Nano-sized 58S Bioactive Glass Enhances Proliferation and Osteogenic Genes Expression of Osteoblast-like Cells

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Objective: *To observe the effects of ionic dissolution products on nano-sized 58S bioactive glass (nano-58S) on proliferation and specific osteogenic genes expression in MG-63 cells.*
Methods: *Ionic dissolution products were prepared by incubating nano-58S or sol-gel bioactive glass 58S (58S) particulates in Dulbecco's modified Eagle's medium (DMEM) at 1% w/v for 24 hr and filtrated through 0.22 μm filters to remove the particulates. MG-63 cells were cultured in the nano-58S extraction, 58S extraction, and DMEM, respectively, for different time periods to assay the proliferation, mRNA expression of alkaline phosphatase (ALP), Cbfa1, Collagen type I (Col-I) and osteocalcin (OCN), as well as ALP staining, activity and matrix mineralisation.*
Results: *In the nano-58S group, cell proliferation and mRNA expression of ALP, Cbfa1 and OCN were significantly enhanced in a time-dependent manner compared with the control group. mRNA expression of Cbfa1 on day 4 and OCN on day 7 was significantly higher than that in the 58S group. Moreover, there was significantly more ALP protein expression and mineralisation in the nano-58S group than in the 58S group.*
Conclusion: *The nano-58S enhanced proliferation, osteogenic markers expression in MG-63 cells and induced stronger mineralization than 58S.*

Key words: *human osteoblast-like cells, nano-sized bioactive glass, gene expression, bone, osteogenesis*

The clinical demands on biomaterials that can induce bone regeneration have been rapidly growing in recent years for treatment of trauma, dental implant and maintenance of quality of life in an aging population. *In vitro* and *in vivo* studies have shown that bioactive glasses are highly biocompatible, osteoproductive and osteoconductive biomaterials that can rapidly form a strong direct bond to bone tissue[1-6]. Previous studies have suggested that the ionic products from bioactive glass (BG) dissolution play an active role in osteoblast behaviour by altering the gene expression relative to osteoblast proliferation[6], differentiation and bone matrix formation[3,5,7-14]. Several *in vitro* studies[15-22] have proved that the osteogenetic-relative genes, such as alkaline phosphatase (ALP), bone sialoprotein (BSP), Runx-2/Cbfa1 and osteocalcin (OCN), were activated by bioactive glasses. The expression of ALP, BSP and OCN were up-regulated when osteoblasts from mouse calvarias were cultured with 58S sol-gel bioactive glass particles at day 18[15]. The dissolution of bioactive glass coating regulated a twofold increase of the Runx-2/Cbfa1 expression in the MC3T3-E1.4 cells[16]. Glass conditioned medium of 45S5 enhanced cell proliferation and the gene expression of ALP, Collagen type I (Col-I) and OCN in MC3T3-E1 cells[17]. Microarray-based gene expression analysis of human osteoblasts has also proved that bioactive glasses

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induced the expression of ALP and Col-I[18]. But some studies showed contrary results. Angela et al reported that MG-63 cells incubated with Consil® particles maintained the similar levels of phenotype markers (Col-I, OCN, proteoglycans and ALP) as controls[19]. Hattar et al did not detect differences in gene expression of Cbfa1, Col-I and ALP between MG-63 cells cultured with 45S5 and with non-reactive 60S glass[20]. Misra et al found a 20% decrease of collagen I in the MC3T3-E1.4 cells cultured with bioactive glass dissolution[16]. As for a cell signal pathway, the signal transduction in the MG-63 cells showed a time-dependent manner. The gene transcription of MAPK and c-Jun decreased from 4 hr to 24 hr and subsequently returned to control levels by 7 days[21]. However, Christodoulou confirmed over twofold up-regulation of gp130 and MAPK3[22]. So far there are still many questions that need to be answered in the gene expression regulation mechanism of bioactive glasses.

In recent years, several investigators have studied the submicron/nano-sized sol-gel bioactive glass particles that possess a higher specific surface area and a more regular size. Some studies suggested that submicron/nano-sized sol-gel bioactive glass particles have better mechanical properties and bioactivity[23-26]. So far, nano-sized bioactive glass has been proven to increase interface effects, bioactivity and osteoblast adhesion compared with traditional micron-sized bioactive glass particles[24-27]. These previous studies suggest that nano-sized bioactive glasses might be potential candidates for applications in regenerative medicine. However, the dissolution of nano-sized particles will bring about fine nano-sized particulates in the body, which can penetrate into the cells resulting in raised intracellular silica or calcium concentration. Some nano-sized materials have the ability to interact with DNA, which may lead to a marked effect on cell metabolism, inducing an undesirable effect in the human body[28] and even potential toxicity[29].

To date, there are still many questions to be answered about the molecular mechanism of the effects of bioactive glass on osteoblasts' proliferation, differentiation and matrix mineralisation. Meanwhile, little is known about the biocompatibility of nano-sized bioactive glass particles and their effects on gene expression in osteoblasts. The aim of this study was to observe the effects of ionic dissolution products of a novel nano-sized bioactive glass, nano-sized 58S, on proliferation and specific osteogenic gene expression in MG-63 cells.

Materials and methods

Bioactive glass extraction preparation

Sol-gel bioactive glass 58S (58S) and nano-sized 58S (nano-58S, particulate size between 10 and 100 nm) were prepared using methods as previously described[30]. Ionic dissolution products were prepared by incubation of 1% w/v nano-58S or 58S particulates that had been sterilised in a dry heat oven at 180ºC for 4 hr in Dulbecco's modified Eagle's medium (DMEM, Gibco) for 24 hr at 37ºC. Particulates were removed by filtration through a 0.22 μm filter (Millipore). Calcium (Ca), silicon (Si) and phosphorus (P) in the solutions were detected by inductively coupled plasma optical emission spectroscopy (ICP-OES) analysis (iCAP 6300, Thermo Electron Corporation). The solution was diluted with DMEM to adjust the Si concentration to the range between 15 μg/ml and 20 μg/ml followed by another ICP analysis to verify the Si concentration. The final concentrations of Ca, Si and P are shown in Table 1, and the diluted medium supplemented with 10% foetal bovine serum (FBS), 100 mg/ml streptomycin, 100 U/ml penicillin and 2 mM L-glutamine was used for the current experiments.

Cell culture and treatments

Human osteoblast-like cell line MG-63 (ATCC) was grown in DMEM containing 10% (v/v) heat-inactivated FBS and 2 mmol/L L-glutamine in an atmosphere of 5% CO_2 at 37ºC. When cells attached onto the well after 24 hr of incubation, the medium was replaced with nano-58S extraction (nano-58S group), 58S extraction (58S group) and complete DMEM growth medium (control group), and cultured and evaluated at selected time points.

Assessment of cell proliferation

Cells were seeded at a density of 2,000 cells/well onto the 96-well plate and cultured with control medium, 58S and nano-58S extractions, respectively, for 10 days. The culture medium was changed every 2 days. The cell proliferation was assessed by 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Extraction of RNA and real-time reverse transcriptase polymerase chain reaction

To quantify the levels of gene expression, real-time reverse transcriptase polymerase chain reaction (real-

Table 1 Concentration of Si, Ca and P in the Extractions and control medium

	Si (μg/ml)	Ca (μg/ml)	P (μg/ml)
DMEM	0.57	75.33	29.18
58S BG	17.32	85.75	25.65
Nano-58S BG	16.23	98.1	25.65

Note: DMEM = Dulbecco's modified Eagle's medium; BG = bioactive glass.

Table 2 The Primers Sequence used for real time RT-PCR

Target gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
GAPDH	CAACGGATTTGGTCGTATTGG	GCAACAATATCCACTTTACCAGAGTTAA
Alkaline phosphatase	AGCACTCCCACTTCATCTGGAA	GAGACCCAATAGGTAGTCCACATTG
Cbfa1	ACCCAGAAGGCACAGACAGAAG	AGGAATGCGCCCTAAATCACT
Collagen type I	CGAAGACATCCCACCAATCAC	TGTCGCAGACGCAGAT
Osteocalcin	AGGGCAGCGAGGTAGTGA	CCTGAAAGCCGATGTGGT

time RT-PCR) was utilised. Cells were cultured in three groups of medium in 35 mm dishes with the density of 5,000 cells/cm^2. On day 2, 4 and 7, the total RNA were extracted using Trizol® reagent (Invitrogen) and the phenol/chloroform method. The concentration and purity of total RNA of each sample was determined by absorbance at 260 nm and by calculating the A260/A280 ratio, respectively. RNA was reversed transcribed into complementary DNA (cDNA) by the use of the Reverse Transcription System (Promega) according to the manufacturer's instructions.

The quantitative PCR was carried out using Applied Biosystem 7500 thermal cycler (Applied Biosystems). The forward and reverse primers used for real-time PCR were designed by Primer 5.0 according to the published cDNA sequences available in GeneBank, and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) was chosen to be the housekeeping gene for the quantification of gene relative expression (Table 2).

Data acquisition was performed with the Applied Biosystem 7500 thermal cycler software package. Two parallel samples were tested and the cycle threshold (Ct) values were the corresponding mean. The experiment was triplicated. The fold change was defined as the relative expression compared with the value of control group on day 2, calculated as $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct_{sample} - Ct_{GAPDH}$ and $\Delta\Delta Ct = Ct_{sample} - Ct_{con\ day2}$. Data are reported as variation folds and calculated taking the values of control cells on day 2 as on day 1.

Alkaline phosphatase staining and activity assay

MG-63 cells were seeded at the density of 5,000 cells/cm^2 onto 12-well plates and cultured in nano-58S extraction, 58S extraction and control medium, respectively, for 7 days or 14 days. After being washed twice in phosphate-buffered saline (PBS), the cells were fixed in 95% ethanol for 20 min and washed with PBS twice. The cells were stained with the BCIP (5-Bromo-4chlro-3-indolyl phosphate)/NBT Alkaline Phosphatase Colour Development Kit (Kangweishiji) and imaged under a light microscope (TE2000-U). ALP activity was determined using Alkaline Phosphatase Assay Kit (Jiancheng). In brief, cells were rinsed with cold PBS and scraped into 1.5 ml Eppendorf tubes in 1 mL 0.1% Triton X-100, and then homogenised by ultrasonication on ice and centrifuged at 18,000 g for 15 min. In total, 30 μl of the supernatant was added with the reagents and incubated for 30 min according to the instructions. The absorbance at 490 nm was measured spectrophotometrically with a

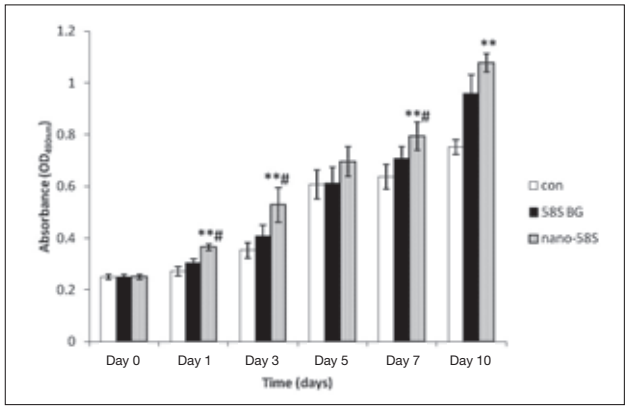

Fig 1 Effect of nano-58S and 58S BG on MG-63 cells proliferation (ANOVA, $^{**}P < 0.01$ versus control group; $\#P < 0.05$ versus 58S BG group).

microreader (ELx 800; Bio Tek). A standard curve of known concentrations was generated concurrently and the protein concentrations were determined using the BCA protein assay (Pierce). The ALP activity was normalised to per gram of protein.

Matrix mineralisation assay

Alizarin red S staining was used to assess the matrix mineralisation. Cells were seeded at 5,000 cells/cm^2 and cultured in nano-58S extraction, 58S extraction and control medium, respectively, for 7 days or 14 days. Samples were rinsed three times in PBS and fixed in 4% paraformaldehyde for 20 min and washed with deionised water for three times, and stained with 40mM Alizarin red S solution (Sigma; pH = 4.2) for 15 min at room temperature. After the incubation, cells were washed with deionised water to remove non-specific staining and then observed under a light microscope (TE2000-U).

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Two-way and one-way analysis of variance (ANOVA) testing was conducted to compare the different effects (58S, nano-58S, CON) on AN-63 cells using SPSS Statistics 17.0 software for Microsoft Windows. Differences were considered significant when $P < 0.05$.

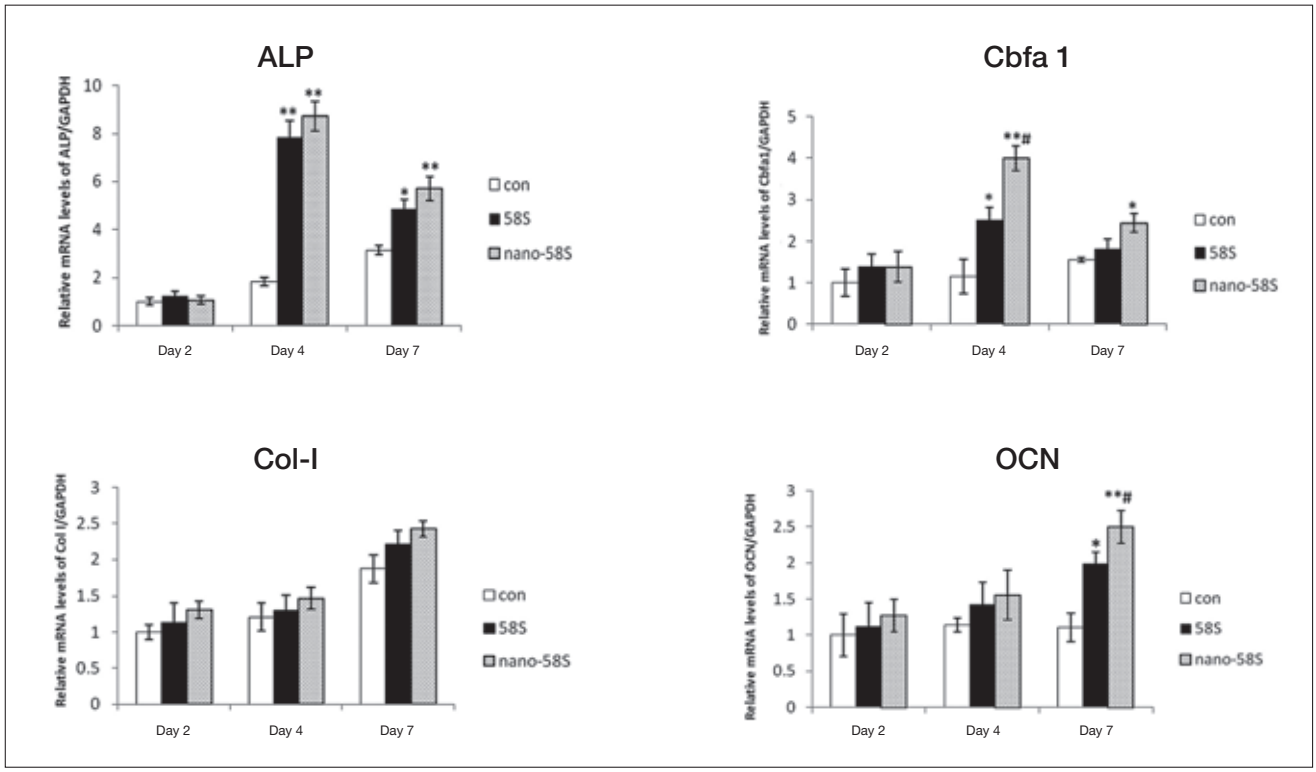

Fig 2 Gene expression profile of MG-63 cells cultured in control medium, 58S and nano-58S extractions. Data are relative expression levels of genes of interest and are showed as fold difference compare to control group on day 2 and have been normalised to the expression of housekeeping gene GAPDH. The data are mean values ± standard deviation of 3 individual experiments (each treatment in duplicate) and the real-time PCR was run twice for each sample ($^{*}P < 0.05$ versus control group; $^{**}P < 0.01$ versus control group; $\#P < 0.05$ versus 58S BG group).

Results

Effects of nano-58S and 58S on MG-63 cells proliferation

Both nano-58S and 58S enhanced the proliferation of MG-63 cells (Fig 1). The proliferation of the nano-58S group was significantly higher than the 58S group ($P < 0.05$).

Effects of nano-58S and 58S on osteogenic genes expression of MG-63 cells

mRNA expression of ALP, Cbfa1, Col-I and OCN in MG-63 cells cultured in nano-58S and 58S extractions on day 2, 4 and 7 was significantly higher than the control group (Fig 2). There was no difference of mRNA expression of ALP between the 58S group and nano-58S group. On day 7, Cbfa1 mRNA expression in the nano-58S and 58S groups fell back close to the level of the control group, with Cbfa1 mRNA expression in nano-58S still higher than that of control group. mRNA expression of OCN in nano-58S groups showed significant higher expression than the 58S group and 2.5-fold higher expression than that of control group. Although Col-I mRNA levels showed a trend of continuous increase during the observation time period, there was no statistical difference among the groups.

Effects of nano-58S and 58S on alkaline phosphatase staining and activity of MG-63 cells

On day 7 there was significantly stronger staining of ALP protein in nano-58S group than in 58S group, while the control group did not show obvious ALP protein staining (Fig 3). Consistently, ALP activity was significantly elevated in the nano-58S group than in the 58S group and in the control group on day 7 (Fig 4). On day 14, ALP activity was not different among the groups (Fig 4).

Effects of nano-58S and 58S on mineralization of MG-63 cells

Alizarin red staining assay showed that calcium deposition was only observed in the 58S and nano-58S groups on day 7, but with much more in the nano-58S group than in the 58S group. The initial formation of mineralised nodules were induced in the nano-58S group as early as day 14, however, there was no obvious mineralisation in the 58S group and control group except for early calcium deposition (Fig 5).

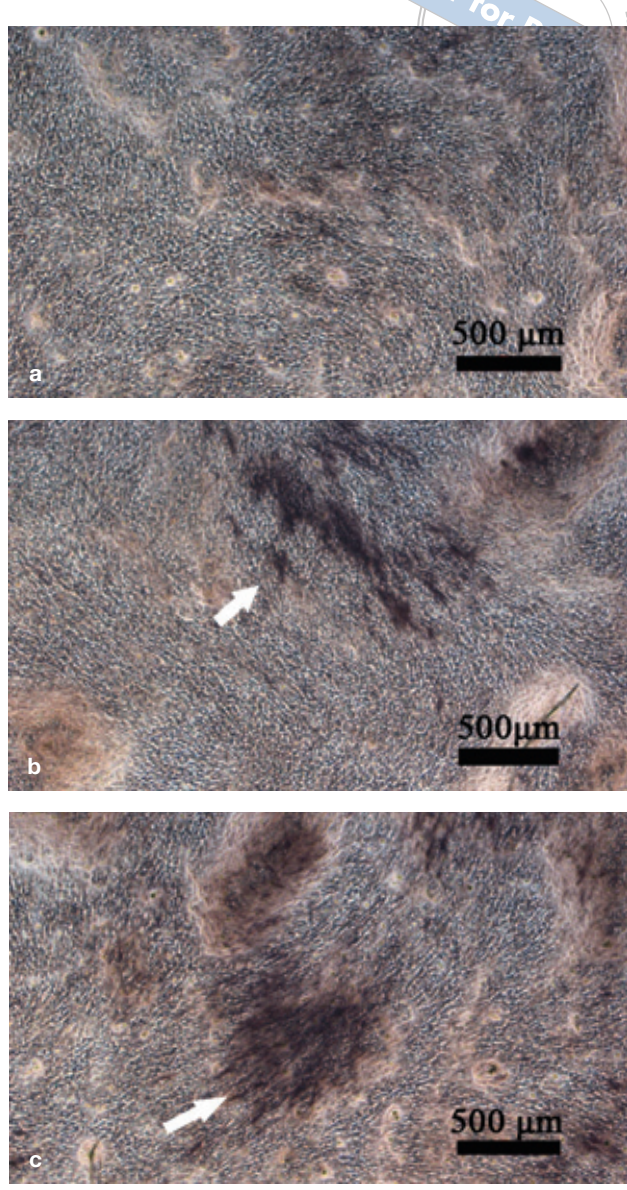

Fig 3 Expression of ALP protein (arrows) of MG-63 cells cultured in a) control; b) 58S; c) nano-58S on day 7 under light microscope.

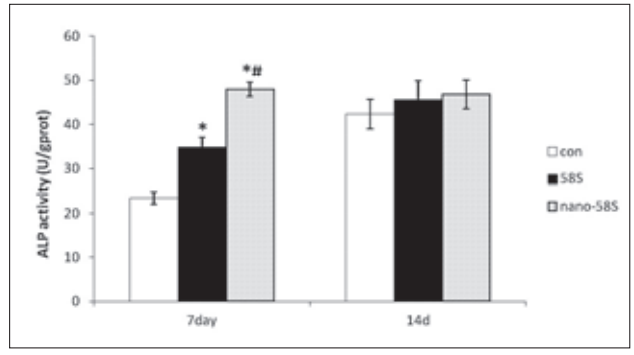

Fig 4 Effect of nano-58S and 58S BG on ALP activity (ANOVA, *$P < 0.05$ versus control group; #$P < 0.05$ versus 58S BG group).

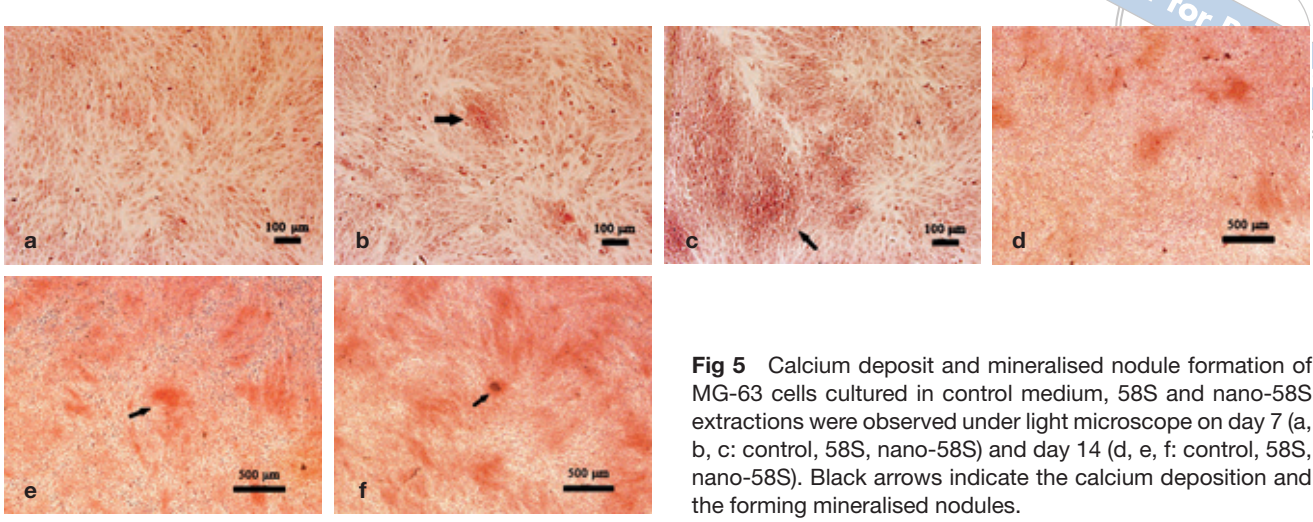

Fig 5 Calcium deposit and mineralised nodule formation of MG-63 cells cultured in control medium, 58S and nano-58S extractions were observed under light microscope on day 7 (a, b, c: control, 58S, nano-58S) and day 14 (d, e, f: control, 58S, nano-58S). Black arrows indicate the calcium deposition and the forming mineralised nodules.

Discussion

Xynos et al[1] found that the Si concentration – around 16.6 ppm in 45S5 bioactive glass conditioned medium – stimulated osteoblasts gene expression. Previous studies have also proved that exposing cells directly to a high concentration of Si inhibits the cell viability and proliferation[19,24]. The result of our preliminary experiment showed that Si ionic concentrations in 58S and nano-58S extractions between 15 and 20 μg/mL induced the best bioactivity, therefore in the present study similar concentrations were used. These results are in agreement with other studies that Si concentrations ranging from 15 to 20 μg/ml showed excellent bioactivity and ability of enhancing cell proliferation[31].

In this study, the effect of nano-sized bioactive glass particles on cell viability and proliferation was investigated. The results showed that ionic products of nano-58S enhanced the proliferation and viability of MG-63 cells significantly, compared to the traditional 58S sol-gel bioactive glass and DMEM without bioactive glass. These results suggested that nano-sized bioactive glass had better biocompatibility.

The MG-63 cell line has been used in several studies due to its ideal osteoblastic characteristics, including the expression of BMP[32], ALP[33, 34], Cbfa1 as well as OCN. In this study, essential differentiation-relative markers and transcript factors including ALP, Cbfa1, OCN and the major bone matrix, such as Col-I, were investigated using real-time RT-PCR. The results showed that mRNA levels of ALP in the nano-58S group and 58S group were significantly higher than the control group; however, there was no statistical difference between the two BG groups. Similarly, the protein staining of ALP verified the downstream effect of higher expression of the mRNA in the BG groups. ALP is one of the most commonly studied markers relative to the mature osteoblastic phenotype and extracellular matrix maturation[35]. The enhanced expression of ALP is in favour of the early and quick beginning of osteogenesis.

The expression of Cbfa1 in the nano-58S group on day 4 was significantly higher than the 58S group ($P < 0.05$) than the control group ($P < 0.01$). This result also suggested the promotion of cells differentiation by nano-58S. Cbfa1 is an essential transcript factor and master gene of osteoblast differentiation. The early up-regulation of the expression of Cbfa1 is in favour of the enhanced expression of downstream transcript factors and osteogenic markers. Studies have proven that activation and expression of Cbfa1 is a key step in bone generation. Without Cbfa1, bone formation would be totally blocked[36].

The expression of OCN in nano-58S group was up-regulated significantly to 2.5 folds compared with the 58S group (1.99 folds) and control group. These findings indicated the late differentiation of MG-63 cells and the beginning of mineralization. OCN is a late osteoblast differentiation marker that can bond calcium with bone matrix and play an important role in the mineralised nodule formation.

The effect of bioactive glass on osteoblast gene activation has been studied extensively and the findings are diverse, even controversial[3,6,11,15,17,19,22,31,37-40]. The major reasons for inconsistent results are the differences in cell models, culture conditions (particles direct connection or ionic products), ionic concentra-

tions and test time points. Similar to the present study, Au et al studied the bioactivity of Consil® Bioglass® particles on MG-63 cells[19,21]. Their results suggested that Consil® Bioglass® particles can enhance the proliferation of MG-63 cells, which is in agreement with our study[19,21]. However, for gene expression, Consil® Bioglass® particles maintained the expression levels of ALP, OCN and Col-I similar to controls, which was different from our study. The reason may be the use of different bioactive glass extracts and the selected time points. In Au's study, the final ionic concentration of Si and Ca was also different from the range of our study. In their study, Consil® Bioglass® particles (500 μg/ml) were added directly to the cells and incubated with control medium. The quick release of Si and Ca from bioactive glass particles exposed to the culture medium may have led to a short period of ionic concentration peak affecting the final ionic concentration of Si and Ca and resulted in different ranges compared to our study.

The Alizarin Red S staining assessment of calcium deposition and matrix mineralisation confirmed the gene expression regulation that promoted osteogenesis in the nano-58S group. Hench and Xynos have also shown the earlier mineralisation of osteoblasts[8,41]. Xynos[8] found that the mineralised nodule develops as early as day 6 in BG group, while no mineralised nodule was found in the control group. Similar results were observed in our study. The calcium began to accumulate at day 7, and the extract of nano-58S advanced the mineralised nodules formation earlier than 58S group and control group at day 14. The early increased calcium deposition was probably attributed to the regulations of gene expression of relative transcription factors and the protein production downstream, which led to mineralised nodules formation.

In summary, the present study results have shown that the novel nano-58S were biocompatible and promoted MG-63 cells proliferation more effectively. Both nano-58S and 58S were able to affect osteoblast gene expression indirectly through their ionic products. Nano-58S induced an eightfold higher expression of ALP and fourfold higher expression of Cbfa1 on day 4 as well as 2.5-fold up regulation of OCN on day 7. Specially, nano-58S induced significantly higher Cbfa1 expression than 58S on day 4 and OCN on day 7. Mineralisation levels and expression of ALP in the nano-58S group were higher than the control, which verified the osteogenic gene activation.

The present study showed that the novel nano-58S more enhanced proliferation, osteogenic genes expression in MG-63 cells than 58S. Further work will still be needed to elucidate the detailed mechanism of nano-58S bioactive glass on osteogenesis.

Acknowledgments

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