast microscope.

Cell proliferation assay. The MSCs were plated at 10⁴ cells/well in 96-well plates and cultured for 2 days in 10% FBS-DMEM. Then they were serum-deprived overnight and incubated with control medium, BSA (200 μ g/ml) or AOPPs (50-400 μ g/ml) for 3 days, or with AOPPs (200 μ g/ml) for different periods of time (0, 24, 48, 72 h). MTT assay was used to evaluate the viability of the MSCs. In brief, MSCs in 96-well plates were washed twice with 0.01 M PBS, then incubated in 100 μ l of FBS-free DMEM supplemented with 10 μ l of 5 mg/ml MTT solution at 37°C. After 4 h, the supernatant was carefully removed, and the crystals were dissolved by incubation with 150 μ l of DMSO for 20 min. The plates were shaken for 5 min and the absorbance at 570 nm was measured

in a microplate reader. The results are expressed as absorbance (OD at 570 nm).

Alkaline phosphatase (ALP) activity assay. The MSCs were seeded at 10^5 cells/well in 24-well plates and stimulated with osteogenic differentiation medium for 2 weeks. The cells were then serum-deprived overnight and incubated under various culture conditions as described above for 3 days. Cell extract was then prepared using RIPA lysis buffer. ALP activity in the cell lysates was assayed at the end of the incubation time with 10 mM p-nitrophenyl phosphate in 0.15 M sodium carbonate buffer (pH 10.3) and 1 mM $MgCl_2$ as previously described (24). The enzyme activity was normalized against the cellular protein concentration and expressed as U/g protein. Protein concentrations were determined using the BCA assay kit.

Determination of ROS generation. Intracellular ROS generation was measured by flow cytometry with the probe DCFH-DA. Briefly, MSCs grown in 10-cm plates were subjected to various culture conditions as described above for 2 h. The medium was replaced by control medium with 10 μ M DCFH-DA for 30 min in the dark. Intracellular ROS generation was visualized under a fluorescent microscope (Olympus, Tokyo, Japan). DCF fluorescence was measured on a flow cytometer. Data were normalized to the control values.

Gene expression by real-time polymerase chain reaction (PCR). MSCs for examining ALP and collagen I mRNA levels were stimulated with osteogenic differentiation medium for 1 week; they were then serum-deprived overnight and subjected to various culture conditions as described above for 3 days. Cells for RAGE were only subjected to various culture conditions as described above for 3 days. After this period, total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. cDNA was reverse-transcribed from 0.8 μ g of total RNA using the PrimeScript one step RT-PCR kit. The primers used were as follows: ALP forward, 5'-AGATGGACAAGT TCCCTTTG-3' and reverse, 5'-ACACAAGTAGGCAGTG GCAGT-3'; collagen I forward, 5'-GTGGAAACCTGATGTAT GCTTG-3' and reverse, 5'-ATGACTTCTGCGTCTGCGTCTG GTGATA-3'; RAGE forward, 5'-ACTCACAGCCAATGTCCC TAA-3' and reverse, 5'-CTTTGCCATCAGGAATCAGAG-3'; and β -actin forward, 5'-TTCTACAATGAGCTGCGTGT GGC-3' and reverse, 5'-CTCATAGCTCTTCTCCAGGGA GGA-3'. Real-time PCR was carried out in a Real-Time PCR System (Stratagene/Agilent Technologies, Wilmington, DE, USA) using SYBR Premix Ex Taq II. The cycling conditions were as follows: 95°C for 2 min and 40 cycles of 95°C for 5 sec, 60°C for 30 sec. The value of $2^{-\Delta\Delta Ct}$ represents the relative level of target gene expression.

Western blot analysis of RAGE. The MSCs were stimulated with various culture conditions as described above for 3 days following starvation overnight in serum-free medium. Proteins were then extracted from the MSCs using RIPA lysis buffer followed by centrifugation at 4°C, 12,000 rpm for 20 min. Protein concentration was determined by BCA assay. Proteins were then separated by SDS-PAGE and electrotransferred onto a PVDF membrane. The membrane was incubated with mouse anti-RAGE (1:500) and mouse anti-GAPDH antibodies (1:500) overnight at 4°C. The

membrane was washed 3 times with TBST and incubated with HRP-conjugated secondary IgG for 50 min. The bands were detected using the ECL chemiluminescence detection system.

Statistical analysis. All values are expressed as the mean \pm SD. Each experiment was repeated at least 3 times. One-way ANOVA followed by the Scheffe's test or Dunnett's T3 (equal variances not assumed) was performed for multiple comparisons. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Isolation and identification of MSCs. MSCs were isolated from rat femur bone marrow and formed elongated spindle or polygon shapes. Passage 3 MSCs were detected by flow cytometry and the results revealed that the cells were negative for CD34 (Fig. 1A) and positive for CD29 (Fig. 1B). The former is a marker of hematopoietic stem cells and the latter is a characteristic cell surface marker of MSCs. Following stimulation in adipogenic differentiation and osteogenic differentiation medium, lipid droplets in the cells (Fig. 1C) and bone nodules (Fig. 1D) were observed.

Effects of AOPPs on proliferation of MSCs. MSC proliferation was evaluated by MTT assay. As shown in Fig. 2A, AOPPs (50–400 μ g/ml) significantly inhibited the proliferation of the MSCs within 3 days compared with the control medium and the unmodified BSA preparation. Furthermore, the inhibitory effects on proliferation were observed in the MSCs stimulated with 200 μ g/ml of AOPPs for 24–72 h (Fig. 2B). These results indicated that AOPPs inhibited MSC proliferation in a dose- and time-dependent manner.

Effects of AOPPs on osteogenic differentiation of MSCs. To investigate the effect of AOPPs on MSC osteogenic differentiation, ALP activity and the mRNA expression of ALP and collagen I were detected. The cells were stimulated as described above. As shown in Fig. 3A, AOPPs (100–400 μ g/ml) decreased ALP activity in the MSCs; a time-dependent decrease in ALP activity was observed in the cells exposed to AOPPs (Fig. 3B) compared with those cultured in the control medium and BSA. We also found that the exposure of MSCs to AOPPs resulted in a significant decrease in the mRNA expression of ALP (Fig. 4A) and collagen I (Fig. 4B). Although bone nodule formation was observed in the control group, only slight bone nodule formation was observed in the cells exposed to AOPPs (Fig. 5) after alizarin red staining. These data indicate that AOPPs inhibit the osteogenic differentiation of MSCs.

Effects of AOPPs on ROS generation in MSCs. ROS generation was examined by the detection of DCF fluorescence by flow-cytometry analysis. The increase in ROS generation in the MSCs was observed visually in the cells exposed to AOPPs compared with the control and BSA-treated cells (Fig. 6A–F); this increase in ROS occurred in a dose-dependent manner (Fig. 6G).

Effects of AOPPs on RAGE mRNA and protein expression in MSCs. RAGE is considered a receptor of AOPPs, and oxidative stress may be activated with their binding (7). The mRNA

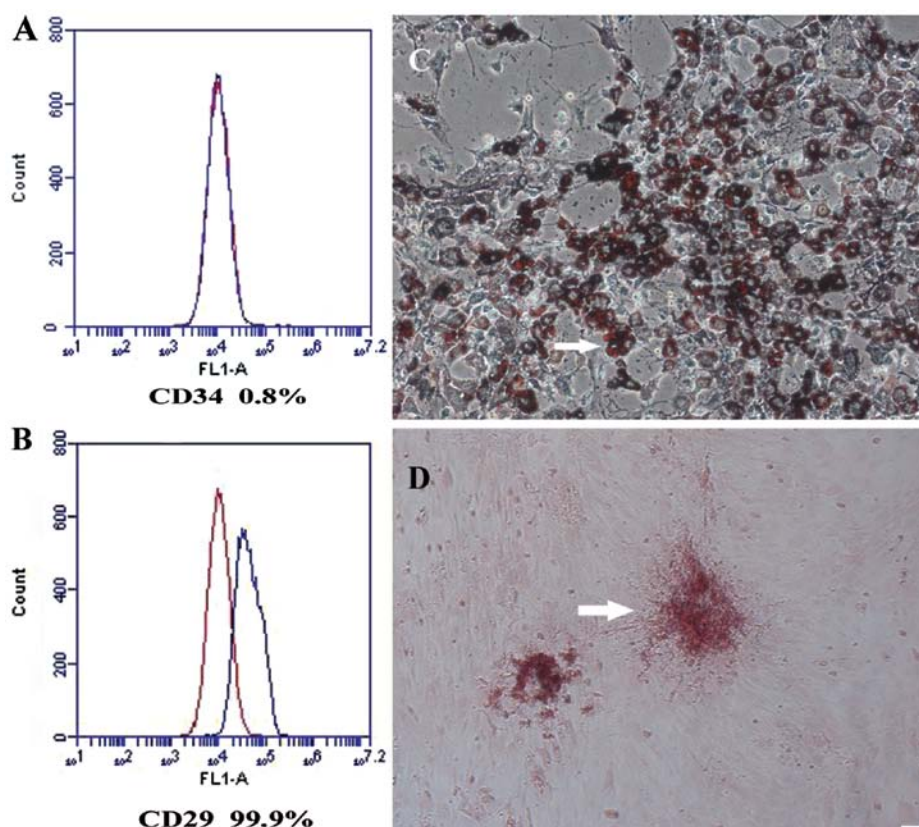


Figure 1. Identification of mesenchymal stem cells (MSCs). The surface markers, CD34 and CD29, in the MSCs were detected by flow cytometry. MSCs were stimulated with adipogenic and osteogenic differentiation medium for 3 weeks. Lipid droplets were detected by Oil Red O staining. Bone nodule formation was examined by alizarin red staining. The MSCs were (A) negative for CD34 and (B) positive for CD29. (C) Lipid droplets (arrows) and (D) bone nodules (arrows) were also observed following stimulation. Scale bar, 100 μ m.

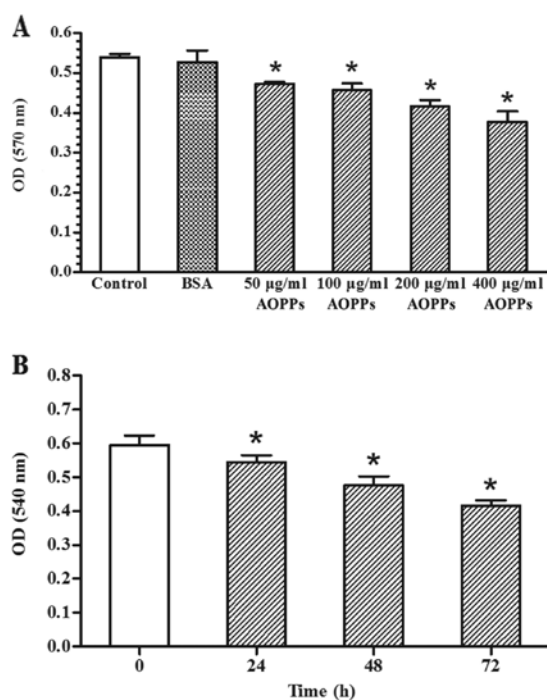


Figure 2. Effect of advanced oxidation protein products (AOPPs) on the proliferation of mesenchymal stem cells (MSCs). MSCs were incubated with control medium, bovine serum albumin (BSA) or indicated concentrations of AOPPs for the indicated periods of time. MTT assay was used for detecting cell proliferation. AOPPs inhibited MSC proliferation in (A) a concentration- and (B) a time-dependent manner. Data are presented as the means \pm SD. $P < 0.01$, * $P < 0.01$ vs. control.

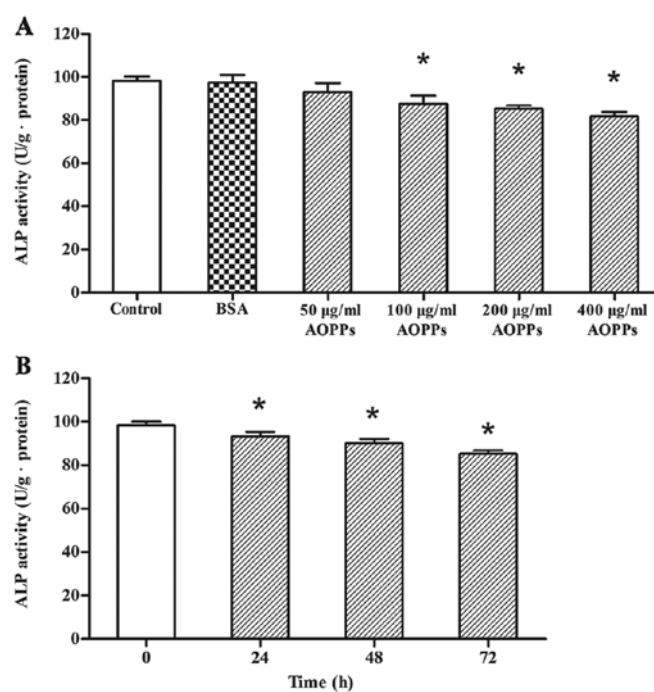


Figure 3. Effect of advanced oxidation protein products (AOPPs) on alkaline phosphatase (ALP) activity in mesenchymal stem cells (MSCs). MSCs were stimulated with osteogenic differentiation medium for 2 weeks, then incubated with control medium, bovine serum albumin (BSA) or indicated concentrations of AOPPs for 3 days. (A) AOPPs (100–400 μ g/ml) decreased ALP activity in the MSCs; (B) AOPPs decreased ALP activity in a time-dependent manner. Data are presented as the means \pm SD. $P < 0.01$, * $P < 0.01$ vs. control.

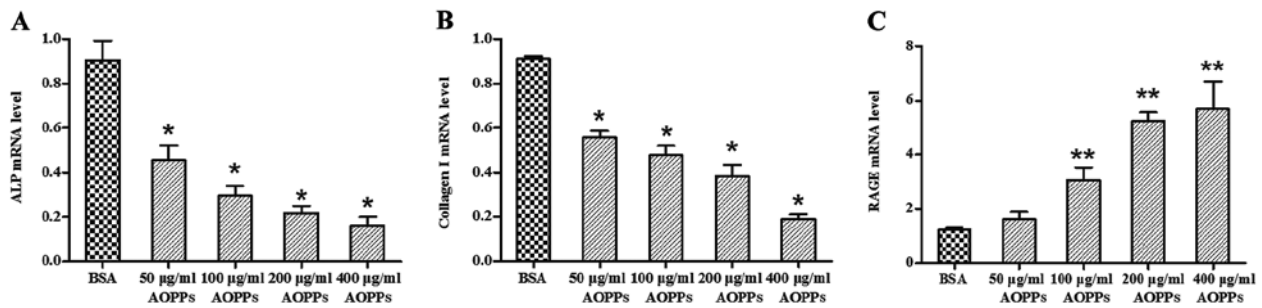


Figure 4. Effect of advanced oxidation protein products (AOPPs) on the expression of alkaline phosphatase (ALP), collagen I, RAGE mRNA. Mesenchymal stem cells (MSCs) were incubated with control medium, bovine serum albumin (BSA) or indicated concentrations of AOPPs for the indicated periods of time. mRNA levels were detected by real-time PCR. AOPPs inhibited the mRNA expression of (A) ALP, (B) collagen I and (C) RAGE. Data are presented as the means \pm SD. (A and B) $P < 0.01$, (C) $P < 0.05$, * $P < 0.01$ vs. control, ** $P < 0.05$ vs. control.

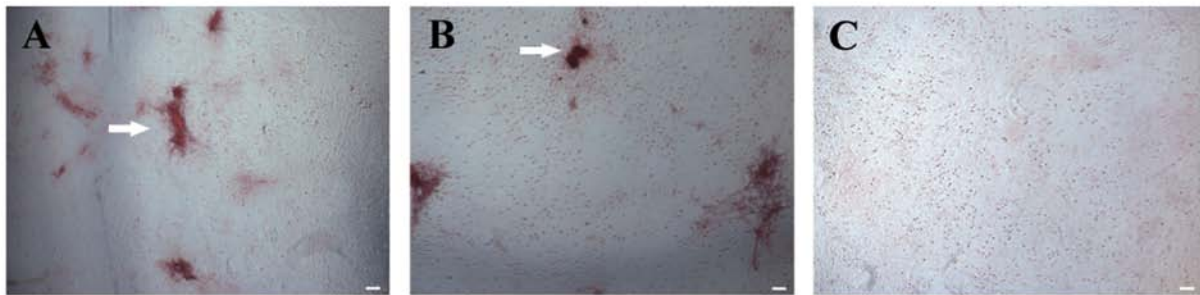


Figure 5. Effect of advanced oxidation protein products (AOPPs) on bone nodule formation of mesenchymal stem cells (MSCs). MSCs were cultured in induction medium for 2 weeks, and then stimulated in medium with the addition of bovine serum albumin (BSA) (200 µg/ml) or AOPPs (200 µg/ml) for 1 week. Bone nodules were observed by alizarin red staining. Bone nodule formation was observed in (A) the controls and (B) the BSA-treated cells, indicated by arrows; (C) however, only slight bone nodule formation was observed in the cells exposed to AOPPs. Scale bar, 200 µm.

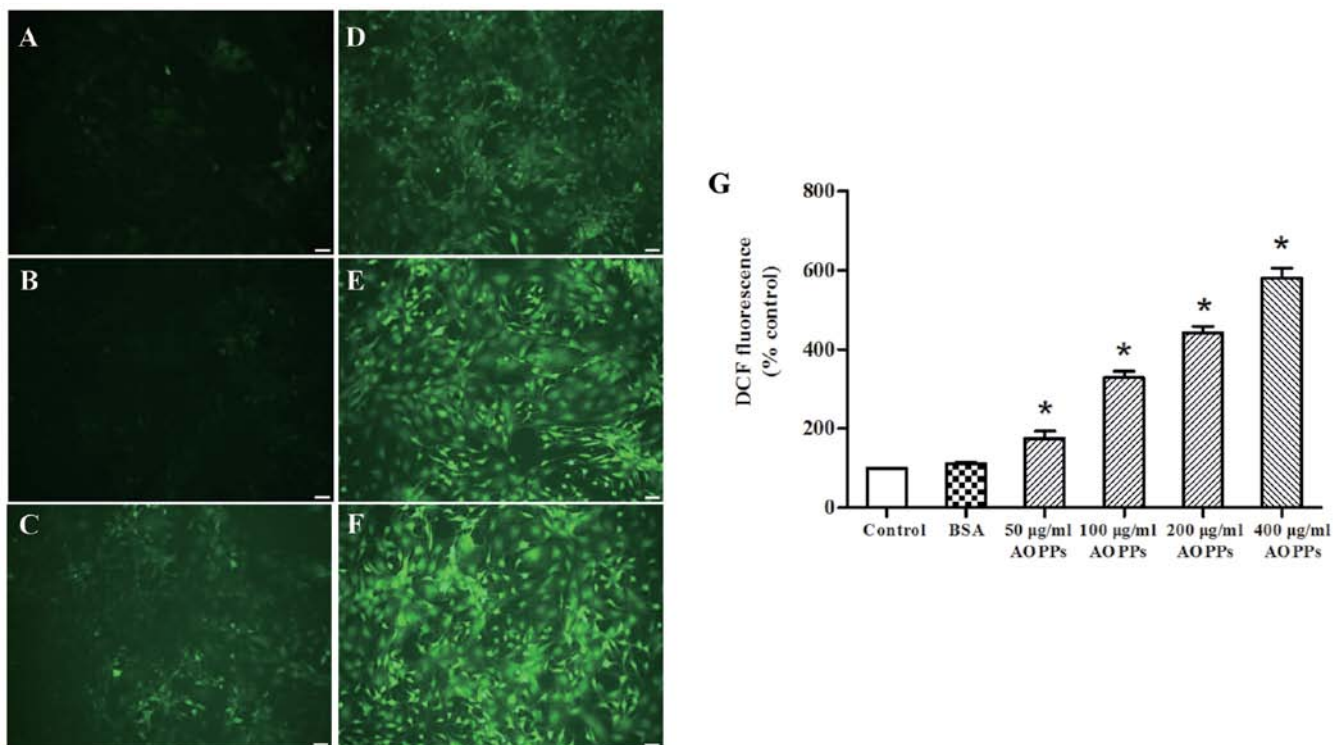


Figure 6. Effect of advanced oxidation protein products (AOPPs) on the generation of reactive oxygen species (ROS) in mesenchymal stem cells (MSCs). MSCs were incubated with control medium, bovine serum albumin (BSA) or indicated concentrations of AOPPs for 2 h. Intracellular ROS generation was measured with the probe DCFH-DA, and visualized using a fluorescent microscope. (A) Control; (B) BSA; (C) 50 µg/ml AOPPs; (D) 100 µg/ml AOPPs; (E) 200 µg/ml AOPPs; (F) 400 µg/ml AOPPs. DCF fluorescence was measured on a flow cytometer. AOPPs induced ROS generation (as visualized by green fluorescence) compared with the control and BSA (A-F), in a dose-dependent manner (G). Data are presented as the means \pm SD. $P < 0.01$, * $P < 0.01$ vs. control. Scale bar, 100 µm.

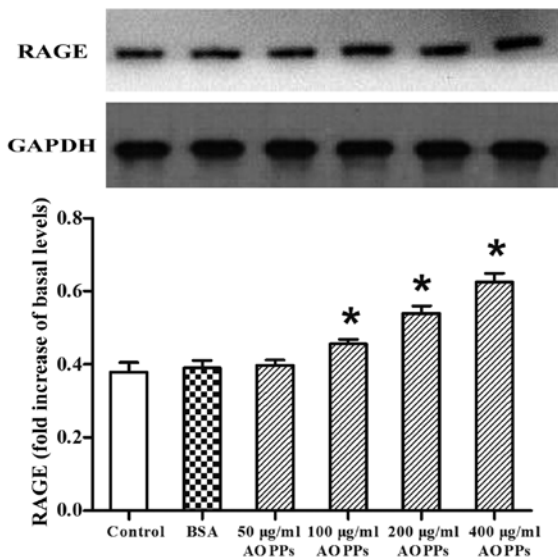


Figure 7. Effect of advanced oxidation protein products (AOPPs) on the protein expression of RAGE. Mesenchymal stem cells (MSCs) were incubated with control medium, bovine serum albumin (BSA) or indicated concentrations of AOPPs for 3 days. Protein levels were detected by western blot analysis. AOPPs (100–400 µg/ml) upregulated the protein level of RAGE. Data are presented as the means ± SD. * $P < 0.05$, * $P < 0.05$ vs. control.

and protein expression of RAGE in the MSCs was measured by real-time PCR and western blot analysis, respectively. As shown in Fig. 4C and 7, exposure to AOPPs, as opposed to exposure to BSA, increased the mRNA and protein expression of RAGE.

Discussion

Bone mass is maintained locally by the balance between osteoclastic bone resorption and osteoblastic bone formation. The latter process is carried out by osteoblasts whose number and activity is determined by the proliferation and differentiation of osteoblast precursors derived from MSCs (25). Hence, MSCs play an important role in the development of osteoporosis. The cells used in this study were positive for the cell-surface marker, CD29, but negative for CD34, and had the potential to differentiate into adipose cells and osteoblasts, suggesting that these cells were MSCs.

Increasing evidence indicates that AOPPs, as a novel marker of oxidative stress, are involved in various diseases, such as renal failure (5), diabetes mellitus (9), coronary artery disease (12) and osteoporosis (14), and have various biological activities. The data presented in this study demonstrate that AOPPs inhibit the proliferation and osteogenic differentiation of MSCs. We found that AOPPs inhibited the proliferation and ALP activity in MSCs, as well as ALP and collagen I gene expression. In addition, AOPPs increased the mRNA and protein expression of RAGE, as well as the generation of ROS. These results suggest that AOPPs exert adverse effects on MSCs through the RAGE-ROS pathway.

It has been reported that AOPPs inhibit cell proliferation in human gingival fibroblasts (26) and osteoblasts (17); however, it also has been shown that AOPPs (50–400 µg/ml) induce adventitial fibroblast proliferation (27). In the present study, we found that AOPPs inhibited MSC proliferation in a dose- and time-dependent manner. These results indicate that AOPPs react differently according to different cell types and doses.

The developmental sequence of osteoblast differentiation can be characterized in 3 stages, including cell proliferation, extracellular matrix production and mineralization. During proliferation, the production of collagen I initially occurs, followed by an increased in the expression of ALP and calcium deposition (28). Collagen I comprises approximately 90% of the organic material as the most abundant protein component of the bone matrix. The enhanced expression of ALP metabolizes calcium phosphate into insoluble phosphate salts, thus mediating calcification (29). In this study, AOPPs significantly decreased ALP and collagen I mRNA levels and inhibited ALP secretion by MSCs in a dose- and time-dependent manner. Using alizarin red staining, we also observed that dystrophic mineralization occurred in the MSCs exposed to AOPPs compared with the controls. These data indicate that AOPPs inhibit MSC osteogenic differentiation.

AOPPs have similar biological characteristics to those of advanced glycation end-products (AGEs), and bind to the same receptor, RAGE (8). To investigate the potential mechanisms behind the adverse effects AOPPs on MSCs, we examined ROS levels and RAGE expression in the MSCs exposed to AOPPs. ROS are products of oxidative stress, such as $O_2^{\cdot -}$, H_2O_2 and $\cdot OH$, which have been implicated in the regulation of diverse cellular functions, including intracellular signaling, transcriptional activation, proliferation and apoptosis. A number of studies have suggested that oxidative stress is involved in the progression of osteoporosis. Oxidative stress suppresses the osteoblastic differentiation of MSCs through ERK and NF- κB (30). Receptor activator of NF- κB ligand (RANKL)-mediated ROS production may promote the differentiation of MSCs into osteoclasts as an intracellular signal mediator (31). A reduction in ROS permits the restoration of osteoblastic markers, specifically the induction of osteoprotegerin and osteocalcin (32). Our results also demonstrated that AOPPs increased ROS generation in the MSCs in a dose-dependent manner, indicating that ROS production induced by AOPPs may participate in the inhibition of proliferation and the osteogenic differentiation of MSCs.

RAGE is capable of binding to multiple ligands, including AGEs, AOPPs, high-mobility group box-1 and β -sheet fibrils (33). AGEs-RAGE interaction induces the generation of ROS through NADPH oxidase, resulting in the apoptosis of osteoblasts/MSCs (34) and in the inhibition of the proliferation and differentiation of osteoblasts/MSCs (35). RAGE overexpression by lentiviral transfection has been shown to inhibit osteoblast proliferation through the suppression of the Wnt, PI3K and ERK pathways (36). Studies using RAGE knockout mice have also shown increased bone mass and bone biomechanical strength and a decreased number of osteoclasts in RAGE $^{-/-}$ mice compared with wild-type mice (37). These data indicate that RAGE plays a modulatory role in the development of osteoporosis. In this study, we also observed an increased expression of RAGE at the mRNA and protein level as the dose of AOPPs increased. Therefore, taking all these data into consideration, we hypothesized that AOPPs may inhibit the proliferation and osteogenic differentiation of MSCs by upregulating RAGE expression following an increase in ROS production.

In conclusion, our data indicate that AOPPs inhibit the proliferation and osteogenic differentiation of MSCs. We also observed an increase in RAGE expression and ROS generation. Although the association between the increased expression of

RAGE and increased ROS generation induced by AOPPs was not examined in detail, considering the similar biological characteristics of AOPPs and AGEs and taking into account the data from previous studies, it can be concluded that AOPPs inhibit the proliferation and osteogenic differentiation of MSCs through a AOPPs-RAGE-ROS pathway. This may provide a new perspective as to the development of osteoporosis, and further suggests that AOPPs may be an effective clinical indicator of osteoporosis.

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