Original Research Article

Low-dose aspirin promotes osteogenic differentiation and osteogenic activity in osteoporotic rats by regulating Opg/Rankl/Rank Axis

Haijiao Zhao[1], Lei Zhao[2]*

[1]Department of Stomatology, The Fourth Affiliated Hospital of School of Medicine, Zhejiang University, [2]Department of Oral Implantology, Yiwu Stomatological Hospital, Yiwu 322000, PR China

*For correspondence: **Email:** hbpn60@163.com

Abstract

Purpose: To investigate the effect of low-dose aspirin (ASP) on osteogenic differentiation and osteogenic effects in osteoporotic rats, and the associated mechanism.
Methods: The bone marrow mesenchymal stem cells were randomly divided into 4 groups: control group and 3 drug groups treated with graded doses of ASP i.e. 0.5, 1 and 2 mmol/L, with 4 wells in each group. The OPG, RANKL protein expression levels, OPG/RANKL ratio, alkaline phosphatase (ALP) level, and ALP secretion at days 3, 5 and 7 were compared amongst the groups.
Results: ALP secretion in BMSCs was markedly higher in the 1 mmol/L ASP group than in control ($p < 0.05$). The OPG protein concentration and OPG/RANKL ratio were markedly higher in ASP-treated BMSCs than in control, while RANKL protein expression level was significantly lower than that in control ($p < 0.05$). In BMSCs treated with ASP at doses of 0.5 and 2 mmol/L, OPG protein expression levels and ratio of OPG/RANKL were markedly lower than those in 1 mmol/L ASP group, while the mean level of RANKL protein expression was markedly higher than that in 1 mmol/L ASP group ($p < 0.05$).
Conclusion: Low-dose aspirin increases the expression of ALP, and promotes calcification which relates to upregulation of OPG and inhibition of the OPG/RANKL/RANK axis. This provides new leads for the development of new anti-osteoporosis drugs.

Keywords: Aspirin, OPG, RANKL, RANK, Osteoporosis

INTRODUCTION

Postmenopausal osteoporosis has become a global public health problem, and its prevention and treatment have attracted a lot of attention from clinical researchers [1]. China is one of the countries with a high incidence of osteoporosis. A survey has shown markedly higher risk of osteoporotic hip fracture in women than the total risks of breast cancer, ovarian cancer and endometrial cancer [2]. In clinical practice, drugs such as hormone-stimulating calcitonin, and parathyroid hormone are used to treat postmenopausal osteoporosis. However, these treatment methods have certain disadvantages such as slow onset time, long treatment time, numerous adverse reactions, and high financial cost [3].

Therefore, it is important to evolve new, effective, and highly safe drugs for osteoporosis treatment. Osteoprotegerin (OPG) is formed in osteoblasts, the number of which can increase with cell differentiation, and is currently the only negative regulatory factor found in osteoclasts [4]. The function of receptor activator of nuclear factor-κB (RANK) is similar to that of OPG to a certain extent, and it partially blocks the ligand of RANKL, thereby significantly inhibiting osteoclast bone resorption [5].

Aspirin (ASP), a drug commonly used for middle-aged and elderly people, is easily affordable, and it has very few adverse reactions. Moreover, it is used in a wide range of treatments. Ingestion of low-dose aspirin for more than 1 year resulted in significant increase in BMD, especially in elderly females and post-menopausal women who took aspirin daily for 4 years [6]. Some studies have used ASP to treat osteoporotic rat models after ovariectomy, and found that the drug significantly improved trabecular bone and cortical bone density in rats [7]. Therefore, in this study, it was hypothesized that ASP has an anti-osteoporosis effect. However, ASP has not been used clinically to treat osteoporotic lesions. This study was carried out to determine the influence of ASP on postmenopausal osteoporosis, and the process involved.

EXPERIMENTAL

Materials

Twelve female Sprague Dawley rats aged 3 weeks (mean weight = 245 ± 25 g) were purchased from Guangzhou Focus Biotechnology Co. Ltd. The rats were raised in separate cages (4 in each cage), and were permitted *ad libitum* availability of feed and water which were changed once every 12 hours. Adaptive feeding was done 7 days before the start of the study.

This research was approved by the Animal Ethical Committee of Yiwu Stomatological Hospital (approval no. 20190421), and conducted according to the guidelines of Principles of Laboratory Animal Care [8].

Main reagents and equipment

Aspirin (ASP) was purchased from Wuxi Asiapeptide Biotechnology Co. Ltd. Alkaline phosphatase detection kit was purchased from Shanghai Xin Yu Biotech Co. Ltd. Alkaline phosphatase staining kit was bought from Shanghai Duma Biotechnology Co. Ltd. Alizarin red calcium nodule staining solution was product of Beijing Chreagen Biotechnology Co. Ltd. ELISA kits for RANKL were purchased from Wuhan Vicset Technology Co. Ltd, while OPG ELISA kits were obtained from Shanghai Jingkang Biological Engineering Co. Ltd.

Cell culture flasks were purchased from Greiner Biotechnology (Shanghai) Co. Ltd. Cell culture plates (24 wells) were obtained from Beijing BioDee Biotechnology Co. Ltd. Conventional surgical instruments were produced by Hunan YX Biology Co. Ltd. Enzyme-linked immunoassay kits were purchased from Guangzhou Vipotion Biotechnology Co. Ltd. Ultra-clean workbench was supplied by Jinan Bohang Scientific Instrument Co. Ltd. Medical centrifuge was product of Beijing Taize Jiaye Technology Development Co. Ltd., while inverted microscope was purchased from Dongguan Spectral Lab Equipment Technology Co. Ltd.

Treatments

The rats were anesthetized with intraperitoneal injection of 1% pentobarbital sodium, and their ovaries were removed so as to establish ovariectomized rat model. After 12 weeks, the ovariectomized rats were sacrificed via atlas dislocation, and BMSC osteoblast-inducing cells were isolated. The cells were subjected to primary culture and subculture.

Then, 3 to 6 generation BMSCs were seeded in a 24-well plate at a density of 1×10^6 cells/well. Four groups of cells were used: control group, and 3 groups of BMSCs treated with ASP at doses of 0.5, 1 and 2 mmol/L, with 4 wells for each group. The BMSCs in the control group were untreated.

Alkaline phosphatase (ALP) assay kit was used to assay ALP secretion in each group of BMSCs at days 3, 5 and 7; while ALP staining kit was used to detect ALP in each group of BMSCs on day 7. After 21 days, BMSCs in each group were stained with alizarin so as to observe the number of calcium nodules and calculate the percentage of calcified area. With respect to the latter, 3 specimens were randomly selected from each specimen under high magnification, and the mean area was obtained. Using the control group as the standard, the % of calcified area was calculated.

The expression levels of OPG and RANKL in BMSCs of each group were determined after 14 days using ELISA kits, and the OPG/RANKL ratio was calculated.

Statistical analysis

Data analysis was done using SPSS version 22.0. Measurement data for ALP secretion and calcium nodules in BMSCs in each group are expressed as mean ± standard deviation (SD). Student's *t*-test was used for two-group comparison, while one-way analysis of variance (ANOVA) was used for comparison amongst multiple groups. Values of *p* < 0.05 indicated significant differences.

RESULTS

ALP secretion by BMSCs

On day 3, there was no significant difference in ALP levels between BMSC groups treated with ASP and untreated control group (*p* > 0.05). However, after 5 days, ALP secretion was markedly higher in BMSCs in the 1 mmol/L ASP group than in control. A similar result was obtained after 7 days in ALP groups, at which period the BMSCs in each group had differentiated and active osteoblasts with positive cytoplasmic reaction and small amounts of brown particles were present in the cytoplasm. These results are shown in Figure 1.

Table 1: BMSCs ALP secretion of ALP (mean ± SD)

Group	ALP secretion (U/100 mL)		
	Day 3	*Day 5*	*Day 7*
Control	0.33 ± 0.03	0.65 ± 0.04	1.01 ± 0.04
ASP			
0.5 mmol/L	0.32 ± 0.03	0.67 ± 0.04	1.08 ± 0.05[*]
1 mmol/L	0.33 ± 0.03	0.78 ± 0.05[*]	1.30 ± 0.06[*]
2mmol/L	0.32 ± 0.03	0.70 ± 0.05	1.15 ± 0.05[*]

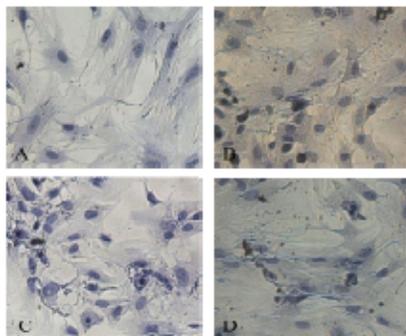**P* < 0.05, vs control.

Figure 1: ALP staining of BMSCs in each group on day 7. A: Control group; B: ASP at a dose of 0.5 mmol/L; C: ASP at a dose of 1 mmol/L; D: ASP at a dose of 2 mmol/L

Number and area of BMSCs calcium nodules

The number and area of calcium nodules in BMSCs in ASP groups were significantly higher than those in control group. The number and area of calcium nodules in BMSCs treated with ASP at doses of 0.5 and 2 mmol/L were markedly lower than those in 1 mmol/L ASP group (*p* < 0.05). These results are presented in Table 2 and Figure 2.

Table 2: Percentage of calcified area of BMSCs in each group (mean ± SD)

Group	Calcified area (%)
Control	101.23 ± 22.56
ASP	
0.5 mmol/L	148.17 ± 23.89[*#]
1 mmol/L	169.47 ± 28.15[*]
2 mmol/L	155.12 ± 227.19[*#]

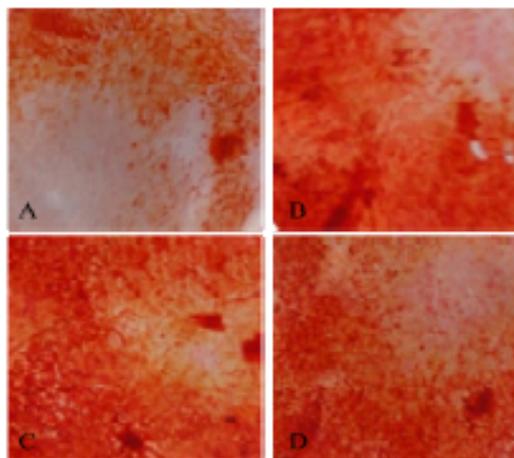**P* < 0.05, vs control, #*p* < 0.05, vs ASP dose of 1 mmol/L group

Figure 2: Calcium nodule staining of BMSCs in each group. A: Control group; B: ASP at a dose of 0.5 mmol/L; B: ASP at a dose of 0.5 mmol/L; C: ASP at a dose of 1 mmol/L; D: ASP at a dose of 2 mmol/L

OPG and RANKL proteins in BMSCs

The OPG protein levels and OPG/RANKL ratio were markedly increased in the ASP-treated BMSCs, relative to control, while the RANKL protein expression was markedly decreased, when compared to control (*p* < 0.05). The expression levels of OPG protein and OPG/RANKL ratio in BMSCs treated with ACP at doses of 0.5 and 2 mmol/L were significantly lower than those in BMSCs treated with ASP at a dose of 1 mmol/L, while RANKL protein expression was significantly higher (*p* < 0.05). These results are shown on Table 3.

Table 3: Comparison of OPG and RANKL protein expression levels and OPG/RANKL ratio of BMSCs amongst the groups (mean ± SD)

Group	OPG	RANKL	OPG/RANKL
Control ASP	0.33 ± 0.05	0.74 ± 0.08	0.46 ± 0.05
0.5 mmol/L	0.45 ± 0.07*#	0.60 ± 0.06*#	0.82 ± 0.53*#
1 mmol/L	0.67 ± 0.08*	0.50 ± 0.06*	1.44 ± 0.06*
2 mmol/L	0.53 ± 0.06*#	0.57 ± 0.06*#	1.10 ± 0.08*#

*$P < 0.05$, vs control; #$p < 0.05$, vs 1 mmol/L ASP

DISCUSSION

Postmenopausal osteoporosis is a primary osteoporosis which manifests as systemic bone loss, degeneration of bone microstructure, increased bone fragility and increased risk of fracture [9]. Postmenopausal osteoporosis is a serious problem which deserves research attention. Thus, the focus of this research was to evolve a safe and more easily available drug for protecting against postmenopausal osteoporotic lesions, and for treating same. Clinical studies have shown that ASP increases the osteogenic gene opening and mineral accumulation of normal human BMSCs. Another study found that when BMSCs of mice were transplanted subcutaneously in nude mice, the bone formation ability of the ASP intervention group was significantly increased, when compared with the control group, indicating that ASP promotes bone formation and mitigates osteoporosis [10]. Alkaline phosphatase (ALP) is an important marker enzyme for differentiation of BMSCs. It is present in the cytoplasm of mature and active osteoblasts. The higher the activity of ALP, the greater the degree of osteogenic differentiation [11]. In this study, it was found that the secretion of ALP was markedly higher in BMSCs treated with ASP at a dose of 1 mmol/L than in control after 5 days, and after 7 days, the secretion of ALP in all ASP-exposed BMSCs was markedly higher than that of control. These results suggest that low-dose ASP increases ALP activity. Calcified nodule is used as an important indicator of osteogenic properties. Type I collagen is produced in osteoblasts and appears in the form of collagen. Type 1 collagen is rapidly cleaved in the matrix, resulting in the release of C-terminal and N-terminal peptides, thereby producing mature Type I collagen, which is one of the important components of bone organic matter. The results of this study revealed that the population and area of calcium nodules were markedly higher in the BMSCs of the ASP dose group than in control, and the expression was most significant when the ASP dose was 1 mmol/L. This suggests that low-dose ASP significantly promotes calcium salt deposition. Clinical studies have shown that OPG suppressed differentiation of osteoclast precursor cells and mature osteoclasts to form bone resorption pockets, and promoted osteoclast apoptosis. Moreover, it hindered osteoclast-mediated bone resorption and improved cortical bone and cancellous bone density [12]. Animal studies have shown that after knocking out the OPG gene in mice, the mice suffered from severe osteoporosis, and that excessively increased levels of OPG expression in mice resulted in severe osteoporosis [13]. Therefore, many scholars believe that OPG can be used as a physiological inhibitor of osteoclast differentiation and function, which is beneficial for maintenance of balance between bone resorption and bone formation. Many studies have shown that the expression of OPG in bone tissue is closely related to OPG/RANKL/RANK pathways mediated by the maturation and activation of osteoblasts, and is key to the onset of postmenopausal osteoporosis [14]. Clinical studies have shown that after menopause, there are many RANKs on the surface of osteoclasts which can be combined with RANKL to activate osteoclasts, eventually leading to a "high absorption" state [15,16]. This study showed that the OPG protein expression level and OPG/RANKL ratio were markedly elevated in the BMSCs of the ASP dose group, relative to control, while the RANKL protein expression level was markedly lower. Moreover, ASP at a dose of 1 mmol/L ASP was more effective than at any other dose, suggesting that it can induce BMSCs to release OPG, competitively bind RANKL to block RANKL/RANK binding, and ultimately inhibit BMSCs activation and reduce bone resorption. This study has shown that low-dose aspirin significantly increased osteogenic differentiation and osteogenic effect in osteoporotic rats, increased the secretion of ALP, and promoted calcification, with the most significant effects produced at a dose of 1mmol/L. These effects were most likely exerted through a mechanism involving increased expression level of OPG, resulting in inhibition of the RANKL/RANK interaction, which in turn affected the OPG/RANKL/RANK axis. This research provides scientific basis for the innovative application of ASP in clinical prevention and treatment of postmenopausal osteoporosis.

DECLARATIONS

(no. Y201942167). The foundation is from the Ministry of Education in Zhejiang Province.

Conflict of interest

No conflict of interest is associated with this work.

Authors' contributions

This study was done by the authors named in this article, and the authors accept all liabilities resulting from claims which relate to this article and its contents. The study was conceived and designed by Lei Zhao; Haijiao Zhao and Lei Zhao collected and analyzed the data; while Haijiao Zhao wrote the manuscript. All authors read and approved the manuscript for publication.

Open Access

This is an Open Access article that uses a funding model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0) and the Budapest Open Access Initiative (http://www.budapestopenaccessinitiative.org/read), which permit unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

REFERENCES

1. Cano A, Chedraui P, Goulis DG, Lopes P, Mishra G, Mueck A, Senturk LM, Simoncini T, Stevenson JC, Stute P, et al. Calcium in the prevention of postmenopausal osteoporosis: EMAS clinical guide. Maturitas 2018; 107(4): 7-12.
2. Boyacioglu O, Orenay-Boyacioglu S, Yildirim H, Korkmaz M. Boron intake, osteocalcin polymorphism and serum level in postmenopausal osteoporosis. J Trace Elem Med Biol 2018; 48(7): 52-56.
3. Bone HG, Cosman F, Miller PD, Williams GC, Hattersley G, Hu MY, Fitzpatrick LA, Mitlak B, Papapoulos S, Rizzoli R, et al. ACTIVExtend: 24 Months of Alendronate After 18 Months of Abaloparatide or Placebo for Postmenopausal Osteoporosis. J Clin Endocrinol Metab 2018; 103(8): 8-12.
4. Lee DW, Kwon JY, Kim HK, Lee HJ, Kim ES, Kim HJ, Kim HJ, Lee HB. Propofol attenuates osteoclastogenesis by lowering RANKL/OPG ratio in mouse osteoblasts. Int J Med Sci 2018; 15(7): 723-729.
5. Omi M, Kaartinen V, Mishina Y. Activin A receptor type 1–mediated BMP signaling regulates RANKL-induced osteoclastogenesis via canonical SMAD-signaling pathway. J Biol Chem 2019; 3(1): 294-298.
6. Mardente S, Mari E, Massimi I, Tafani M, Guerriero R, Morsilli O, Pulcinelli FM, Bianchi ME, Zicari A. From Human Megakaryocytes to Platelets: Effects of Aspirin on High-Mobility Group Box 1/Receptor for Advanced Glycation End Products Axis. Front Immunol 2018; 8(6): 1946-1957.
7. Kachhadia PK, Doshi AS, Joshi HS. Validated Column High-Performance Liquid Chromatographic Method for Determination of Aspirin and Clopidogrel in Combined Tablets in the Presence of Degradation Products Formed Under ICH Recommended Stress Conditions. J Aoac Int 2009; 92(1): 1-7.
8. World Health Organization. Principles of laboratory animal care. WHO Chron 1985; 39: 51-56.
9. Yang XW, Wang F, Qin RZ, Zhou QL, Huang HX. Elevated serum CCL4/MIP-1β levels in postmenopausal osteoporosis patients are linked with disease severity. Biomark Med 2019; 13(1): 17-25.
10. Heidemann DR, Schulenberg ES, Smith WH. Determination of Aspirin and Salicylic Acid by Reverse-Phase Liquid Chromatography. J Assoc Off Anal Chem 1987; 70(6): 6-12.
11. Panteghini M, Pagani F. Reference intervals for two bone-derived enzyme activities in serum: bone isoenzyme of alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TR-ACP). Clin Chem 1989; 35(1): 1-5.
12. Olsson B, Corso PFCL, Meger MN, Petean I, Costa DJ, Rebellato NLB, Kluppel LE, Sousa-Neto MD, Kuchler EC, Scariot R. Single nucleotide polymorphism in opg raises 10 times the chances for developing temporomandibular joint ankylosis. Int J Oral Maxillofac Surg 2019; 48(3): 173-178.
13. Cassuto J, Folestad A, Göthlin J, Malchau H, Kärrholm J. The key role of proinflammatory cytokines, matrix proteins, RANKL/OPG and Wnt/β-catenin in bone healing of hip arthroplasty patients. Bone, 2018; 107(1): 66-77.
14. Wu F, Zhou D, Shen G, Cui Y, Lv Q, Wei F. Association of VDR and OPG gene polymorphism with osteoporosis risk in Chinese postmenopausal women. Climacteric 2019; 22(2): 1-5.
15. Shin HJ, Choi BK, Trinh PTH, Lee HS, Kang JS, Van TTT, Lee HS, Lee JS, Lee YJ, Lee J. Suppression of RANKL-Induced Osteoclastogenesis by the Metabolites from the Marine Fungus Aspergillus flocculosus Isolated from a Sponge Stylissa sp. Mar Drugs 2018; 16(1): 14-16.
16. Chen H, Guo T, Wang D, Qin R. Vaccaria hypaphorine impairs RANKL-induced osteoclastogenesis by inhibition of ERK, p38, JNK and NF-κB pathway and prevents inflammatory bone loss in mice. Biomed Pharmacother 2018; 97(2): 1155-1163.