Lower dosage of aspirin promotes cell growth and osteogenic differentiation in murine bone marrow stromal cells

Mi Du, Wan Pan, Xiaoqi Duan, Pishan Yang, Shaohua Ge*

Shandong Provincial Key Laboratory of Oral Tissue Regeneration, Department of Periodontology, School of Stomatology, Shandong University, Jinan, PR China

Received 4 December 2015; Final revision received 18 March 2016
Available online 13 May 2016

Abstract Background/purpose: The effect of aspirin on bone regeneration remains controversial. This study aimed to determine the effect of various concentrations of aspirin on cell viability, osteogenic differentiation, cell cycle, and apoptosis on ST2 cells to find an effective range of aspirin for bone regeneration induction.

Materials and methods: Cell viability was measured with MTT assay after being stimulated with aspirin for 1 day, 2 days, 3 days, 5 days, and 7 days. Alkaline phosphatase (ALP) activity was measured after cells were treated for 1 day, 3 days, and 7 days. Expression of runt-related transcription factor 2 (Runx-2) was evaluated using Western-blot analysis at 3 days and 7 days. Flow cytometry was used for cell cycle and apoptosis measurement after cells were treated for 48 hours.

Results: Lower concentrations of aspirin (1μM and 10μM) promoted cell growth and increased ALP levels and Runx-2 expression, while higher concentrations (100μM and 1000μM) inhibited cell growth (P < 0.05), and lost their effect on ALP activity after 3 days, while even showing an inhibitory effect on the expression of Runx-2. Aspirin at a concentration of 100μM promoted cell mitosis from the S phase to the G2/M phase, and 1000μM arrested the cell cycle in the resting phase G0/G1 (P < 0.05). Parallel apoptosis/necrosis studies showed the percentage of cells in apoptosis decreased dramatically at any dose of aspirin.

Conclusion: A lower dosage of aspirin could promote ST2 cell growth, osteogenic differentiation, and inhibit their apoptosis which indicates that aspirin can be used as an alternative for bone regeneration.

Copyright © 2016, Association for Dental Sciences of the Republic of China. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
Introduction

Periodontitis is an infectious disease of the periodontal supportive tissues usually accompanied with bone loss. The current therapy of periodontitis centers on the reduction of the bacterial load by mechanical and antimicrobial treatment, as well as the regeneration of lost periodontal tissues. However, bone regeneration is still one of the more challenging problems in the field of periodontal therapy. It is reported that nonsteroidal anti-inflammatory drugs (NSAIDs) may lighten the severity of tissue destruction and bone loss resulting from periodontal diseases. Recently, a study showed that some antiplatelet regents, such as clopidogrel, can enhance periodontal repair by decreasing inflammation. As a member of the NSAID family, aspirin (acetyl salicylic acid; ASA) is a common drug used for its analgesic, antipyretic, and anti-inflammatory effects. In addition to its inhibitory effect on cyclooxygenases and antiplatelet aggregators, high doses of ASA such as 1 mM, 5 mM, and 10 mM have also been shown to inhibit smooth muscle proliferation, endothelial proliferation/angiogenesis, and pro-inflammation. The effects of NSAIDs on bone tissue were controversial. Some animal studies showed that NSAIDs could promote bone repair, while several other studies indicated that aspirin/NSAIDs could suppress overall bone remodeling, especially at high doses.

However, it has been advocated that aspirin treatment is beneficial for bone health by improving bone mineral density (BMD) in trabecular and cortical bones, and one clinical study reported that aspirin users who had elevated whole body BMD (+4.2%) and total hip BMD (+4.6%) compared with nonusers. Researchers found aspirin could promote bone marrow stromal cells (BMSCs) to differentiate into osteoblasts. Unlike some other NSAIDs, aspirin has no adverse effects on osteoblast growth with proper doses—growth was only reduced at higher concentrations such as 100 μM and 1000 μM by cell cycle arrest and apoptosis induction. Aspirin has no adverse effects on osteoblast growth with proper doses—growth was only reduced at higher concentrations such as 100 μM and 1000 μM by cell cycle arrest and apoptosis induction. Bone marrow stromal cells (BMSCs) are a source of osteoprogenitors. Thus, BMSCs are candidate cells for evaluating the effects of ASA on bone formation and bone remodeling. Actually, the effect of aspirin on BMSC growth remains unclear. ST2 cells are interstitial cells that are isolated from BC8 mice bone marrow stroma and have the proper differentiation capacities, and the published data indicate that they are widely used in biomedical/bioengineering fields. During adult life, BMSCs are a source of osteoprogenitors. Thus, BMSCs are candidate cells for evaluating the effects of ASA on bone formation and bone remodeling. Actually, the effect of aspirin on BMSC growth remains unclear. ST2 cells are interstitial cells that are isolated from BC8 mice bone marrow stroma and have the potency to differentiate into osteoblast-like cells under appropriate conditions. Therefore, the objective of this study was to analyze the effects of different doses of aspirin on cell viability, osteogenic differentiation, cell cycle, and apoptosis of ST2 cells.

Materials and methods

Cell culture

ST2 cells were purchased from Riken Cell Bank (Riken BioResource Center, Tsukuba, Ibaraki, Japan). Cultures were kept at 37°C in a humidified atmosphere of 95% air and 5% CO₂ and the medium was changed [containing Dulbecco’s modified Eagle’s medium (Hyclone, Logan, UT, USA), 10% fetal bovine serum (Hyclone), and 1% penicillin/streptomycin (Sigma-Aldrich, Saint Louis, MO, USA)] every 2 days. When the adherent cells added up to 80%, cells were treated with a solution of 0.05% trypsin (Sigma-Aldrich) and 0.02% EDTA (Sigma-Aldrich) to subculture by 1:3.

Assessment of cell viability

Cell viability was determined using the MTT method. ST2 cells were seeded at 4 × 10⁴ cells/well into a 96-well plate (Corning, Corelle City, NY, USA) at a final volume of 100 μL Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 1% penicillin/streptomycin and cultured at 37°C for 24 hours. Subsequently, cells were treated with various concentrations of aspirin (0 μM, 1 μM, 10 μM, 100 μM, and 1000 μM; Sigma-Aldrich) for 1 day, 2 days, 3 days, 5 days, and 7 days, respectively. At the end of treatment, 5 mg/mL MTT (Sigma-Aldrich) was added into the medium and incubated for 4 hours. After that, the medium was aspirated and a dark-purple water-insoluble deposit of formazan crystals appeared. Then, 150 μL dimethylsulfoxide (Genview, Beijing, China) was added to dissolve the formazan crystals. Absorbance was measured at 490 nm with a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Osteogenic differentiation assay

Alkaline phosphatase (ALP) activity and runt-related transcription factor 2 (Runx2) were measured for osteogenic differentiation. ST2 cells were plated at 4 × 10⁴ cells/well into six-well plates and treated with aspirin at a dose of 0 μM, 1 μM, 10 μM, 100 μM, and 1000 μM for 1 day, 3 days, and 7 days. After the treatment, the adhered cells were lysed by 0.1% triton-X 100 (US Biological, Swampscott, MA, USA) for 35 minutes. AKP Kit (Nanjing Jiancheng, China) and BCA Protein Kit (Solarbio, Beijing, China) were used to measure ALP activity and concentrations of bovine serum albumin according to the manufacturer’s instructions. Bovine serum albumin is usually used for establishing a standard curve of protein concentration.

For the other osteogenesis marker, Runx2, ST2 cells were cultured in an osteogenesis medium [10-8M dexamethasone (Sigma-Aldrich), 10 mM b-glycerophosphate (Sigma-Aldrich), and 50-ng/mL ascorbic acid (Sigma-Aldrich)] with different doses of aspirin for 3 days and 7 days, then proteins were extracted from the cells with ice-cold radioimmunoprecipitation assay lysis buffer (Solarbio) containing 0.1% phenylmethylsulfonyl fluoride (Solarbio), followed by centrifugation at 12,000 g at 4°C for 10 minutes. Samples were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (GE Amersham, Fairfield, CT, USA) by electroblotting. Filters were then blocked in 5% nonfat milk-Tris buffered saline and 0.05% Tween 20 for 1 hour and incubated with the following primary antibody overnight: rabbit monoclonal anti-Runx2 antibody (1:500 dilution; ab23981, Abcam, Cambridge, UK). The bands corresponding to Runx2 were detected using a chemiluminescence reagent (Millipore, Darmstadt, Germany).
Effects of aspirin on bone marrow stromal cells

Germany). Glyceraldehyde 3-phosphate dehydrogenase (1:10,000) was used as a loading control. Images were collected with Tanon-5200 (Tanon, Shanghai, China).

Cell cycle analysis

Cultured ST2 cells were plated at 3 × 10^5 cells/well into a six-well plate and treated with 0μM, 1μM, 10μM, 100μM, and 1000μM of aspirin for 48 hours. Cells were detached from the culture plate by treatment with a solution of 0.05% trypsin and 0.02% EDTA and transferred into a 15-mL centrifugal tube (Corning). After centrifugation for 10 minutes at 180g, the cells were washed with cold phosphate buffered saline (PBS). Fluorescein Annexin V (annexin V-FITC) Apoptosis Detection Kit (BIOBOX, Nanjing, China) was used according to the manufacturer’s instructions for identifying the apoptosis marker. Briefly, cells were resuspended in 500 μL of binding buffer and stained with 5 μL of annexin V-FITC and 5-μL PI. After incubation at room temperature in the dark for 10 minutes, cells were analyzed with flow cytometry (Beckman Coulter Inc., Pasadena, CA, USA).

Apoptosis analysis

Cultured ST2 cells were plated at 2 × 10^5 cells/well into a six-well plate and treated with 0μM, 1μM, 10μM, 100μM, and 1000μM of aspirin for 48 hours. Cells were then detached from the culture plate and washed with 2-mL cold PBS. Fluorescein Annexin V (annexin V-FITC) Apoptosis Detection Kit (BIOBOX, Nanjing, China) was used according to the manufacturer’s instructions for identifying the apoptosis marker. Briefly, cells were resuspended in 500 μL of binding buffer and stained with 5 μL of annexin V-FITC and 5-μL PI. After incubation at room temperature in the dark for 10 minutes, cells were analyzed with flow cytometry within 1 hour of staining, with single annexin V-FITC or PI staining as positive controls and no staining as a negative control.

Statistical analysis

SPSS (version 17.0; SPSS Inc., Chicago, IL, USA) was used for data analysis. A one-way analysis of variance and Students–Newman–Keuls test were used for examining the effects on cell viability, ALP activity, cell cycle, and apoptosis. The data were given as the mean ± standard deviation for each experiment. For each test, P < 0.05 was considered statistically significant.

Results

Cell morphology observation after recovery

From 4 hours to 6 hours after recovery, ST2 cells were attached to the wall and sprawled out, with a spherical or rounded triangular shape. After 24 hours, the number of synapses increased. ST2 cells loosely arranged with long spindle shapes, and a large intercellular space was observed (Figure 1A). Forty-eight hours after recovery, cells proliferated into a large number and connected with each other into a net. The oval nucleus was observed in the middle of cell body (Figure 1B and 1C).

Effects of aspirin on cell viability

In the present study, ST2 cells were treated with various concentrations of aspirin (0μM, 1μM, 10μM, 100μM, and 1000μM) for 1 day, 2 days, 3 days, 5 days, and 7 days. MTT results demonstrated that proliferation of ST2 cells was affected by aspirin in a dose- and time-dependent manner. Compared with the control group, cell viability was significantly enhanced in aspirin-treated (1μM, 10μM, 100μM, and 1000μM) groups after 1 day and 2 days (P < 0.001 and P < 0.05, respectively). Significant favorable effect of lower doses (1μM and 10μM) on cell growth lasted in the following 3 days, 5 days, and 7 days (P < 0.05). By contrast, higher concentrations (100μM) had no impact on cell growth (P > 0.05) from the 3rd day and cell viability was apparently inhibited by 1000μM of aspirin at the same time point (P < 0.05; Figure 2).

Effects of aspirin on osteogenic differentiation

ALP activity of ST2 cells increased at all assayed doses after treatment for 1 day. All doses of aspirin (1μM, 10μM, 100μM, and 1000μM) promoted ALP activity (7.870 ± 2.245 U/gprot, 9.149 ± 1.423 U/gprot, 9.299 ± 1.425 U/gprot, and 8.978 ± 1.237 U/gprot) compared with the control group (5.413 ± 0.460 U/gprot; P < 0.05). When cultured for 3 days, ALP levels kept on increasing for the groups treated with 1μM and 10μM aspirin, while higher concentrations (100μM and 1000μM) of aspirin showed no significant effect on ALP levels at this time point (Figure 3). Not surprisingly, lower concentrations (1μM and 10μM) of aspirin significantly increased ALP activity compared with the higher ones and

Figure 1 Cell morphology observation after recovery. (A) Twenty-four hours after recovery (×100), cells were in long spindle shapes and large intercellular space was observed; (B) 48h after recovery (×200); (C) 48h after recovery (×100) cells connected each other into a net, the oval nucleus was in the middle.
control group when treated for 7 days (P < 0.05). Western-blot analysis showed that in Day 3, all doses of aspirin promoted Runx-2 expression, while on Day 7 only lower concentrations of aspirin increased Runx-2 levels compared with the control, especially 10 μM aspirin. By contrast, the higher concentrations (100 μM and 1000 μM) had an inhibitory effect on the expression of Runx-2 (Figure 4).

Effects of aspirin on cell cycle

The percentage of cells in each cell cycle phase (G0/G1, G2/M, and S) was determined with flow cytometry (Figures 5 and 6). No significant effect on the ST2 cell cycle (P > 0.05) was observed at the lower doses (1 μM and 10 μM). Aspirin at a concentration of 100 μM induced a higher percentage in the division phase (G2/M) than the proportions of cells in the G2/M phase in the control group and the 100 μM treated group were 8.125 ± 0.075% and 9.33 ± 0.07%, respectively (P < 0.05). However, 1000 μM aspirin arrested the cell cycle in resting phase (G0/G1) and induced a decrease in the proliferative phase (S phase). The mean percentage of cells in the G0/G1 phase was 75.0 ± 0.9% in control cultures versus 78.55 ± 1.35% in the 1000 μM treated group (P < 0.01; Figure 5). A typical result for cell cycle distribution treated with different doses of aspirin [0 μM (A), 1 μM (B), 10 μM (C), 100 μM (D), and 1000 μM (E)] is described in Figure 6.
Effects of aspirin on cell apoptosis

Annexin V and PI were used to measure the number of the apoptotic cells after culture for 48 hours. The means, standard deviations, and P values of viable and apoptotic cells under different doses of aspirin are described in Table 1. The percentage of cells in apoptosis decreased dramatically after treatment with aspirin for 48 hours (P < 0.05). Figure 7 typically showed the cell percentage in each quadrant under different conditions [0 μM (A), 1 μM (B), 10 μM (C), 100 μM (D), and 1000 μM (E)]. The number of viable cells (Ann V− and PI−) was counted in the lower left quadrant (C3), and the percentages of cells in early apoptosis (Ann V+, PI−, lower right quadrant C4), late apoptosis (Ann V+, PI+, upper right quadrant C2), and necrosis (Ann V−, PI+, upper left quadrant C1) were also determined. These results indicated that all concentrations of aspirin inhibited cell apoptosis in a short amount of time.

Discussion

Periodontitis is a common chronic inflammatory disease initiated by bacteria which is characterized by the destruction of connective tissue and alveolar bone. Many studies have shown that some NSAIDs are commonly used in bone tissue repair, especially for bone healing and the treatment of bone fractures.9–12 However, a recently published systematic review demonstrated an adverse effect of NSAIDs on osteoblasts proliferation.12 It was previously reported that the dose of NSAIDs is a key factor in the effect on cell proliferation as well as on cell differentiation and migration.13

Table 1  Effect of aspirin on cell apoptosis rate of ST2 cells (n = 4). Data for the percentage of viable ST2 cells and apoptic cells treated with aspirin for 48 hour are shown numerically.

|                        | Mean | SD    | P     |
|------------------------|------|-------|-------|
| Control                | Early ap. 11.5 | 0.9 | –     |
|                        | Negative 86.05 | 2.45 | –     |
| Aspirin 1 μM           | Early ap. 5.3** | 0.1 | 0.0003|
|                        | Negative 94.3** | 0.2 | 0.0044|
| Aspirin 10 μM          | Early ap. 5.5** | 0.1 | 0.0003|
|                        | Negative 93.3** | 0.3 | 0.0070|
| Aspirin 100 μM         | Early ap. 4.9** | 0.9 | 0.0009|
|                        | Negative 93.8** | 0.9 | 0.0068|
| Aspirin 1000 μM        | Early ap. 7.95** | 0.25 | 0.0028|
|                        | Negative 90.55* | 0.55 | 0.0361|

* P < 0.05 compared with control (0 μM aspirin treatment).
** P < 0.01 compared with control (0 μM aspirin treatment).
ap. = apoptosis; SD = standard deviation.
In our study, MTT assays showed the proliferative capacity of ST2 cells from murine bone marrow was significantly promoted by 1μM and 10μM of aspirin. However, higher concentrations of aspirin (1000μM) inhibited cell proliferation when treated for >3 days. This result was in agreement with observations on the effects of aspirin on endothelial cell proliferation in vitro.7 Moreover, low concentrations of aspirin (0.1–1000μM) promoted migration and adhesion of late endothelial progenitor cells (EPC) while the high concentrations of aspirin (10,000μM) decreased EPC proliferation and the migratory capacity of EPC after treatment for 24 hours.20

According to the acting mechanism, a previous study indicated that 1mM, 5mM, and 10mM of aspirin inhibited MSC proliferation and the downregulation of the Wnt/beta-catenin signal pathway may be involved in growth inhibition.21 Therefore, lower doses of aspirin have a favorable effect on cell viability, while higher doses may have an adverse effect on cell proliferation increasing with time. Since ALP and Runx-2 are well recognized as biochemical markers for osteogenesis activity, we examined the change of ALP and Runx-2 levels of ST2 cells in response to aspirin. ALP activity increased in a short amount of time treated with all concentrations of aspirin, but higher concentrations (100μM and 1000μM) lost their effect when cultured for 3 days. It was the same with the expression of Runx-2—the inhibition effect of higher doses of aspirin appeared at Day 7. These results demonstrated that lower doses of aspirin stimulated ST2 cell differentiation into osteoblast-like cells but not at higher doses.

The effect of aspirin on the growth of ST2 cells is closely related to the effects on the cell cycle. Cell numbers in the proliferative phase (G2/M phase) significantly increased when treated with 100μM aspirin, which indicated that 100μM aspirin could promote cell proliferation by proceeding cell mitosis from the S phase to the G2/M phase. By contrast, the results showed that cell numbers in the resting phase (G0/G1 phase) were higher than that in the control group and decreased dramatically in the S phase after treatment with 1000μM aspirin for 48 hours; thus, the negative effect of 1000μM aspirin on cell viability can be explained by cell cycle arrestment in the phase G0/G1. Annexin V and PI staining showed aspirin could inhibit cell apoptosis at all concentrations when treated for 48 hours which is consistent with the result of the MTT assays. However, apoptosis is almost present in proliferating cells and molecules acting on cells in the late G1 phase are required for apoptosis,22 which may be the basis for the inhibitory effect of 1000μM aspirin in the longer times.

In addition, inflammation in the bone microenvironment is known to contribute to bone loss. Therefore, decreasing inflammation in bone marrow might be helpful for inhibiting bone loss. Aspirin remains the most commonly used medication for the treatment of inflammation. A well-known

Figure 7  (A) Control group; (B) typical result for the effect on cell apoptosis rate of 1μM; (C) 10μM; (D) 100μM; and (E) 1000μM of aspirin with flow cytometry. The number of viable cells was counted in the lower left quadrant (C3), early apoptosis in lower right quadrant (C4), late apoptosis in upper right quadrant (C2), and necrosis in upper left quadrant (C1).
mechanism of aspirin anti-inflammatory effect is inhibition of prostaglandins (PGs), which play a central role in the inflammatory response, produced through the cyclooxygenase (COX) pathway. Recent research has suggested that aspirin directly modifies the action of cyclooxygenase 2, changing its activity towards the lipoxygenase pathway, resulting in the formation of aspirin-triggered lipoxins. Lipoxins have emerged as mediators of endogenous anti-inflammatory events and are known to inhibit neutrophil chemotaxis, superoxide generation, and secretion of proinflammatory cytokines and proteolytic molecules including PGs. By contrast, some researchers thought PGs (PGE2 in particular) stimulated bone formation in vivo, and the suppressive effects of NSAIDs on bone repair are due to the inhibitory effect of PGs in bone cells. Thus, inhibition of PGs as an anti-inflammatory pathway might not contribute to bone healing and deserves further research.

Interestingly, some studies demonstrated that aspirin inhibited inflammatory responses in various cell lines, including endothelial cells, fibroblasts, and bone marrow mesenchymal stem cells (BMMSCs) through the inhibition of reactive oxygen species generation or NF-kappaB activation.

Moreover, aspirin could promote the immunomodulatory function of BMMSCs by upregulation of regulatory T cells and downregulation of Th17 cells via 15-deoxy-delta-12,14-prostaglandin J2/peroxisome proliferator-activated receptor-γ transforming growth factor-beta pathway. By contrast, a study which demonstrated that the pharmacologic regulation of BMMSCs by aspirin might offer an approach for estrogen-deficient osteoporosis treatment and showed activated T lymphocytes induced BMMSCs apoptosis through the Fas/FasL pathway, but aspirin treatment could induce activated T-cell death in vitro, and promote osteogenesis of BMMSCs by upregulating telomerase activity. This research also suggested long-term aspirin treatment could improve BMD in ovariectomy mice, and inhibit osteoclast activities.

From a clinical standpoint, a cross-sectional investigation suggest that long-term (>6 months) low dose (75 mg/d) aspirin therapy for humans may reduce the risk of periodontal attachment loss. The highest plasma drug concentration of 75 mg/d is 40 μM, which is in the concentration range for promoting cell proliferation and differentiation in our study. A randomized trial showed that low-dose aspirin (81 mg/d) contributed to improving moderate periodontitis and gingival inflammation supplemented with docosahexaenoic. These indicated a therapeutic dose range (1–100 μM) used in the clinic for bone cell proliferation as well as differentiation.

In conclusion, this study provided clear evidence that aspirin at lower doses promotes BMSC growth and enhances osteogenic differentiation of ST2 cells. Cell growth only decreased at higher doses by cell cycle arrest and showed a trend of apoptosis induction. Together with its anti-inflammatory effect, ASA could be applied in the treatment of periodontitis with bone loss. However, we need further research to examine the effect of aspirin on BMSC adhesion and migration and to evaluate the accurate doses of aspirin to promote bone regeneration in vivo studies.

**Conflicts of interest**

The authors have no conflicts of interest relevant to this article.

**Acknowledgments**

This study was supported by the National Natural Science Foundation of China (Numbers 81100756 and 81371157) and Science and Technology Program of Shandong Province (Number 2014GSF118075). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We would like to acknowledge the support of Dr Faliang Zhu with the flow cytometry.

**References**

1. Slots J, Ting M. Systemic antibiotics in the treatment of periodontal disease. Periodontol 2000 2002;28:106–76.
2. Elkhouri AM. The efficacy of host response modulation therapy (omega-3 plus low-dose aspirin) as an adjunctive treatment of chronic periodontitis (clinical and biochemical study). J Periodontal Res 2011;46:261–8.
3. Shiloah J, Bland PS, Scarbacz M, Patters MR, Stein SH, Tipton DA. The effect of long-term aspirin intake on the outcome of nonsurgical periodontal therapy in smokers: a double-blind, randomized pilot study. J Periodontal Res 2014;49:102–9.
4. Coimbra LS, Steffens JP, Rossa Jr C, Graves DT, Spolidoro LC. Clopidogrel enhances periodontal repair in rats through decreased inflammation. J Clin Periodontol 2014;41:295–302.
5. Khaidakov M, Szwoj J, Mitra S, et al. Antiangiogenic and antimitotic effects of aspirin in hypoxia-reoxygenation modulation of the LOX-1 NADPH oxidase axis as a potential mechanism. J Cardiovasc Pharmacol 2010;56:635–41.
6. Marra DE, Simoncini T, Liao JK. Inhibition of vascular smooth muscle cell proliferation by sodium salicylate mediated by upregulation of p21 (Waf1) and p27 (Kip1). Circulation 2000;102:2124–30.
7. Pearce HR, Kalia N, Bardhan KD, Brown NJ. Effects of aspirin and indomethacin on endothelial cell proliferation in vitro. J Gastroenterol Hepatol 2003;18:1180–7.
8. Lack WD, Fredericks D, Petersen E, et al. Effect of aspirin on bone healing in a rabbit ulnar osteotomy model. J Bone Joint Surg Am 2013;95:488–96.
9. Krayer JW, Leite RS. Nonsurgical chemotherapeutic treatment strategies for the management of periodontal disease. Dent Clin North Am 2010;54:13–33.
10. Aspalli SS, Shetty VS, Parab PG, Nagappa G, Devnoorkar A, Devarathamma MV. Osteoporosis and periodontitis: Is there a possible link? Indian J Dent Res 2014;25:316–20.
11. Wei JS, Zeng R, Chen SY, Lin H, Wu SK, Zheng JC. Effects of aspirin on fracture healing in QPF rats. Asian Pac J Trop Med 2014;7:801–5.
12. Pountos I, Georgouli T, Calori GM, Giannoudis PV. Do nonsteroidal anti-inflammatory drugs affect bone healing? A critical analysis. Sci World J 2012;2012:606404.
13. Muller M, Raabe O, Addicks K, Wenisch S, Arnhold S. Effects of nonsteroidal anti-inflammatory drugs on proliferation, differentiation and migration in equine mesenchymal stem cells. Cell Biol Int 2011;35:235–48.
14. Bauer DC, Orwoll ES, Fox KM, et al. Aspirin and NSAID use in older women: effect on bone mineral density and fracture risk. Study of osteoporotic fractures research group. *J Bone Miner Res* 1996;11:29–35.

15. Carbone LD, Tylavsky FA, Cauley JA, et al. Association between bone mineral density and the use of nonsteroidal anti-inflammatory drugs and aspirin: impact of cyclooxygenase selectivity. *J Bone Miner Res* 2003;18:1795–802.

16. Papathanasopoulos A, Kouroupis D, Henshaw K, McGonagle D, Jones EA, Giannoudis PV. Effects of antithrombotic drugs fondaparinux and tinzaparin on in vitro proliferation and osteogenic and chondrogenic differentiation of bone-derived mesenchymal stem cells. *J Orthop Res* 2011;29:1327–35.

17. De Luna-Bertos E, Ramos-Torrecillas J, Garcia-Martinez O, Diaz-Rodriguez L, Ruiz C. Effect of aspirin on cell growth of human MG-63 osteosarcoma line. *Sci World J* 2012;2012:834246.

18. Bradamante S, Barenghi L, Maier JA. Stem cells toward the future: the space challenge. *Life (Basel)* 2014;4:267–80.

19. Itoh F, Aoyagi S, Furihata-Komatsu H, et al. Clodronate stimulates osteoblast differentiation in ST2 and MC3T3-E1 cells and rat organ cultures. *Eur J Pharmacol* 2003;477:9–16.

20. Liu ZZ, Li GQ, Liu M, Sun SX, An GY, Dong NZ. Effect of aspirin on function of human umbilical cord blood-derived late endothelial progenitor cell. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 2013;21:1032–7.

21. Wang Y, Chen X, Zhu W, Zhang H, Hu S, Cong X. Growth inhibition of mesenchymal stem cells by aspirin: involvement of the WNT/beta-catenin signal pathway. *Clin Exp Pharmacol Physiol* 2006;33:696–701.

22. Alenzi FQ. Links between apoptosis, proliferation and the cell cycle. *Br J Biomed Sci* 2004;61:99–102.

23. Yao C, Yang D, Wan Z, et al. Aspirin-triggered lipoxin A4 attenuates lipopolysaccharide induced inflammatory response in primary astrocytes. *Int Immunopharmacol* 2014;18:85–9.

24. Ho ML, Chang JK, Chuang LY, Hsu HK, Wang GJ. Effects of nonsteroidal anti-inflammatory drugs and prostaglandins on osteoblastic functions. *Biochem Pharmacol* 1999;58:983–90.

25. Wang X, Lu J, Khaidakov M, et al. Aspirin suppresses cardiac fibroblast proliferation and collagen formation through down-regulation of angiotensin type 1 receptor transcription. *Toxicol Appl Pharmacol* 2012;259:346–54.

26. Wu Y, Zhai H, Wang Y, et al. Aspirin-triggered lipoxin A4 attenuates lipopolysaccharide-induced intracellular ROS in BV2 microglia cells by inhibiting the function of NADPH oxidase. *Neurochem Res* 2012;37:1690–6.

27. Zhang F, Lu M, Wang H, Ren T. Aspirin attenuates angiotensin II-induced inflammation in bone marrow mesenchymal stem cells via the inhibition of ERK1/2 and NF-kB activation. *Biomed Rep* 2013;1:930–4.

28. Tang J, Xiong J, Wu T, et al. Aspirin treatment improved mesenchymal stem cell immunomodulatory properties via the 15d-PGJ2/PPARY/TGF-b1 pathway. *Stem Cells Dev* 2014;23:2093–103.

29. Yamaza T, Miura Y, Bi Y, et al. Pharmacologic stem cell based intervention as a new approach to osteoporosis treatment in rodents. *PLoS One* 2008;3:e2615.

30. Faizuddin M, Tarannum F, Korla N, Swamy S. Association between long-term aspirin use and periodontal attachment level in humans: a cross-sectional investigation. *Aust Dent J* 2012;57:45–50.

31. Naqvi AZ, Hasturk H, Mu L, et al. Docosahexaenoic acid and periodontitis in adults: a randomized controlled trial. *J Dent Res* 2014;93:767–73.