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ORIGINAL ARTICLE

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

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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.
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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.[1,2] 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.[2,3] Recently, a study showed that some antiplatelet regents, such as clopidogrel, can enhance periodontal repair by decreasing inflammation.[4] 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 aggregator effects, high doses of ASA such as 1mM, 5mM, and 10mM have also been shown to inhibit smooth muscle proliferation, endothelial proliferation/angiogenesis, and pro-inflammation.[5–7] 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.[8–13] However, it has been advocated that aspirin treatment is beneficial for bone health by improving bone mineral density (BMD) in trabecular and cortical bones,[14] and one clinical study reported that aspirin users had elevated whole body BMD (+4.2%) and total hip BMD (+4.6%) compared with nonusers.[15] Researchers found aspirin could promote bone marrow stromal cells (BMSCs) to differentiate into osteoblasts.[16] Unlike some other NSAIDs, aspirin has no adverse effects on osteoblast growth with proper doses—growth was only reduced at higher concentrations such as 100μM and 1000μM by cell cycle arrest and apoptosis induction.[17]

BMSCs are characterized by their self-renewal and multipotent differentiation capacities, and the published data indicate that they are widely used in biomedical/bioengineering fields.[18] 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.[19] 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 Bio-Resource Center, Tsukuba, Ibaraki, Japan). Cultures were kept at 37°C in a humidified atmosphere of 95% air and 5% CO_2 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 4×10^3 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 (0mM, 1mM, 10mM, 100mM, and 1000μM; 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 (Runx-2) were measured for osteogenic differentiation. ST2 cells were plated at 4×10^4 cells/well into six-well plates and treated with aspirin at a dose of 0μM, 1μM, 10μM, 100μM, and 1000μM 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^{-8}M dexamethasone (Sigma-Aldrich), 10mM 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 12,000g 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 Runx-2 were detected using a chemiluminescence reagent (Millipore, Darmstadt,

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) once. Subsequently, cells were fixed by ice-cold 70% ethanol for 24 hours at 4°C and harvested by centrifugation and then resuspended in 2-mL cold PBS. Cells were then incubated at 37°C for 30 minutes with ribonuclease and propidium iodide (PI) according to the instructions. Finally, samples were analyzed using 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 cells 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 ± 0.558 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
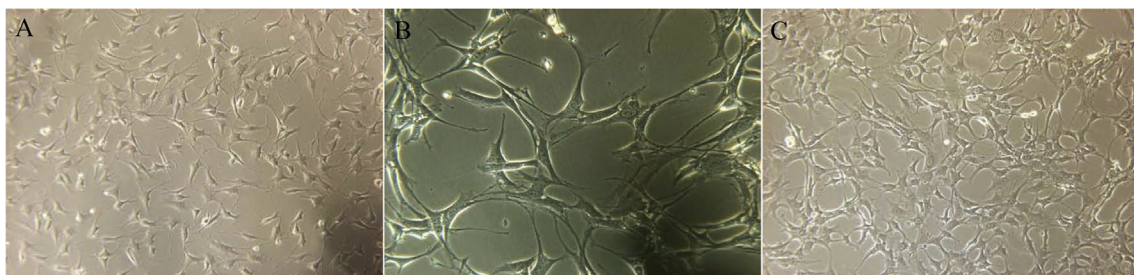

Figure 1 Cell morphology observation after recovery. (A) Twenty-four hours after recovery (×100), cells were in long spindle shapes and large intercellular space was observed; (B) 48h after recovery (×200); (C) 48h after recovery (×100) cells connected each other into a net, the oval nucleus was in the middle.
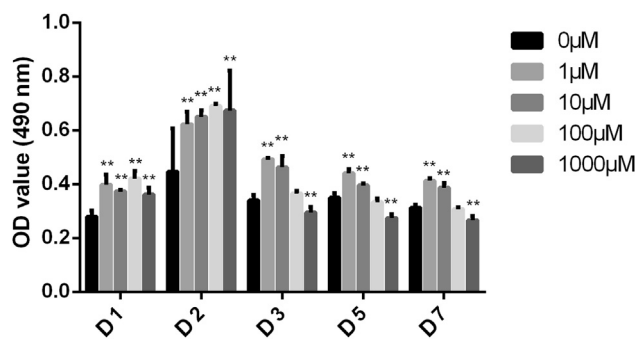
Figure 2 Effect of different doses of aspirin on viability of ST2 cells ($n = 6$). ST2 cells were incubated with various concentrations of aspirin for 1 day, 2 days, 3 days, 5 days, and 7 days, respectively. The proliferation of ST2 cells was measured with MTT assay (490 nm wavelength). Data represents means $\pm$ standard deviation ($n = 6$, six replicates per time point for each experimental condition). ** $P < 0.01$ compared with control (0μM aspirin treatment). OD = optical density.

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 10μM aspirin. By contrast, the higher concentrations (100μM and 1000μM) had an inhibition 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
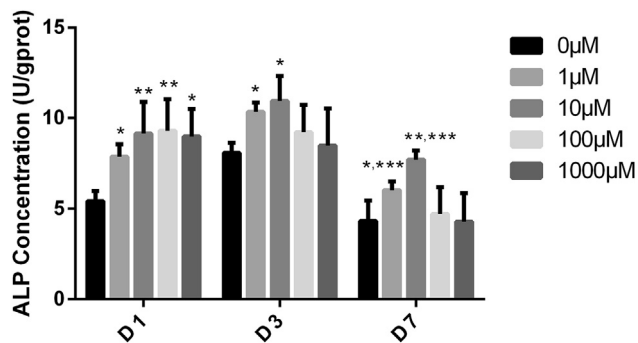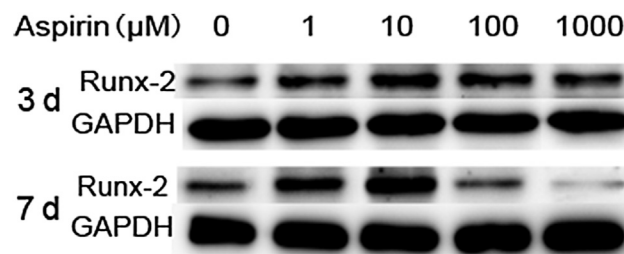

Figure 4 (A) Effect of aspirin on runt-related transcription factor 2 (Runx-2) expression of ST2 cells. ST2 cells were cultured in an osteogenesis medium with different doses of aspirin for 3 days and 7 days; (B)western-blot analysis showed that on Day 3, all doses of aspirin promoted Runx-2 expression. On Day 7, only 1μM and 10μM of aspirin increased Runx-2 levels compared with the control, the higher ones (100μM and 1000μM) showed an inhibitory effect on the contrary. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as a loading control.

($P > 0.05$) was observed at the lower doses (1μM and 10μM). Aspirin at a concentration of 100μM induced a higher percentage in the division phase (G2/M)—the proportions of cells in the G2/M phase in the control group and the 100μM treated group were $8.125 \pm 0.075\%$ and $9.33 \pm 0.07\%$, respectively ($P < 0.05$). However, 1000μM 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 $75.0 \pm 0.9\%$ in control cultures versus $78.55 \pm 1.35\%$ in the 1000μM treated group ($P < 0.05$), and $16.95 \pm 0.75\%$ versus $14.6 \pm 0.30\%$ for the S phase ($P < 0.01$; Figure 5). A typical result for cell cycle distribution treated with different doses of aspirin [0μM (A), 1μM (B), 10μM (C), 100μM (D), and 1000μM (E)] is described in Figure 6.

Figure 3 Effect of different doses of aspirin on (alkaline phosphatase) ALP activity of ST2 cells. ST2 cells were cultured with various concentrations of aspirin for 1 day, 3 days, and 7 days. Data were normalized for total protein content to account for the effects. The ALP activity incubated with 1μM and 10μM aspirin significantly increased. Data represents means $\pm$ standard deviation ($n = 6$, 6 replicates per time-point for each experimental condition). By one-way analysis of variance and Students—Newman—Keuls test, significant differences were shown. * $P < 0.05$ compared with control. ** $P < 0.01$ compared with control. *** $P < 0.05$ versus 100μM and 1000μM.
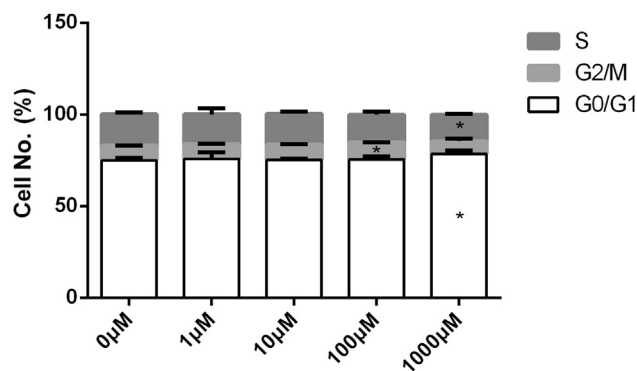

Figure 5 Effect of aspirin on cell cycle distribution of ST2 cells. ST2 cells were cultured in serum-free medium for 24 hours and then treated with the indicated concentrations of aspirin for 48 hours. Flow cytometric analysis showed that 100μM aspirin significantly increased the proportion of cells in the G2/M phase and G0/G1 phase for 1000μM aspirin. Data was shown as means $\pm$ standard deviation ($n = 4$, 4 replicates per time-point for each experimental condition). By one-way analysis of variance and Students—Newman—Keuls test, significant differences were shown. * $P < 0.05$ compared with the same phase of cell cycle of control.
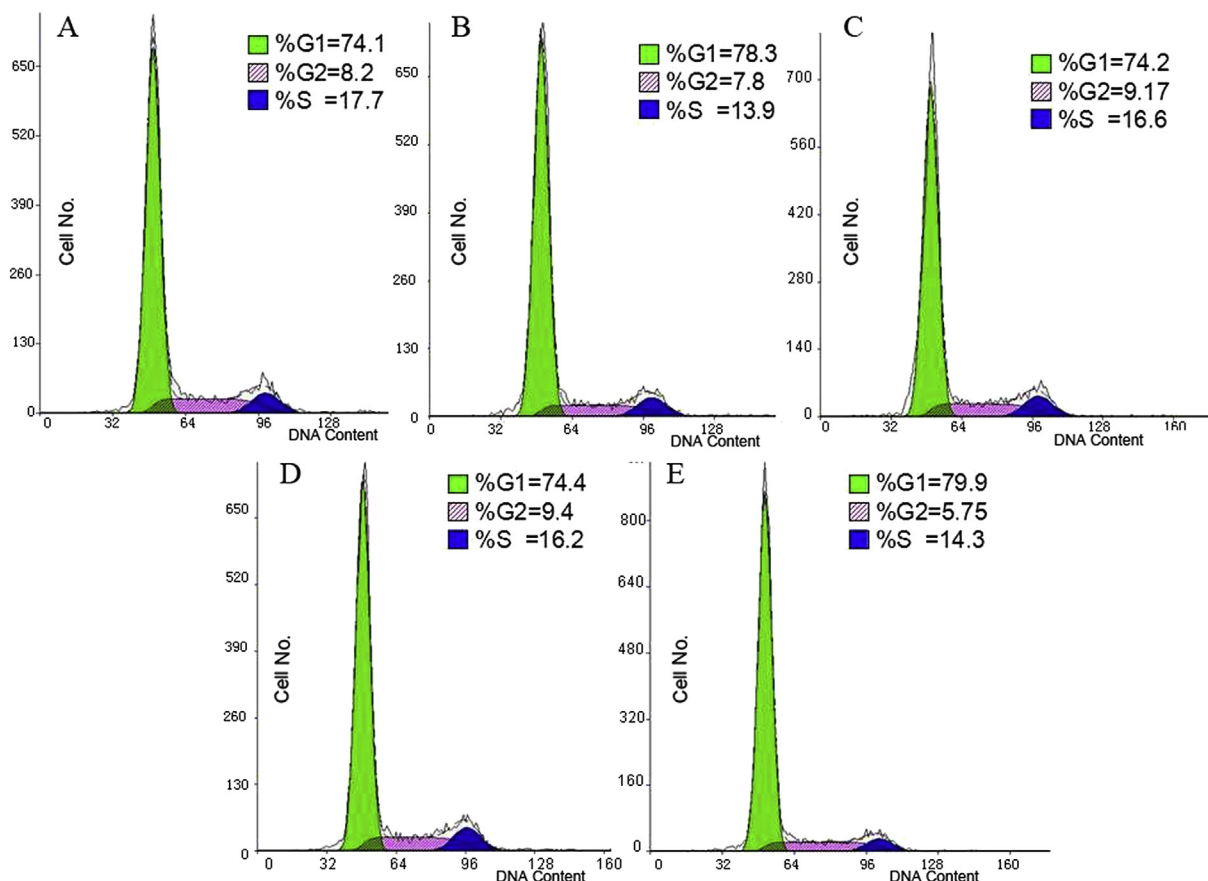
Figure 6 (A) Control group; (B) typical result for the effect on cell cycle of 1μM; (C) 10μM; (D) 100μM; and (E) 1000μM of aspirin with flow cytometry.

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 [0μM (A), 1μM (B), 10μM (C), 100μM (D), and 1000μM (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 1μM	Early ap.	5.3**	0.1	0.0003
	Negative	94.3**	0.2	0.0044
Aspirin 10μM	Early ap.	5.5**	0.1	0.0003
	Negative	93.3**	0.3	0.0070
Aspirin 100μM	Early ap.	4.9**	0.9	0.0009
	Negative	93.8**	0.9	0.0068
Aspirin 1000μM	Early ap.	7.95**	0.25	0.0028
	Negative	90.55*	0.55	0.0361

* P < 0.05 compared with control (0μM aspirin treatment).
** P < 0.01 compared with control (0μM aspirin treatment).
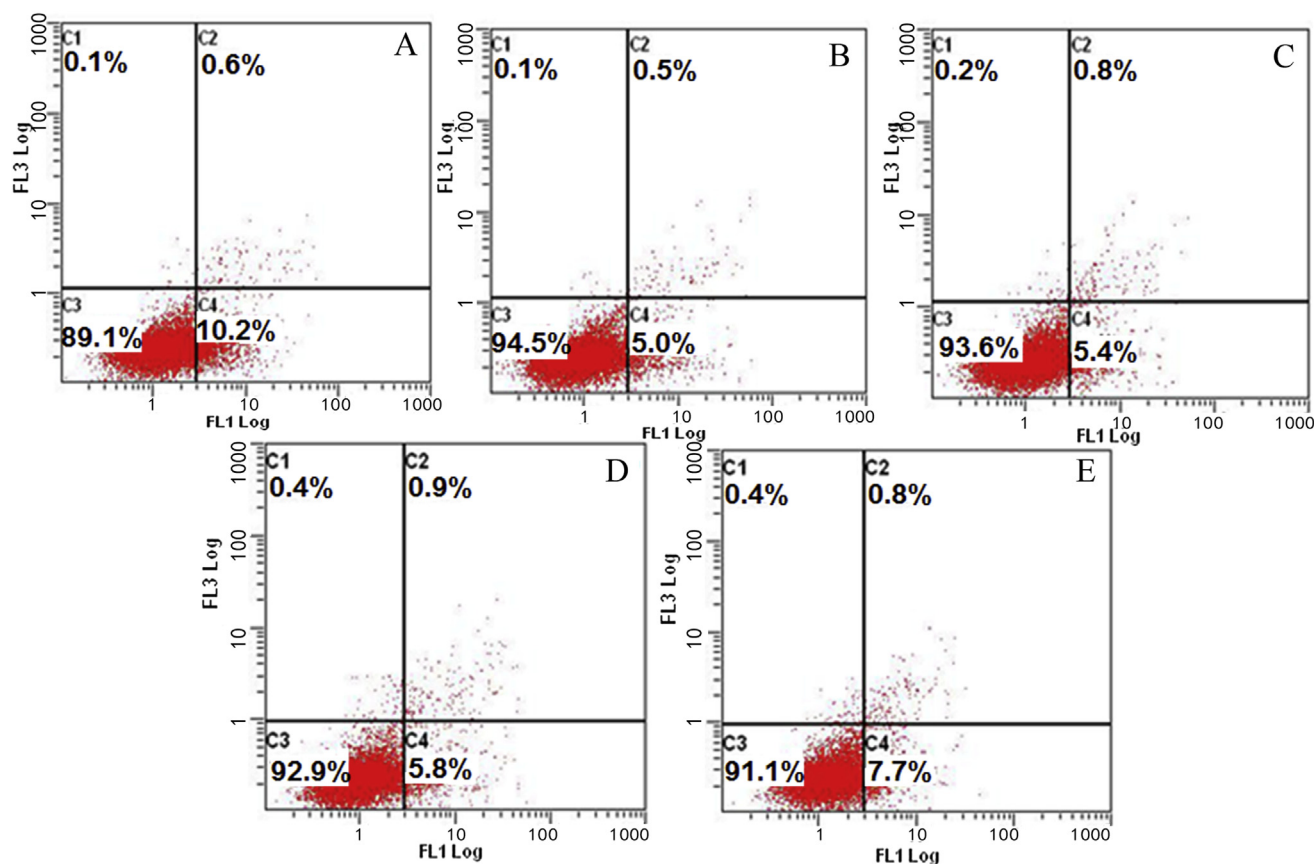ap. = apoptosis; SD = standard deviation.

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).

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

mechanism of aspirin anti-inflammatory effect is inhibition of prostaglandins (PGs), which play a central role in the inflammatory response, produced through the cyclo-oxygenase (COX) pathway. Recent research has suggested that aspirin directly modifies the action of cyclo-oxygenase 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.[23] 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.[24] 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.[25–27]

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-β1 pathway.[28] 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.[29]

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.[30] The highest plasma drug concentration of 75 mg/d is 40μM, 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.[31] These indicated a therapeutic dose range (1–100μM) 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 *in vivo* studies.

Conflicts of interest

Acknowledgments

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