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Dose-Dependent Effects of Nicotine on Proliferation and Differentiation of Human Bone Marrow Stromal Cells and the Antagonistic Action of Vitamin C

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ABSTRACT

A range of biological and molecular effects caused by nicotine are considered to effect bone metabolism. Vitamin C functions as a biological antioxidant. This study was to evaluate the in vitro effects of nicotine on human bone marrow stromal cells and whether Vitamin C supplementation show the antagonism action to high concentration nicotine. We used CCK-8, alkaline phosphatase (ALP) activity assay, Von Kossa staining, real-time polymerase chain reaction and Western Blot to evaluate the proliferation and osteogenic differentiation. The results indicated that the proliferation of BMSCs increased at the concentration of 50, 100 ng/ml, got inhibited at 1,000 ng/ml. When Vitamin C was added, the OD for proliferation increased. For ALP staining, we found that BMSCs treated with 50 and 100 ng/ml nicotine showed a higher activity compared with the control, and decreased at the 1,000 ng/ml. Bone morphogenetic protein-2 (BMP-2) expression and the calcium depositions decreased at 100 and 1,000 ng/ml nicotine, while the addition of Vitamin C reversed the down regulation. By real-time PCR, we detected that the mRNA expression of collagen type I (COL-I) and ALP were also increased in 50 and 100 ng/ml nicotine groups ($P < 0.05$), while reduced at 1,000 ng/ml ($P < 0.05$). When it came to osteocalcin (OCN), the changes were similar. Taken all together, it is found that nicotine has a two-phase effect on human BMSCs, showing that low level of nicotine could promote the proliferation and osteogenic differentiation while the high level display the opposite effect. Vitamin C could antagonize the inhibitory effect of higher concentration of nicotine partly. J. Cell. Biochem. 114: 1720–1728, 2013.

KEY WORDS: NICOTINE; VITAMIN C; HUMAN BMSCS; PROLIFERATION; OSTEOGENIC DIFFERENTIATION

Cigarette smoking, which is considered a risk factor for skeletal disease such as osteoporosis [Johnell and Kanis, 2005], was demonstrated by numerous case-control studies. For example, it can be seen from the research of Aloia et al. [1985], Jensen et al. [1985], and Riggs and Melton [1986]. But those studies were all based on the smokers who were exposed large amount of tobaccos. That means that the relationship between a light tobacco consumption and osteoporosis has not been clarified yet. On the other hand, a study implied that there was no association between smoking and lower premenopausal bone mineral density [Bainbridge et al., 2004]. It is

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no doubt that a high concentration of nicotine (above 200 ng/ml) profoundly showed the effects that inhibits osteoblasts [Fang et al., 1991] and delay bone healing [Porter and Hanley, 2001]. However, a stimulatory effect of nicotine (10–100 ng/ml) has been noted in cultures of osteoblast-like cells [Kim et al., 2012] and gingival fibroblasts [Tipton and Dabbous, 1995]. Especially, in the latest study of Rothem et al. [2009], they found that nicotine (20–120 ng/ml) affects proliferation of human osteosarcoma cells (MG63) in a biphasic manner, including toxic effects at high levels and stimulatory effects at low levels. Moreover, low levels of nicotine upregulated gene expression related to osteogenic differentiation.

It is showed that nicotine exposure is associated with an imbalance in the cellular oxidant-antioxidant system [Ashakumary and Vijayammal, 1996]. Vitamin C functions as a biological antioxidant, reducing the oxidative properties of toxic substances. On this basis, it has been suggested that smokers in particular would benefit from increasing their dietary intake of antioxidants [Schectman et al., 1991]. On the other hand, Vitamin C may play an important role in the process of bone metablism and skeletal maintenance [Morton et al., 2001]. Kaptoge et al. [2003] reported that Vitamin C can reduce bone loss and show as a protective factor of fracture. In particular, from the study of Carinci et al. [2005], Vitamin C show the effect to induce stem cell to osteogenic cell.

From those above, we can speculate that the direct effects of low level nicotine on skeletal tissue maybe protective and those components other than nicotine may account for the deleterious effect of cigarette smoking on bone repair. However, the evidence of very low level nicotine effect and the action of Vitamin C added into high level nicotine to human BMSCs are lacking. The purpose of this study was to evaluate the direct effect of nicotine with different concentrations (5–1,000 ng/ml) on osteogenic differentiation of human BMSCs and whether Vitamin C (60 mg/L) supplementation show the antagonism action to high concentration of nicotine.

MATERIALS AND METHODS

ISOLATION AND CULTURE OF HUMAN BONE MARROW STROMAL CELLS (BMSCS)

Bone marrow (5 ml) aspirates were isolated from the posterior iliac crest of three healthy volunteers, aged 35–60 years (two males and one female). Then bone marrow was washed with growth culture medium (DMEM, Gibco) supplemented with 10% (V/V) fetal bovine serum (FBS, Gibco), 1% (V/V) penicillin and streptomycin (Gibco). The mixture of bone marrow and medium was gently added to the 50% Percoll solution (Sigma) and centrifuged at 3,000 rpm for 30 min. Then, we acquired the cell suspension between the layer of Percoll and the supernatant liquid layer. Cells were plated and then incubated in a humidified atmosphere of 5% CO_2 at 37°C. They were passaged every 3–4 days using 0.25% (w/v) trypsin–EDTA solution (Gibco) and the third or fourth passage cells were used in our experiments. To induce osteoblastic differentiation, the BMSCs were cultured in an osteogenic medium (growth culture medium supplemented with 10^{-8} M dexamethasone (Sigma), 50 μg/ml ascorbic acid (Sigma) and 5 mM β-glycerol phosphate (Sigma)) at an initial density of 1×10^4 cells/cm^2. The medium was changed every 4 days during osteogenesis. Full ethical consent was obtained from all patients and the study was granted ethical approval by the Medical Ethical Committee of the Second Affiliated Hospital, Wenzhou Medical College.

CELL PROLIFERATION BY CELL COUNTING KIT-8 (CCK8) ASSAY

For the cell proliferation assays, human BMSCs were cultured in 96-well plates at 1×10^4 cells per well with growth culture medium. Twenty-four hours later, cells were exposed to various concentrations of nicotine (0, 5, 10, 50, 100, 1,000 ng/ml) containing media for 1, 4, 7, and 14 days. In another 96-well plates, Vitamin C (60 mg/L) was added into the group with highest concentration of nicotine (0, 1,000 ng/ml + 60 mg/L, 1,000). The proliferation of human BMSCs was determined by the Cell Counting Kit-8 (Dojindo, Japan) and measured by microplate reader scanning at 450 nm.

ALKALINE PHOSPHATASE ASSAY

The cells treated with the same concentrations of nicotine showed above. ALP staining was performed at 7th day. Cells which were cultured in osteogenic medium were rinsed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde. ALP substrate mixture (ALP staining kit, Sigma) was then added and incubated for 10 min. The ALP activity was determined at 405 nm using p-nitrophenyl phosphate (pNPP; Sigma) as the substrate and the total protein contents were determined with the BCA method, which is described previously in the elsewhere literature [Sun et al., 2006].

QUANTITATION OF MINERALIZED NODULE FORMATION

In order to qualitatively assess cell-mediated calcium deposition, human BMSCs which were cultured in osteogenic medium were visualized by Von Kossa staining. Twenty-four hours after cells cultured in 24-well tissue culture plates at a cell density of 1×10^4, cells were treated with the same concentrations of nicotine showed above. Von Kossa staining was performed at 21st day. Cells in the well plates were fixed in 4% paraformaldehyde, and then stained with 1% silver nitrate, placed under a UV lamp for 20 min and rinsed with distilled water before treatment with 5% sodium thiosulfate for 2 min. Von Kossa-positive (black) deposits was observed after alcohol washing. Calcium deposition was measured using the Quantichrom calcium assay kit (Reebio Products, Ningbo, Zhejiang, China). Briefly, the supernatants of the human BMSCs cultures were prepared and mixed with working reagent. The mixtures were incubated for 3 min and the amount of calcium in the supernatants was quantified using a spectrometer at 640 nm.

REAL-TIME PCR ASSAY

Real-time PCR was used to detect the expression of several osteogenic differentiation related marker genes (COL-I, ALP, OCN) at 4th and 7th day, respectively. Total RNA was extracted using TriZol (Invitrogen) according to the manufacturer's instructions and quantified. Its concentration was determined spectrophotometrically at 260 nm (HP 8452A Diode Array Spectrophotometer). First strand complementary DNAs (cDNAs) were synthesized from 3 ug of the isolated RNA by oligo (deoxythymidine; oligo [dT]) using

TABLE I. Primers for Real-Time PCR

Gene	Primer sequences (5′– 3′)
COL-I	Forward: GAG AGC ATG ACC GAT GGA T Reverse: ATG TTT TGG TGG TTC AGG AGG
ALP	Forward: CGC TGT GTC AAC TCC ACC T Reverse: CCA GAA GGT TCT GTT AAC TTG
OCN	Forward: GTG CAG CCT TTG TGT CCA AG Reverse: GTC AGC CAA CTC GTC ACA GT
GAPDH	Forward: CCT CAA GAT CAT CAG CAA T Reverse: CCA TCC ACA GTC TTC TGG GT

DyNamoTM cDNA Synthesis Kit (Fermentas), and used as templates for real-time PCR. The expression of mRNAs was determined quantitatively using DyNamo SYBR1 Green qPCR kit (Takara, Japan).The PCR was performed on a final volume of 25 ml containing 2 ml cDNA, 7.5 pmol of each primer, 1 ml ROX reference dye and 12.5 ml of SYBR Green Master mix (TIANGEN),with ABI Prism 7300 (Applied Biosystems, Foster City, CA). The samples underwent 40 cycles consisting of the following steps: initial denaturation at 95°C for 5 min, followed by a set cycle of denaturation at 94°C for 10 s, different annealing temperatures for each pair of primers (ranging between 53°C and 62°C)for 10 s, extension at 72°C for 27 s and a final elongation at 72°C for 5 min. Fold increment of any assayed gene was calculated by normalizing its expression level to that of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, which was used as an internal control. Each gene analysis was performed in triplicate. Primer's sequences of the targeted genes were listed in Table I.

WESTERN BLOT ANALYSIS FOR BMP-2

At the end of this incubation, cells were washed with ice-cold PBS and lysed in NP 40 lysis buffer (20 mmol/L Tris–HCl (pH 7.4), 100 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 5 mmol/L MgCl2, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 10 mg/ml of protease inhibitor mixture). Protein was extracted using Mammalian Protein Extraction Reagent (Pierce, Inc., Rockford, IL) and its concentration was determined by BCA (Pierce) assay. Proteins (30 μg) were separated in 10–15% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a polyphorylated difluoride (PVDF) membrane. Membranes were incubated with primary antibody overnight at 4°C, and then with the respective secondary antibodies. Immunoreactive bands were detected by the enhanced chemiluminescence (ECL) kit for Western blotting detection with hyper-ECL film. The same membrane was reprobed with the anti-β-actin antibody, which was used as an internal control for protein loading.

STATISTICAL ANALYSIS

All experiments reported in this study were performed independently at least three times. 18 replicates were used per experiment ($n = 18$). Results are expressed as mean ± standard deviation. Student's *t*-test was used to determine statistical significance, values of $P < 0.05$ were considered significant.

RESULTS

CELL PROLIFERATION IN VITRO

The proliferation of human BMSCs showed that a slight increase in relative cell number (value of OD) were observed at 5, 10 ng/ml concentrations of nicotine, but the difference was not statistically significant, while the at 50, 100 ng/ml, the increase was meaningful ($P < 0.05$). However, we found at the concentration of 1,000 ng/ml, the BMSCs numbers were decreased significantly compared to the control ($P < 0.01$; Fig. 1A). When the Vitamin C (60 mg/L) was added into the group with 1,000 ng/ml nicotine, we found increase of the

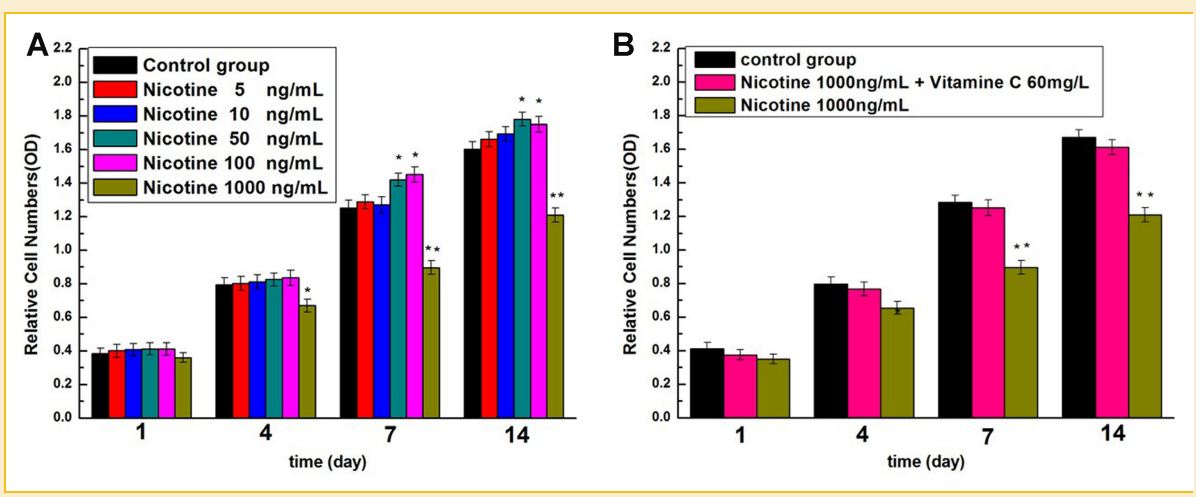

Fig. 1. The proliferation of BMSCs at days 1, 4, 7, and 14. The BMSCs number increased at the 50 and 100 ng/ml level. While the cell numbers were decreased significantly compared to the control throughout the duration of the concentration of 1,000 ng/ml nicotine experiment. When the Vitamin C (60 mg/L) was added, the decrease compared to the control was not significant ($P > 0.05$). The data are represented as mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$.

number of the human BMSCs compared to the 1,000 ng/ml nicotine only group, while the decrease compared to the control was not significant ($P > 0.05$; Fig. 1B).

ALKALINE PHOSPHATASE ACTIVITY

The ALP activity of BMSCs was measured with the pNPP assay at 7th day. Cells treated with 50 and 100 ng/ml nicotine presented a higher activity ($P < 0.05$). And no significant difference was found in the ALP activity of BMSCs at the concentration of 5 or 10 ng/ml ($P > 0.05$). Also, lower ALP activity was found in the group of 1,000 ng/ml nicotine ($P < 0.05$; Fig. 2A). Vitamin C (60 mg/L) can reverse the decrease of ALP activity when added into the highest concentration nicotine group (Fig. 2B).

QUANTITATION OF THE DEPOSITION OF CALCIUM

Compared to control group, there were no significant change of calcium depositions observed in 5, 10, and 50 ng/ml nicotine treatment groups ($P > 0.05$). However, the calcium depositions were reduced at the concentration of 100 and 1,000 ng/ml treatment groups; especially in 1,000 ng/ml treatment group, the reduction was significant ($P < 0.01$; Fig. 3A). When the Vitamin C (60 mg/L) was added, the calcium depositions of 1,000 ng/ml treatment group increased a little but it is still significantly ($P < 0.05$; Fig. 3B).

THE MRNA EXPRESSION OF SEVERAL OSTEOGENIC DIFFERENTIATION RELATED MARKER GENES

Real-time PCR (Fig. 4) was used to detect the expression of several osteogenic differentiation related marker genes when the human BMSCs were cultured in osteogenic medium supplemented with different concentration of nicotine for 4 and 7 days. The expression of COL-I and ALP was significantly increased in BMSCs cultured with nicotine (50 and 100 ng/ml) at 4th and 7th day ($P < 0.05$), but at the high level of 1,000 ng/ml, the decrease of COL-I and ALP was significant ($P < 0.05$; Fig. 4A,B). Incubation of cells with nicotine from 5 to 1,000 ng/ml, the expression of OCN did not change significantly compared to control at 4th day ($P > 0.05$; Fig. 4C). At 7th day, only 1,000 ng/ml treatment group decreased ($P < 0.05$) and the change at the concentrations 5, 10, 50, and 100 ng/ml was insignificant respectively. When the Vitamin C (60 mg/L) was added, the level of COL-I and ALP mRNA expression increased at 1,000 ng/ml treatment group after 4 and 7 days, respectively (Fig. 4D,E). However, the decrease of the OCN mRNA expression was significant compared to control at 7th day ($P < 0.05$) at the group of 60 mg/L Vitamin C + 1,000 ng/ml nicotine group (Fig. 4F).

THE CHANGE OF BMP-2 EXPRESSION BY THE EFFECT OF NICOTINE AND VITAMIN C

Western blot was used to detect the change of BMP-2 by the effect of nicotine and Vitamin C. The expression of BMP-2 increased by the treatment with nicotine (5,10, and 50 ng/ml), but at the high level of 100 and 1,000 ng/ml, BMP-2 got down regulation (Fig. 5A,B). The fold change of BMP-2 was 1.2, 1.34, and 0.94 at the nicotine concentration 5, 10, and 50 ng/ml, but decrease to 0.81 and 0.71 at the high level of 100 and 1,000 ng/ml. The addition of the Vitamin C (60 mg/L) favors the BMP-2 expression compared with the 1,000 ng/ml nicotine group.

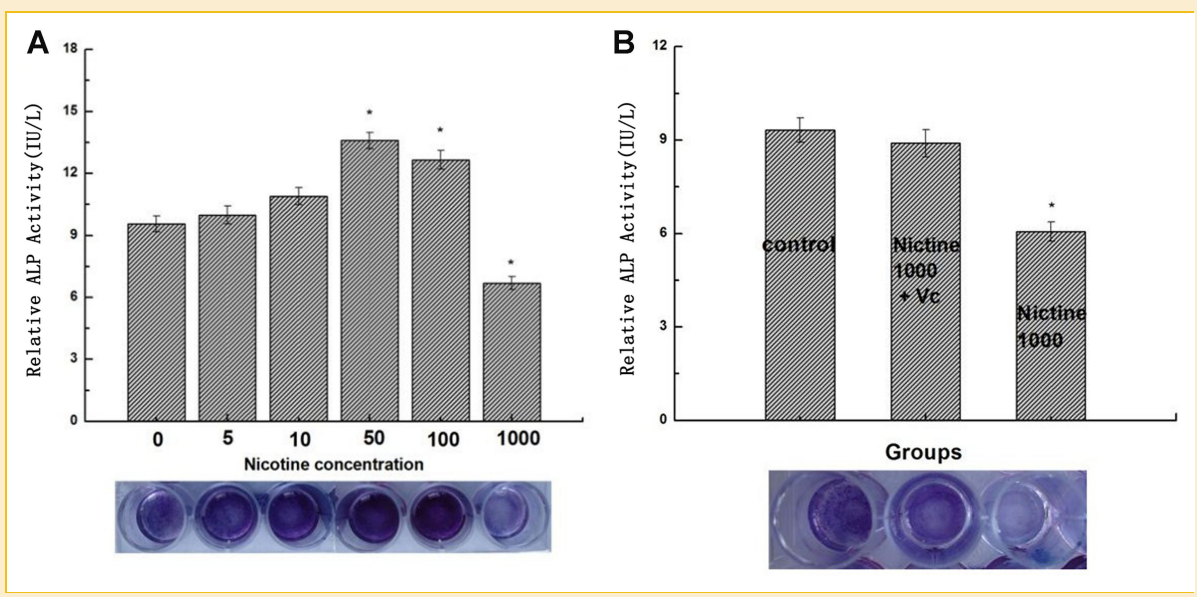

Fig. 2. To assess the effects of nicotine on the alkaline phosphatase activity of BMSCs, alkaline phosphatase (ALP) staining was performed at 7th day after cells were cultured in 24-well tissue culture plates at a cell density of 1×10^4.The ALP activity of BMSCs was measured with the pNPP assay. Cells treated with 50 and 100 ng/ml presented the higher activity compared with control. At 1,000 ng/ml, lower ALP activity was found ($P < 0.05$). Vitamin C (60 mg/L) addition can reverse the decrease of ALP activity. The data are represented as mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$.

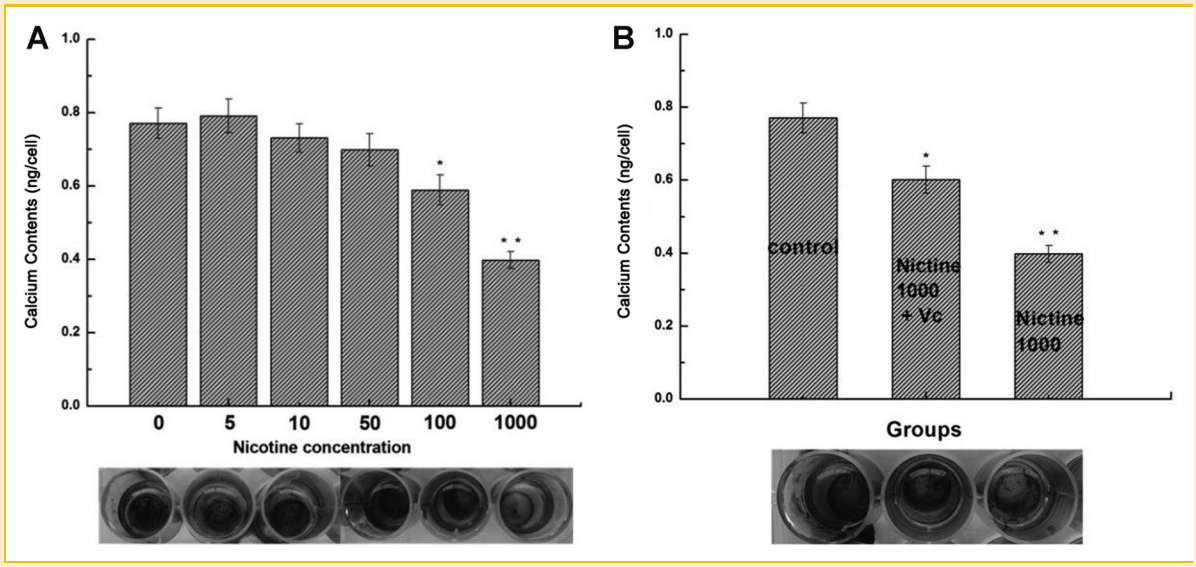

Fig. 3. To qualitatively assess cell-mediated calcium deposition, Von Kossa staining was performed at 21st day after cells were cultured in 24-well tissue culture plates at a cell density of 1×10^4. Compared to the untreated control group, decreased calcium depositions were observed in 100 and 1,000 ng/ml nicotine treatment groups, which was significant. When the Vitamin C (60 mg/L) was added into 1,000 ng/ml treatment group, the difference compared to control is still significantly. The data are represented as mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$.

DISCUSSION

Tobacco smoking, containing more than 150 known toxic agents to human, is divided into two different kinds of free radicals, 92% in the gas phase and 8% as particulates [Rodgman et al., 2000]. During the predominant volatile phase, nearly 500 different gases are released such as nitrogen, carbon monoxide, and hydrogen cyanide. In the particulate phase, almost 3,500 chemicals are released, including nicotine, which was the most physiological active substance in the smoking. Well established as an ideal source of cell-based therapy, human BMSCs have been used in the field of bone tissue engineering. It might be convincing to choose human BMSCs and detect the proliferation and osteogenic differentiation to explain the effect of nicotine and Vitamin C. In this study, present results indicated that nicotine had a significant positive effect on cell proliferation at levels from 50 to 100 ng/ml, and the increase was significant. Similar nicotine-induced stimulation was noted by others in bone cells [Kim et al., 2012], chondrocyte [Gullahorn et al., 2005], endothelial cells [Zimmerman and McGeachie, 1985], and epithelial cells [Mazhari et al., 2003], but at much higher doses. But, it is not consistent with some studies in the present. Kawakita et al. [2008] discovered that nicotine, from cigarette smoking, acted directly via alpha7 nicotinic acetylcholine receptors (nAChR), leading to delay skeletal growth. Jaiswal et al. [2009] concluded that patients who smoke had worse preoperative function and obtained less benefit from this procedure than non-smokers after a case-controlled study. However, the key is the level of the nicotine concentration. In habitual smokers, high levels of nicotine (above 200 ng/ml) are found and have been shown to have inhibitory proliferation effects on bone turnover in vivo [Krall and Dawson-Hughes, 1991]. Habitual smokers often suffer loss of bone mass [Krall and Dawson-Hughes, 1991; Kayed et al., 2007], increased risk of fracture [Krall and Dawson-Hughes, 1991, 1999], prolonged fracture repair, and increased nonunion rates. In our study, we get the similar result about the adverse effect of high level nicotine in the 1,000 ng/ml group (Fig. 1A).

As known to all, smokers suffer increased oxidant load from free radicals generated in cigarette smoke and from reactive oxidants created by smoke-induced activation of the inflammatory-immune system. This suggests that increased oxidant damage may be one of the mechanisms by which cigarette smoke causes smoking-related diseases. Vitamin C, also known as ascorbic acid (AA), is a cofactor required for the function of several hydroxylases. AA acts as a free radical scavenger and repair lymphocytic DNA damage caused by smoking [Newman et al., 2002]. Many studies have shown that the proliferation and differentiation of mesenchymal stem cell into bone tissues needs AA as an essential medium component. Takamizawa et al. [2004] studied the effect of AA and the Asc2-P derivative on the proliferation and differentiation of human osteoblast-like cells. Both AA and Asc-2-P were shown to stimulate nascent cell growth and expression of various osteoblast differentiation markers, such as collagen synthesis and ALP activity. Malicev et al. [2000] pointed out that apoptotic changes were found to be minimal in cells cultivated in the absence of AA or with low concentrations (0.05–0.2 mg/ml). A few studies have directly investigated the relationship between nicotine and AA. Recently, it has been shown that AA induces embryonic stem cells and MC3T3 (a well-defined model of pre-osteoblast differentiation) to differentiate into mineralized osteoblasts in vitro [zur Nieden et al., 2003; Hu et al., 2008]. Also, in the study of Harada et al. [1991] and Franceschi and Iyer [1992], AA is responsible for the sustained proliferation of

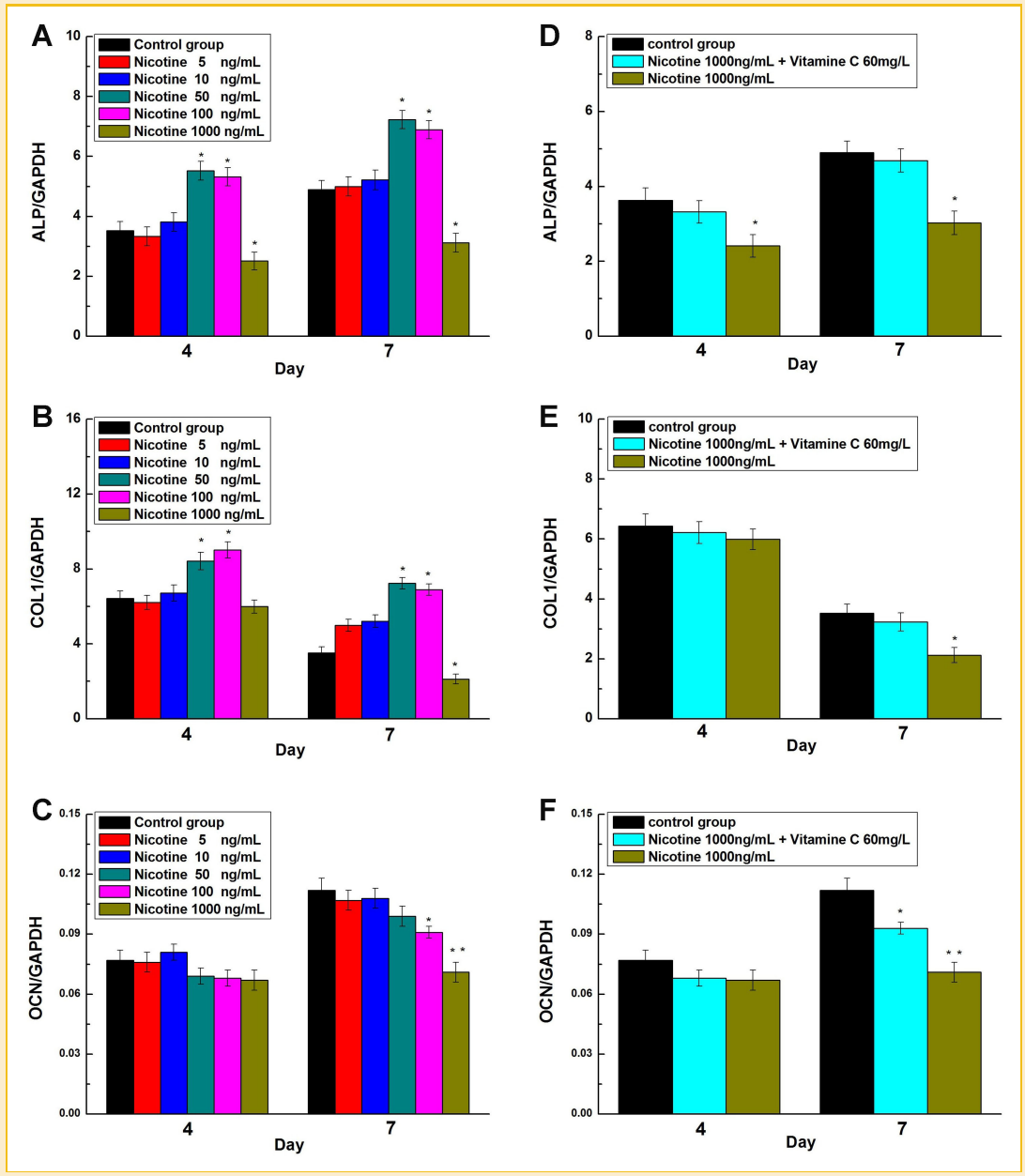

Fig. 4. Real-time PCR reactions were performed using primers for osteoblastic genes, including ALP (a), OCN (b), COLL-I (c), and BMP-7 (d) for 4 and 7 days. Each bar represents the mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$.

MC3T3-E1 cells and the effect is mediated through the synthesis of collagen. Choi et al. [2008] and Fernandes et al. [2010] demonstrated that the proliferation or differentiation for human multipotent mesenchymal stromal cells depends on AA concentration, which they pointed out that the most suitable concentration of AA is 50–150 mg/L. By using DNA micro-arrays containing 15,000 genes, Carinci et al. [2005] identified several genes in MC3T3-E1 cultured with AA for 24 h whose expression was significantly up or down-regulated. AA is able to modulate a broad range of biological processes in pre-osteoblasts as follows: (1) cell growth; (2) metabolism; (3) morphogenesis; (4) cell death; (5) cell communication. Franceschi and Young [1990] reported that AA promoted the cell growth and stimulated the differentiation of these human osteoblast-like cells (MG-63). So it might be an interesting question about what will happen if Vitamin C was added into the solution containing nicotine, especially in the high concentration. In our study, we found that the adverse effect of high level nicotine, but the proliferation got increased when Vitamin C (60 mg/L) was added. And there was no significant decrease compared with the control (Fig. 1B).

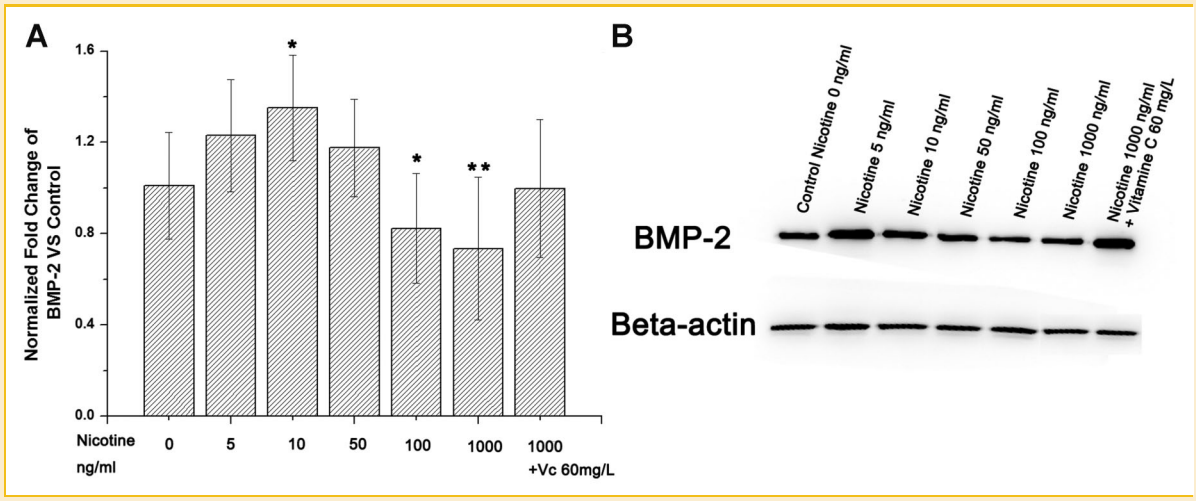

Fig. 5. Western blot depicting alterations were performed to detect the BMP-2 change in different concentration of Nicotine and in the presence of Vitamin C for 1,000 ng/ml nicotine. Each bar represents the mean ± standard deviation. $^{*}P < 0.05$, $^{**}P < 0.01$.

Three of the genes involved in bone metabolism were chosen to be examined for level of gene expression: (1) COL-I, a fibril-forming collagen, is the most abundant protein in bone matrix; it is abundant (about 90%) of the organic bone matrix and high levels of COL-I mRNA expression would be observed during proliferation [Owen et al., 1990], (2) ALP, a cell membrane-associated enzyme linked directly to skeletal defects, appeared early during osteoblast differentiation and was the most widely recognized marker of osteoblastic differentiation [Zou et al., 2008; Serigano et al., 2010]. ALP activity proposed function of matrix formation in osteoblasts prior to the initiation of mineralization. (3) OCN, a small and highly conserved molecule involved in controlling the mineralization process, appeared at a late stage of osteogenic differentiation and was characterized by mature cells of the osteoblastic lineage [Luu et al., 2007]. The expression of these extracellular matrix proteins was estimated by determining the levels of their mRNAs using Real-time PCR. In our study, we detected that the mRNA expression of ALP and COL-I were increased in 50 and 100 ng/ml nicotine treatment groups both in the 4 and 7th day ($P < 0.05$), and they were reduced at the concentration of 1,000 ng/ml groups. In the nicotine added with Vitamin C (60 mg/L) group, the increase of mRNA expression of ALP and COL-I prove that Vitamin C can counterbalance the harmful effect of 1,000 ng/ml nicotine. By ALP staining, we discovered the similar result which showed that human BMSCs treated with extra Vitamin C can present higher ALP activity compared with 1,000 ng/ml nicotine only groups (Fig. 2). In Rothem's [Rothem et al., 2009] study, nicotine, a selective agonist of nicotinic acetylcholine receptor (nAChR) in many cell lines, affects cell proliferation in a biphasic manner, including toxic and antiproliferative effects at high levels of nicotine and stimulatory effects at low levels. However, the situation is different in our study, the mRNA expression of OCN did not get increase at the low level nicotine, while decreased at 100 and 1,000 ng/ml, especially at 1,000 ng/ml nicotine ($P < 0.01$; Fig. 4). The results may be explained by differences in the batches of nicotine and the concentrations, as well as the different cell we use. More important, the OCN in 1,000 ng/ml nicotine group added with Vitamin C also decreased compared with the control group ($P < 0.05$). There may be some unknown mechanism why Vitamin C can not counteract with the decrease of OCN in high level nicotine satisfactorily. The similar result can be seen in the Von Kossa staining (Fig. 3). Bone morphogenetic proteins (BMPs), as members of the TGF- β superfamily, are multifunctional cellular regulators with a pivotal role in numerous biological processes in both the developing embryo and adult. BMP-2 had been reported to induce bone formation, the regeneration of bone in fracture healing, and vertebral arthrodesis [De Biase and Capanna, 2005]. In our study, we detected that the BMP-2 was increased at 10 ng/ml nicotine treatment group respectively, while the 1,000 ng/ml nicotine injure the BMP-2 expression. Interesting, Vitamin could reverse this trend and favors the expression which was injured by 1,000 ng/ml nicotine already. This may be one of the mechanisms applied on osteogenic differentiation of BMSCs.

We should know that smoking and nicotine administration are quite different. Recently, Gullihorn's study [Gullihorn et al., 2005] suggests that nicotine acts as a direct stimulant of bone cell metabolic activity. Smoke condensate containing equivalent levels of nicotine elicits an inhibitory effect. Zhang et al. [2010] give out a probable speculation that the delay in clinical healing of skeletal trauma in smoking patients may be a result of absorption of components of smoke other than nicotine. It was suggested that nicotine at a low dose (20–120 ng/ml) would be a novel topical drug for the clinical application to reduce osteoporosis incidence. For the heavy smokers, increasing the Vitamin C level in their diet may be an effective method to counterbalance the adverse effect of smoking to some extent, if they insist on continuing smoking. Our results suggest that nicotine concentrations at high level of habitual smokers decrease gene expression, osteogenesis, and cell proliferation, and the addition

of Vitamin C might show antagonistic action, which can explain the biological and clinical influence of smoking and Vitamin C on bone healing and osteoporosis. However, further studies upon the mechanism would be performed to bring to light how nicotine and Vitamin C affect human BMSCs proliferation and metabolism. As the results of nicotine and Vitamin C on osteogenic differentiation of BMSCs in vitro cannot be fully representative of which is in vivo, the results still need further validation in animal models.

Taken all together, our study reported that low level of nicotine can promote the proliferation of human BMSCs, which can enhance the related gene expression during osteoblastic differentiation. While the adverse effect of high level nicotine could be counterbalanced by Vitamin C to some extent. The future work needs to be focused on how and where-by these osteogenic differentiation related marker genes modulated by nicotine and Vitamin C, along with the study in vivo and regulation mechanism.

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