# THE ROLE OF NITRIC OXIDE ON THE PROLIFERATION OF A HUMAN OSTEOBLAST CELL LINE STIMULATED WITH HYDROXYAPATITE

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The aim of the present study was to test the hypothesis that the proliferation of a human osteoblast cell line (HOS cells) stimulated with hydroxyapatite (HA) may be regulated by nitric oxide (NO). The cells were cultured on the surface of HA. Medium or cells alone were used as controls. L-arginine, D-arginine, 7-NI (an nNOS inhibitor), L-NIL (an iNOS inhibitor), L-NIO (an eNOS inhibitor) or carboxy PTIO, a NO scavenger, was added in the HA-exposed cell cultures. The cells were also precoated with anti-human integrin αV antibody. The levels of nitrite were determined spectrophotometrically. Cell proliferation was assessed by colorimetric assay. The results showed increased nitrite production and cell proliferation by HA-stimulated HOS cells up to day 3 of cultures. Anti-integrin αV antibody, L-NIO, or carboxy PTIO suppressed, but L-arginine enhanced, nitrite production and cell proliferation of HA-stimulated HOS cells. The results of the present study suggest, therefore, that interaction between HA and HOS cell surface integrin αV molecule may activate eNOS to catalyze NO production which, in turn, may regulate the cell proliferation in an autocrine fashion.

**Key Words:** HOS cells, hydroxyapatite, nitric oxide, osteoblasts, proliferation

## INTRODUCTION

Hydroxyapatite (HA) is known as a ceramic material widely used for orthopaedic and dental implants, since this biomaterial has the ability to stimulate osteoblast functions in vitro and in vivo.[1–3] However, the exact mechanisms by which HA induces osteoblast functions remain unclear. Previous studies indicated that osteoblasts via surface integrin molecules initially attach and spread on protein-coated HA.[4,5] Signal transduction pathways generated by osteoblast integrin-HA interaction may also induce the production of cytokines which may, in turn, regulate cell proliferation and differentiation.[6–8]

Nitric oxide (NO) is a gaseous molecule generated from L-arginine under catalization of nitric oxide synthase (NOS), and it plays a crucial role on the nervous, cardiovascular, qatalization, and immune system.[9] Three isoforms of NOS, ie, neural NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2), and endothelial NOS (eNOS or NOS-3), are recognized.[10] Human osteoblasts have been shown to express all NOS isoforms.[11] However, others found that osteoblasts expressed eNOS and iNOS molecules only.[12,13] Proinflammatory cytokines and bacterial lipopolysaccharides increased iNOS expression,[11,12,14] whereas the eNOS activities could be induced by stimulators such as estradiol,[15] estrogen,[16] and fluid shear stress.[17] These

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studies indicate that iNOS and eNOS may play a role in bone inflammation and physiology, respectively.

The exact mechanism(s) by which NO regulates bone formation remains unclear. Hikiji and colleagues showed that NO directly induces osteoblast proliferation, independent of the presence of cytokines.[18] Others demonstrated, however, that NO stimulates osteoblast proliferation via the induction of prostaglandin $E_2$ ($PGE_2$) production.[19] Further studies suggested that the effect of NO on osteoblast functions may depend on the concentration of this gaseous molecule. Thus, low concentration of NO stimulated osteoblast proliferation, whereas high concentration induced osteoblast apoptosis.[11,20–22] A recent study showed that $PGE_2$ production by HA-stimulated human osteoblast-like cell line (HOS cells) is under regulation by NO.[23] Therefore, the aim of the present study was to test the hypothesis that the proliferation of HA-stimulated HOS cells may be regulated by NO.

## MATERIALS AND METHODS

### Hydroxyapatite

Hydroxyapatite (HA) discs (9% porosity) sintered at 1200°C were cut into pieces with $2 \times 2 \times 2$ mm$^3$ in size. After being autoclaved, each HA disc was placed in 96-well culture plates (Corning, NY).

### Cell cultures

HOS cells were purchased from American Type Culture Collection (Rockville, Md) and grown in Dulbecco's modified Eagle's medium (Sigma, St Louis, Mo) supplemented with 10% heat-inactivated fetal calf serum (Sigma) and 1% penicillin-streptomycin (Sigma) until confluent. After harvesting and washing, a single cell suspension ($1 \times 10^6$ cells/mL) was prepared in the above medium. Two hundred microliters of cell suspension containing $2 \times 10^5$ cells/well in the 96-well culture plates were plated on the surface of the HA-disc and incubated for 3 days at 37°C in a humidified atmosphere and 5% $CO_2$ as previously described.[23] Medium only and cell suspension plated in the wells were used as controls. All cultures were performed in triplicate.

Anti-human integrin αV antibody (Santa Cruz Biotechnology, Santa Cruz, Calif), was dissolved in distilled water to obtain 1 mg/mL of stock solution, and was filter sterilized. One million HOS cells were incubated with 1 mL of the medium containing 5 μg, 10 μg, and 20 μg of the antibody for 1 hour at room temperature. After washing, the cells were resuspended in 1 mL of the culture medium and then cultured in the 96-well culture plates for 3 days as above.

L-arginine and D-arginine (Sigma) were dissolved in the complete culture medium to obtain 1 mM stock solution, and were filter sterilized. One million HOS cells were resuspended in 1 mL of the medium containing 1 μM, 10 μM, and 100 μM L- or D-arginine and then cultured in the 96-well culture plates for 3 days as above.

Carboxy PTIO, a NO scavenger (Sigma), was dissolved in methanol to obtain 1 mM stock solution. One million HOS cells were resuspended in 1 mL of the medium containing 1 μM, 10 μM, and 100 μM carboxy PTIO and then cultured in the 96-well culture plates for 3 days as above. Our preliminary study indicated that the cell morphology and growth in the cultures containing less than 0.01% methanol were not altered (data not shown).

The NOS isoforms were inhibited with 7-NI (an nNOS inhibitor), L-NIL (an iNOS inhibitor), or L-NIO (an eNOS inhibitor), all of which were purchased from Sigma, dissolved in distilled water to obtain 1 mM stock solution and filter sterilized. One million HOS cells were resuspended in 1 mL of the medium containing 1 μM, 10 μM, and 100 μM NOS isoform inhibitors, and then cultured in the 96-well culture plates for 3 days as above.

### NO assay

The levels of nitrite, a stable end-product of NO, were determined from the culture supernatants as previously described.[24] Briefly, 100 μL of the supernatants were mixed with equal volume of the Griess reagent (1% sulfanilamide, 0.1% naphthlethylenediamine dihydrochloride in 2.5% phosphoric acid) and the optical density was read by μQuant spectrophotometer (Biotek-Instrument Inc, Winooski, Vt) at 540 nm. The $OD_{540nm}$ of the unknown was compared to a standard curve generated with known amounts of sodium nitrite. All reagents for NO assays were purchased from Sigma.

### Cell proliferation assay

Cell proliferation was determined by a colorimetric assay as previously described.[25] Briefly, after harvesting the supernatant and gently washing with sterile phosphate buffered saline (PBS), the cells were dehydrated with 100 μL of 20% methanol for 10 minutes, and then the solution was carefully aspirated. The cells were then exposed to 100 μL of 0.5% crystal violet for 5 minutes followed by extensive rinsing with PBS. The dye was released from the cells by adding 100 μL of 0.1 M Na citrate in 50% ethanol. The optical density was read at a wavelength of 540 nm using μQuant spectrophotom-
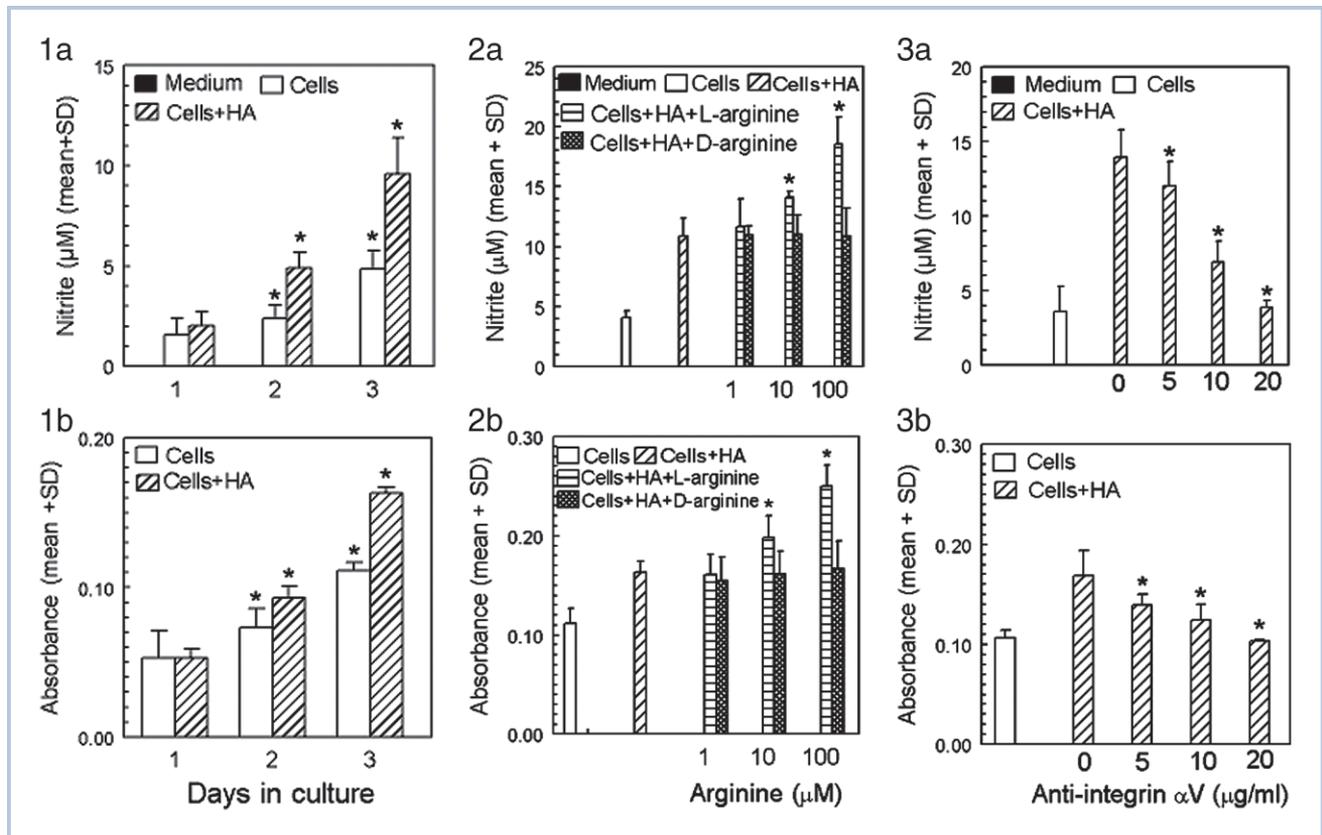
Fɪɢᴜʀᴇꜱ 1–3. Fɪɢᴜʀᴇ 1. (a) Nitrite production and (b) cell proliferation of hydroxyapatite (HA)-stimulated human osteoblast cell line (HOS cells). *Significant difference at $P < .05$. Fɪɢᴜʀᴇ 2. Effect of L-arginine or D-arginine on (a) nitrite production and (b) cell proliferation of HA-stimulated HOS cells. Cells alone or HA-stimulated cells with or without the presence of L-arginine or D-arginine were cultured for 3 days. *Significant difference at $P < .05$. Fɪɢᴜʀᴇ 3. Effect of anti-human integrin αV antibody on (a) nitrite production and (b) cell proliferation of HA-stimulated HOS cells. Prior to stimulation with HA, cells were precoated with anti-human integrin αV antibody. Uncoated cells alone were used as a control. All cultures were incubated for 3 days. *Significant difference at $P < .05$.

eter (Biotek-Instrument). The results were subtracted by the optical density reading of medium only.

### Statistical analysis

The results were analyzed using a repeat measurement test for the data derived from the NO production and cell proliferation of HA-stimulated HOS cells. A 1-way analysis of variance followed by Fisher's least square differences was used to analyze the data from the remaining experiments. The data were analyzed by using a statistical software package (SPSS Co, Chicago, Ill).

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### NO levels and cell proliferation

The cell cultures were incubated for 3 days and both nitrite levels from the culture supernatant and cell proliferation were assessed daily. As seen in Figure 1,

nitrite could be detected in the culture supernatant of cells alone and HA-stimulated HOS cells at day 1 and significantly increased at day 2 and 3 ($P < .05$). There was no significant difference between nitrite levels produced by cells alone and HA-stimulated cells at day 1 ($P > .05$). However, the production of nitrite by HA-stimulated cells at day 2 and 3 in the cultures was higher than that by cells alone ($P < .05$). Furthermore, gradually increased cell proliferation up to day 3 was observed in the cultures of cells alone or HA-stimulated cells ($P < .05$) (Figure 1). Proliferation of HA-stimulated cells at day 2 and 3, but not at day 1, was higher than that of cells alone ($P < .05$).

### The effects of L- and D-arginine

After the addition of 10 μM L-arginine, nitrite levels in the cultures of HA-stimulated cells were higher than those in the cultures of HA-stimulated cells without L-arginine ($P < .05$) (Figure 2). Further enhancement of nitrite production in the cultures of HA-stimulated

cells was observed after the addition of 100 μM L-arginine ($P < .05$). In sharp contrast, no significant difference in the levels of nitrite in the cultures of HA-stimulated cells with and without the presence of D-arginine could be found ($P > .05$) (Figure 2). Furthermore, HA-stimulated cell proliferation in the presence of 10 μM, but not 1 μM, L-arginine was higher than that of HA-stimulated cells alone ($P < .05$) (Figure 2). Adding 100 μM L-arginine in the cultures of HA-stimulated cells induced higher nitrite production than the cultures with 10 μM L-arginine or without L-arginine ($P < .05$). However, increased concentration of exogenous D-arginine failed to augment HA-stimulated cell proliferation ($P > .05$).

### The effect of anti-human integrin αV antibody

Increasing concentration of anti-human integrin αV antibody was paralleled with increased suppression of nitrite production by HA-stimulated cells ($P < .05$) (Figure 3). Interestingly, nitrite levels produced by cells precoated with 20 μg of anti-human integrin αV antibody and then stimulated with HA were similar to those produced by uncoated cells alone ($P > .05$). Likewise, precoating the cells with the antibody resulted in suppression of HA-stimulated cell proliferation ($P < .05$) (Figure 3). When the cells were precoated with 20 μg of anti-human integrin αV, HA-stimulated cell proliferation was not significantly different than that of uncoated cells alone ($P > .05$).

### The effects of NOS inhibitors

The results of the present study showed that increased concentration of 7-NI (nNOS inhibitor) or L-NIL (iNOS inhibitor) did not alter the levels of nitrite in the cultures of HA-stimulated HOS cells ($P > .05$) (Figure 4). However, suppression of nitrite production by HA-stimulated cells after adding L-NIO (eNOS inhibitor) could be seen in a dose-dependent fashion ($P < .05$). One hundred micromolar L-NIO did induce lower nitrite production by HA-stimulated cells than by cells alone ($P < .05$). Furthermore, there was no significant difference between HA-stimulated cell proliferation with or without the presence of increased concentration of 7-NI or L-NIL ($P > .05$) (Figure 5). However, HA-stimulated cell proliferation was suppressed by increased concentration of L-NIO ($P < .05$). The proliferation of HA-stimulated cells in the presence of 100 μM L-NIO was significantly lower than that of cells alone ($P < .05$).

### The effects of carboxy PTIO

As seen in Figure 6, carboxy PTIO suppressed both nitrite production and cell proliferation of HA-stimu-
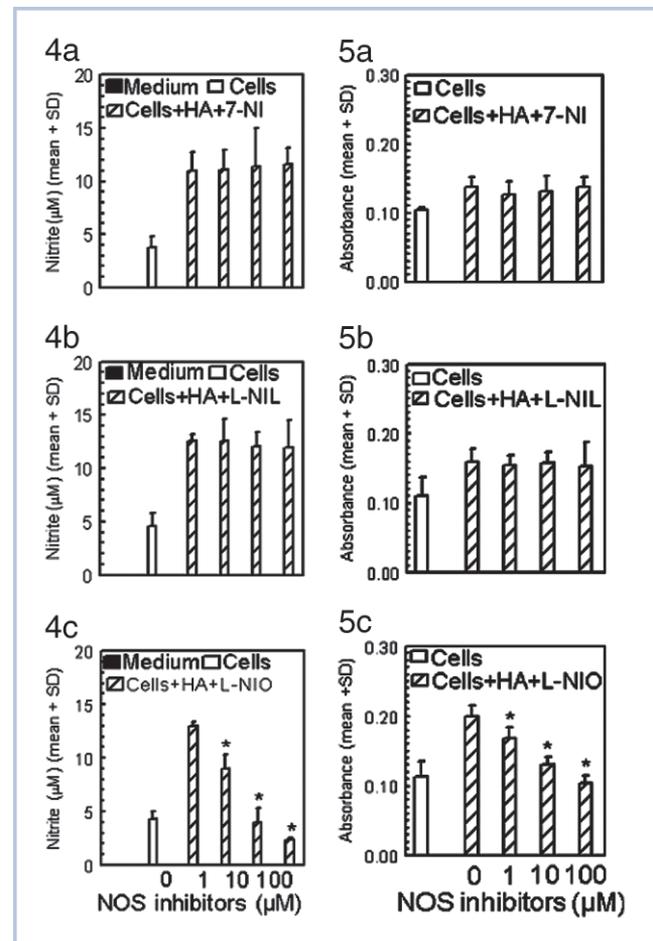


FIGURES 4 AND 5. FIGURE 4. Effect of nitric oxide synthase (NOS) isoform inhibitors on nitrite production by hydroxyapatite (HA)-stimulated human osteoblast cell line (HOS cells). HA-stimulated cell cultures were added with (a) 7-NI (nNOS inhibitor), (b) L-NIL (iNOS inhibitor), and (c) L-NIO (eNOS inhibitor). All cultures were incubated for 3 days. *Significant difference at $P < .05$. FIGURE 5. Effect of NOS isoform inhibitors on HA-stimulated HOS cell proliferation. Notes of figure are similar to those in Figure 4. *Significant difference at $P < .05$.

lated HOS cells, in a dose-dependent mechanism ($P < .05$). Both nitrite production and cell proliferation of HA-stimulated cells in the presence of 100 μM carboxy PTIO were significantly lower than those of cells alone ($P < .05$).

### DISCUSSION

The ability of HA to induce osteoblast functions is well known. For example, the proliferation of osteoblasts exposed to nanophase HA ceramics,[26] HA-coated titanium,[27] or HA cements[28] was higher than osteoblasts alone. Similarly, the present study also showed that HA augmented HOS cell proliferation. Of interest, stimulation with HA may promote the production of
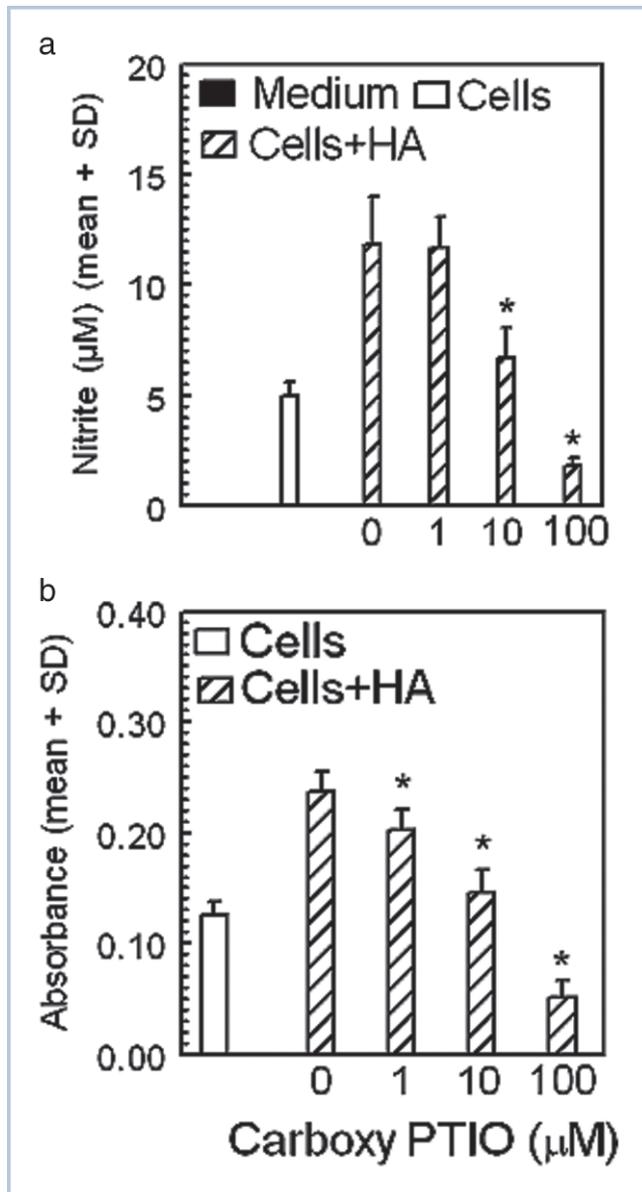
FIGURE 6. Effect of carboxy PTIO on (a) nitrite production and (b) cell proliferation of hydroxyapatite (HA)-stimulated human osteoblast cell line (HOS cells). HA-stimulated cell cultures were added with carboxy PTIO and incubated for 3 days. *Significant difference at $P < .05$.

nitrite by HOS cells. Taken together, the results of the present study demonstrated that HA may stimulate HOS cells to proliferate and produce NO, concomitantly.

L-arginine is an essential amino acid which is oxidized by NOS to generate NO.[9] The present study revealed that exogenous L-arginine up-regulated both nitrite production and proliferation of HA-stimulated HOS cells. The results of the present study are in accordance with previous findings showing that L-arginine modulates osteoblast proliferation and alkaline phosphatase activities as well as NO production.[29,30] The exact mechanism by which exogenous L-arginine elevated cell proliferation and nitrite production in the cultures of HA-stimulated HOS cells seen in the present study is unclear. Previous studies indicated that exogenous L-arginine may stimulate high levels of insulin-like growth factor-I (IGF-I) and the production of intracellular cationic amino acid transporter 2 (CAT2), an essential protein for L-arginine uptake, thereby promoting cell proliferation and NO production, respectively.[31,32] Whether or not the results of the present study might be operated by these pathways remains to be investigated further.

The results in the present study showing that both cell proliferation and nitrite production of HA-stimulated HOS cells may be mediated by integrin αV molecules are supported by the fact that osteoblast attachment and spreading on HA surfaces are initiated by osteoblast-derived integrin αV subunit.[33] Our previous report also indicated that blocking HOS cell-HA interaction with integrin αV antibody inhibited the production of PGE$_2$, regardless of the presence of exogenous NO.[23] Therefore, that the interaction between HOS cell-derived integrin αV and HA may initiate signal transduction leading to induce cell proliferation and nitrite production seen in the present study cannot be ruled out and needs to be further verified.

The present study also demonstrated that eNOS, but not nNOS and iNOS, may play a crucial role on both cell proliferation and nitrite production of HA-stimulated HOS cells. The activities of eNOS play a major role during bone formation as seen by the fact that lack of bone formation was observed in eNOS deficient mice.[34] Osteoblast proliferation and differentiation induced by estradiol[15] and fluid shear stress[17] were also dependent upon eNOS activities. Thus, one may assume that following HOS cell-HA interaction, signal transduction initiated from integrin αV molecules may activate eNOS to catalyze NO production and stimulate cell proliferation. This hypothesis remains to be further tested, however.

Carboxy PTIO is known as a NO scavenger.[35,36] Therefore, suppression of HA-stimulated HOS cell proliferation by carboxy PTIO as seen in the present study suggests that HA-stimulated HOS cell proliferation may be due to NO action in an autocrine fashion. These results are supported by the previous studies showing that NO stimulates osteoblast proliferation and differentiation.[18,19]

The extrapolation of the results of the present study in humans remains speculative. Implantation

with HA in humans stimulates rapid bone formation surrounding implanted materials.[3] Therefore, one may assume that following bone implantation with HA, NO produced by HA-stimulated osteoblast may regulate bone formation in an autocrine fashion. A recent report showing that NO seems to play a role during bone remodeling surrounding dental implants highlights the above notion.[37] Interestingly, L-arginine supplementation in humans may have benefits to enhance bone integrity and prevent bone resorptive activities of osteoclasts.[38] If so, that supplementation with this amino acid to the patients receiving HA-containing dental implants may increase the production of osteoblast-derived NO which, in turn, may enhance the efficacy of dental implantation, is of worth to be investigated further.

In conclusion, the results of the present study suggest that interaction between HA and integrin αV molecules on the surfaces of HOS cells may stimulate intracellular eNOS activation to generate NO which, in turn, modulates HOS cell proliferation.

## Acknowledgments

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