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Ionic Dissolution Products of NovaBone® Promote Osteoblastic Proliferation via Influences on the Cell Cycle

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This study investigated the effects of the ionic dissolution products of NovaBone® on osteoblastic proliferation and cell cycle regulation. MG63 osteoblast-like cells were cultured in NovaBone®-conditioned Dulbecco's Modified Eagle's Medium (DMEM) or control DMEM for 10 days. The concentration of silicon ions was significantly higher in NovaBone®-conditioned DMEM than control DMEM. MG63 cells cultured in NovaBone®-conditioned DMEM exhibited greater proliferation on days 1 and 4 than control cells. There were increased proportions of Novabone®-conditioned DMEM-cultured cells in the S and G_2/M phases, and decreased proportions in the G_0/G_1 phase on days 1 and 4 versus control cells, while no differences were observed on days 7 and 10 between the two groups. Bone morphogenic protein 2 production increased in both groups, but was significantly higher for the NovaBone®-conditioned DMEM-cultured cells on day 10 compared with the controls. In conclusion, the NovaBone® ionic dissolution products, particularly the silicon ions, promoted proliferation of MG63 osteoblast-like cells *in vitro* via influences on the cell cycle.

KEY WORDS: Novabone®; Bioactive materials; Bone loss; Osteoblasts; Cell cycle; Proliferation; MG63 osteoblast-like cells

Introduction

Major bone loss due to injuries sustained in road traffic accidents or the surgical resection of bone tumours still presents numerous challenges for orthopaedic surgeons in spite of the successful use of bone grafts to treat bone loss for several years. The use of autografts is limited and bone harvesting, usually from the patient's own iliac crest, can be responsible for serious complications such as donor site morbidity and the need for further surgery.[1] The possible transmission of microbial agents from allografted bone to the recipient is greatly reduced by using a sterilization process, but it cannot be completely avoided.[1] The possibility, therefore, of transmission of diseases such as acquired immune deficiency syndrome still presents a great challenge, as well as the occurrence of immune reactions.[1] Moreover, inconsistent revascularization of allografted bone may

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lead to its resorption and a high rate of failure in the long term, as has been reported by several authors.[2,3]

The newly emerging concept of tissue engineering provides the potential to resolve some of the problems associated with grafting human bone. Bioactive materials seem to provide a good scaffold to support osteoblasts.[4] When grafted to the defective bone areas, these bioactive scaffolds will not only stimulate osteoblast production, but as these materials are also biodegradable they should eventually be substituted by newly-produced bone.[5]

Studies have already shown that calcium oxide–silicon dioxide ($CaO–SiO_2$)-containing materials, such as bioactive glass,[6] possess excellent bioactivity.[7,8] NovaBone®, which is made from Bioglass® 45S5, has been used clinically and has achieved good results in promoting fracture healing.[9] Thus, biomaterials such as NovaBone® could act as potential substitutes for bone grafting.

Regulation of cell proliferation is dependent on the integration of signal transduction systems that are activated by external signal molecules.[10] It is well known that the cell cycle has four discrete phases – the M, S, G_1 and G_2 phases – in cell proliferation. The effects of NovaBone® on the cell cycle of osteoblasts remain unclear so, in order to provide a better understanding of the effects of NovaBone® on osteoblastic proliferation, this study was designed to investigate the effects of the ionic dissolution products of NovaBone® on the cell cycle of MG63 osteoblast-like cells *in vitro*.

Materials and methods

PREPARATION OF NOVABONE®-CONDITIONED CULTURE MEDIUM

NovaBone® was purchased from NovaBone (Jacksonville, FL, USA). It is made from Bioglass® 45S5 and was supplied in a sterilized, ready to use form. NovaBone® (1 g) was powdered and then soaked in 200 ml of Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL, Grand Island, NY, USA) at 37 °C for 2 h in static conditions to prepare the NovaBone®-conditioned DMEM. The pH of the medium was adjusted to 7.0 and then filtered through a sterilized 0.22 µm Millipore filter (Millipore, Billerica, MA, USA). The concentrations of calcium (Ca), silicon (Si) and phosphorous (P) ions released from the Novabone® into the conditioned DMEM were measured by inductively coupled plasma optical emission spectroscopy (ICP-OES).

CELL CULTURES

The MG63 osteoblast-like cells were seeded in DMEM supplemented with 10% fetal bovine serum in 25 cm^2 flasks at 37 °C in a humidified atmosphere with 5% CO_2. After reaching complete confluence, the cells were detached by digestion with a 0.25% solution of trypsin–ethylenediaminetetraacetic acid. They were then subcultured at an initial density of 2.5×10^4 cells/cm^2 and grown in control DMEM or in NovaBone®-conditioned DMEM. During subculture, the medium was replaced every 3 days. At days 1, 4, 7 and 10 samples were collected for cell proliferation and vitality investigation, cell cycle analysis and bone morphogenic protein 2 (BMP2) assay.

MTT ASSAY

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was used to determine cell vitality and proliferation. Briefly, 5×10^3 cells/well were cultured in 96-well plates (Thermo Fisher Scientific [Nunc GmbH & Co. KG], Langenselbold, Germany). At each predetermined time point the supernatant was discarded, the samples were rinsed three times with phosphate buffered saline (PBS; 0.01 M, pH 7.2 – 7.4) to eliminate non-viable cells, 100 µl/well of MTT

solution (1 mg/ml in PBS) was then added and the cells were incubated at 37 °C for 4 h to allow the formation of the formazan crystals. After incubation, the supernatant was discarded and 100 µl of dimethyl sulphoxide (DMSO) was added to each well to dissolve the formazan crystals with gentle shaking. The absorbance was then read on a micro enzyme-linked immunosorbent assay (ELISA) plate reader (Emax Science Corp., Sunnyvale, CA, USA) using a wavelength of 570 nm. The means from three individual experiments were calculated and the results reported as optical density (OD).

CELL CYCLE ANALYSIS

Cells were seeded in control DMEM or NovaBone®-conditioned DMEM, respectively, with the media changed every 3 days and maintained for up to 10 days. After 1, 4, 7 and 10 days of culture, floating dead cells and trypsinized cells were all collected together. Cells from each culture were washed with PBS (0.01 M, pH 7.2 – 7.4) and centrifuged at 1000 rpm for 5 min (KDC-40 low speed centrifuge; Keda Corp., Shanghai, China). The pellets were then resuspended in 500 µl propidium iodide (0.5 µg/ml) and ribonuclease A (100 µg/ml) and incubated at 37 °C for 30 min. Finally, the cells were run through a flow cytometer (Cytomics FC 500; Beckman Coulter, Fullerton, CA, USA) and the data were analysed by MultiCycle AV (WinCycle) for Windows (Phoenix Flow Systems, San Diego, CA, USA).

BMP2 ASSAY

After 1, 4, 7 and 10 days of cell culture, supernatants were collected into Eppendorf tubes and centrifuged to remove the particles, then stored at –80 °C until analysis. The secreted BMP2 protein in supernatants was measured using a commercial ELISA kit (Cayman, Ann Arbor, MI, USA) according to the manufacturer's instructions. Protein concentrations were normalized to the number of cells and expressed as pg/ml per 10^6 cells. All samples were independently assayed in triplicate.

STATISTICAL ANALYSIS

The data are presented as mean ± SD. Results were analysed by SPSS® version 10.0 software (SPSS Inc., Chicago, IL, USA) using a Student's *t*-test with sample numbers of at least three. A *P*-value of < 0.05 was considered to be statistically significant.

Results

ION CONCENTRATIONS IN NOVABONE®-CONDITIONED DMEM

The concentrations of Ca, P and Si ions in the NovaBone®-conditioned DMEM and control DMEM, analysed by ICP-OES, are presented in Table 1. There was a significant increase in the concentrations of Ca and Si ions after

TABLE 1:
The concentration of Ca, P and Si ions released from Novabone® into (Novabone®-conditioned Dulbecco's Modified Eagle's Medium [DMEM]) compared with control DMEM as measured by inductively-coupled plasma optical emission spectroscopy

Ion	Control DMEM (ppm)	Novabone®-conditioned DMEM (ppm)
Ca	66.84 ± 4.05	75.43 ± 3.04**
P	28.04 ± 3.58	23.76 ± 5.10
Si	0.17 ± 0.10	4.36 ± 0.45**

Values are mean ± SD.
**P < 0.01 compared with control DMEM.

1 g of NovaBone® was soaked in DMEM at 37 °C for 2 h ($P < 0.01$ compared with control DMEM), but the concentration of P ions in control DMEM and NovaBone®-conditioned DMEM were not significantly different. In particular, the concentration of Si ion increased by almost 26-fold in the NovaBone®-conditioned DMEM compared with the control DMEM.

CELL VIABILITY AND PROLIFERATION

The MTT assay demonstrated the cell viability and proliferative status of MG63 cells cultured in control DMEM and NovaBone®-conditioned DMEM over 10 days (Fig. 1). The MTT OD values of cells cultured in NovaBone®-conditioned DMEM were significantly higher than those of the control DMEM-cultured cells on days 1 and 4 ($P < 0.05$ for day 1 and $P < 0.01$ for day 4, respectively). There were no significant differences between the two groups on days 7 and 10, although the MTT OD values were higher in the NovaBone®-conditioned DMEM group. The MTT assay, therefore, showed that MG63 cells cultured in NovaBone®-conditioned DMEM proliferated more quickly than the control DMEM-cultured cells in the first 4 days but were similar during the later period of culture.

CELL PERCENTAGE IN CELL CYCLE PHASES

The percentages of MG63 cells in the S, G_0/G_1 and G_2/M phases are shown in Figs 2A – 2C). Statistically significant differences were demonstrated between the two groups in the proportion of MG63 cells in the three different phases on days 1 and 4 ($P < 0.01$ and $P < 0.001$, respectively), although no significant differences existed on days 7 and 10. Specifically, significantly more NovaBone®-conditioned DMEM-cultured MG63 cells were in the S and G_2/M phases of the cell cycle on days 1 and 4 compared with control DMEM-cultured cells (Figs 2A, 2C), whereas a significantly lower percentage of MG63 cells cultured in NovaBone®-conditioned DMED were in the G_0/G_1 phase compared with the cells cultured in control DMEM on days 1 and 4 (Fig. 2B).

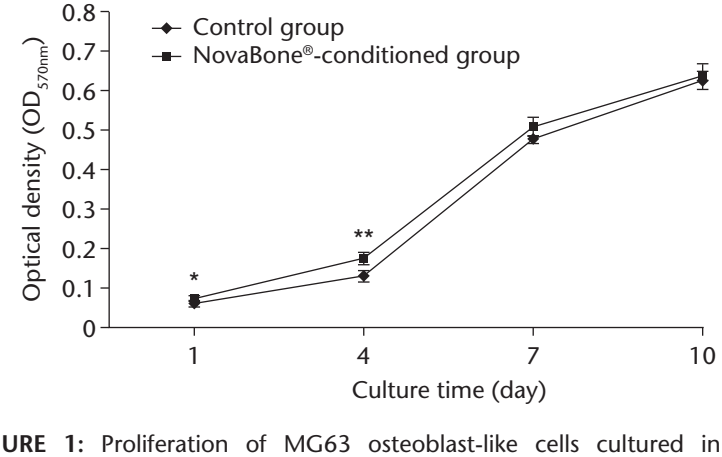

FIGURE 1: Proliferation of MG63 osteoblast-like cells cultured in Novabone®-conditioned Dulbecco's Modified Eagle's Medium (DMEM) compared with control DMEM, as measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (*$P < 0.05$, **$P < 0.01$ between the two groups)

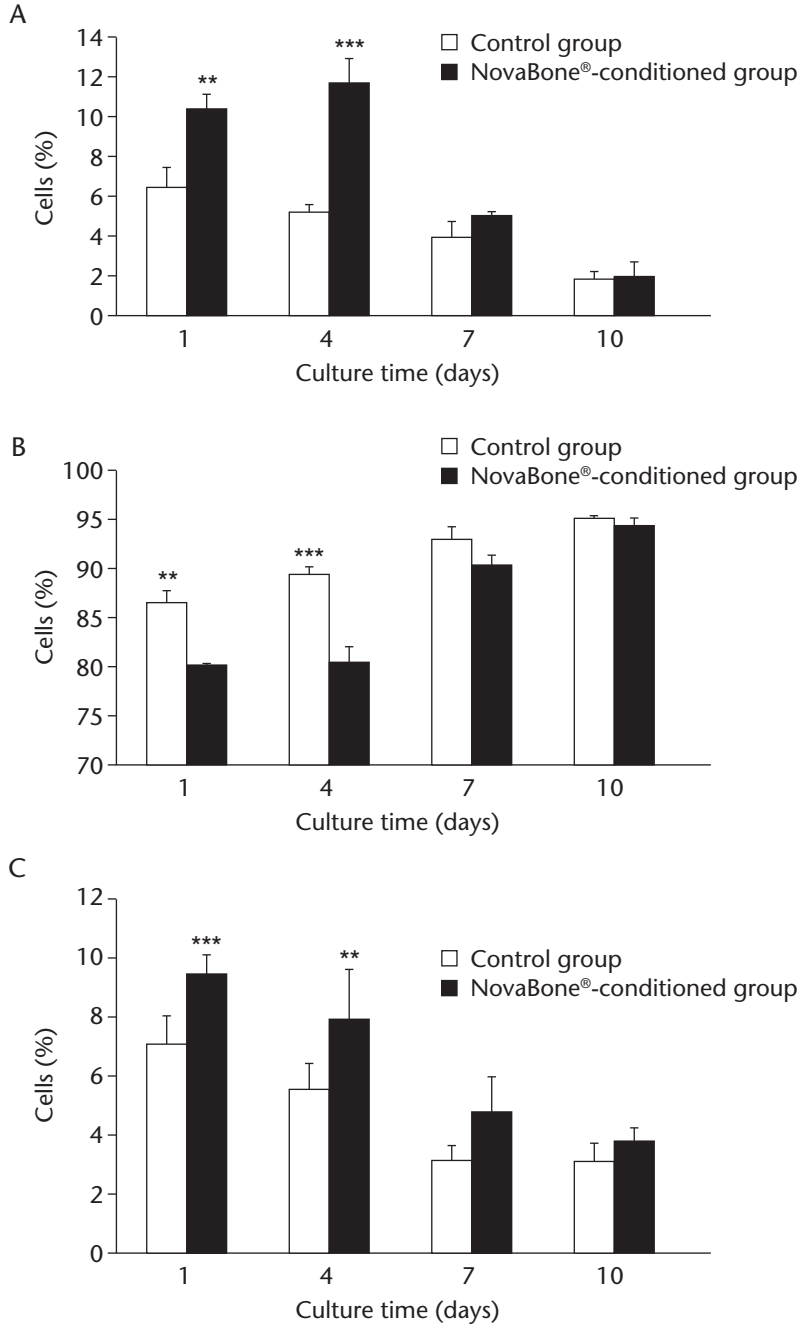

FIGURE 2: Comparison of the percentage of MG63 osteoblast-like cells at various cell cycle phases: (A) S phase; (B) G_0/G_1 phase; and (C) G_2/M phase, following culture in Novabone®-conditioned Dulbecco's Modified Eagle's Medium (DMEM) or control DMEM (**$P < 0.01$, ***$P < 0.001$ for the Novabone®-conditioned group vs control group)

BMP2 CONCENTRATION IN CULTURE MEDIA

The MG63 cells demonstrated an increase in BMP2 production over the 10-day course of the experiment in both groups (Fig. 3). Higher levels of BMP2 were consistently produced by the MG63 cells cultured in NovaBone®-conditioned DMEM compared with control DMEM-cultured cells, although the difference only reached statistical significance on day 10 ($P < 0.01$).

Discussion

It has been suggested that the primary role of biomaterials in osteoblastic metabolism is to release critical concentrations of biologically active ions at the rate needed for cell differentiation.[11] Xynos *et al.*[12] suggested that the presence of the substrate might not necessarily be required to provide the mitogenic stimulus, as the ionic products of bioglass dissolution could also provide an adequate stimulus for cell proliferation. Sun *et al.*[13] hypothesized that the ionic products of bioglass dissolution, especially Si ions, shortened the human osteoblast growth cycle and stimulated osteoblast proliferation.

The result of the MTT assay in the present study showed that the rate of proliferation of MG63 cells cultured in Novabone®-conditioned DMEM was higher than that of the control DMEM-cultured cells on days 1 and 4. The rate of proliferation remained higher on days 7 and 10 in the NovaBone®-conditioned DMEM-cultured cells, although the differences were not statistically significant. These results might indicate that the ionic dissolution products of NovaBone® promote early proliferation of MG63 cells.

In order for new bone to form, it is essential for osteoprogenitor cells to undergo mitosis. In virtually all cells, the cell cycle consists of four discrete phases: the M phase or mitosis; the DNA synthesis or S phase; and the gap phases between these two phases, the G_1 phase between M and S, and the G_2 phase between S and M. The M and S phases are usually not interrupted, in contrast to the gap phases which are used by cells to increase cell mass, but also to control various processes that occur in the preceding phase. Every new cell cycle begins after a cell has completed a preceding round of mitosis. If the local chemical environment is suitable

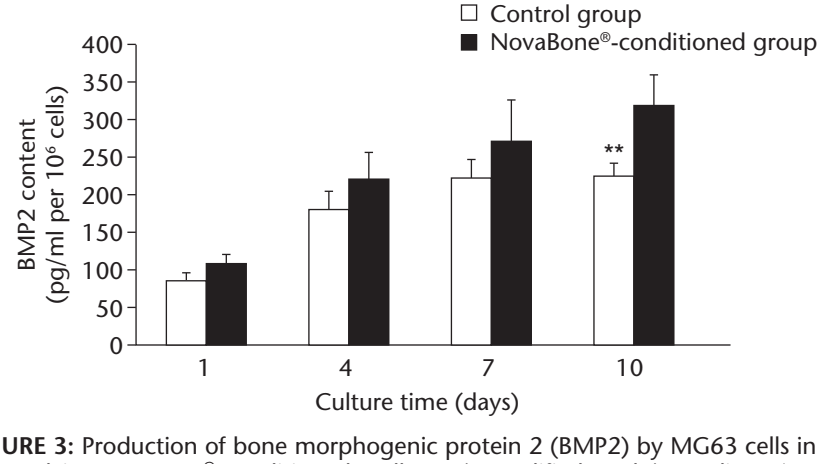

FIGURE 3: Production of bone morphogenic protein 2 (BMP2) by MG63 cells in cells cultured in NovaBone®-conditioned Dulbecco's Modified Eagle's Medium (DMEM) compared with control DMEM (**$P < 0.01$ between the two groups)

and following a critical period of growth in the G_1 phase, the cell enters the S phase when DNA synthesis begins, leading to division of the mother cell.

The present study investigated the cell cycle status of MG63 cells cultured in control DMEM and NovaBone®-conditioned DMEM by flow cytometry, measured the ionic dissolution products released from NovaBone® and the promotion of osteoblast proliferation as shown by the MTT assay. When cultured in NovaBone®-conditioned DMEM, a higher proportion of MG63 cells were shown to be in the S and G_2/M phases, particularly on days 1 and 4, while the proportion of cells in the G_0/G_1 phase decreased accordingly. When the results of the MTT assay and the cell cycle analysis were compared, it was found that the MTT OD values were in accordance with the cell cycle changes. These results indicated that the ionic dissolution products of NovaBone® were able to promote early osteoblastic proliferation by increasing the proportion of MG63 cells in the S and G_2/M phases and decreasing the proportions of cells in the G_0/G_1 phase of the cell cycle.

It is now well known that osteoprogenitor cells must receive the correct chemical stimuli from their local environment in order to enter the active phases of the cell cycle. In accordance with previous studies,[14 – 16] a significant, almost 26-fold increase in the concentration of Si ions in NovaBone®-conditioned DMEM was observed in the present study compared with control DMEM ($P < 0.01$), which was also much higher than the normal Si concentration in plasma.[17] There was no significant difference in the concentration of P ions between the two media and, although the concentration of Ca in the NovaBone®-conditioned DMEM was significantly higher than that of the control DMEM, the literature has indicated that Ca ions may not be important in influencing osteoblastic metabolism.[18] Based on this we suggest that the increased proliferation and changes in the osteoblastic cell cycle observed in the present study resulted from the higher Si ion concentration following NovaBone® conditioning of DMEM. Hence NovaBone®, a product made from Bioglass® 45S5, appears to promote osteoblastic proliferation via its ionic dissolution products, mainly Si, and its influences on the osteoblastic cell cycle.

Osteoblastic metabolism is a complicated process regulated by various mediators such as cytokines and growth factors.[19,20] An important question to consider is whether the ionic products released by NovaBone® act directly or via the induction of other cytokines to regulate osteoblastic metabolism. Several previous studies have explored the possible molecular mechanisms of biomaterials on osteoblastic metabolism. For example, cDNA microarray analysis by Xynos *et al.*[12] showed that the expression of a potent osteoblast mitogenic growth factor, insulin-like growth factor II, was increased to 300% by exposure of osteoblasts to the bioactive glass stimuli. Other studies also indicated that Si-based biomaterials stimulated transforming growth factor-β1 (TGF-β1) and BMP2 production.[21,22] Such results suggest that biomaterials might be able to achieve their biological effects via production of different cytokines, which then produce autocrine/paracrine effects in osteoblasts.

The BMPs belong to the TGF-β family and are important regulators of bone regeneration and repair. The present study examined BMP2 production in the culture medium since it is uniquely required for fracture healing.[23] It was found that BMP2 protein levels were higher in cells cultured in NovaBone®-conditioned DMEM than in control cultures

and that this reached statistical significance on day 10. These results indicated that NovaBone®-conditioned DMEM was capable of inducing BMP2 production in MG63 cells, which may be due to the presence of higher levels of Si ions in the NovaBone®-conditioned DMEM. As the Si concentration in NovaBone®-conditioned DMEM in the present study was not as high as in some previous studies,[15] it is not clear whether a higher concentration of Si ions would lead to a higher production of BMP2. We believe that using NovaBone®-conditioned DMEM with different concentrations of ionic dissolution products would help to determine the effects of NovaBone® on osteoblastic metabolism, as well as their effects on the production of cytokines such as BMP2.

In conclusion, the Si concentration was markedly higher in the NovaBone®-conditioned DMEM than in the control DMEM. The NovaBone®-conditioned medium increased the early proliferation of MG63 osteoblast-like cells, which was in accordance with the increased proportions of cells observed in the S and G_2/M phases of the cell cycle, and the concomitant decreased proportion of cells in the G_0/G_1 phase. Furthermore, BMP2 protein production was increased in both groups of cells over the 10-day culture period, with cells cultured in NovaBone®-conditioned DMEM producing significantly higher levels by day 10. These results, therefore, suggest that the ionic products released from NovaBone®, in particular the Si ions, have beneficial effects on the proliferation of MG63 osteoblast-like cells via influences on the cell cycle, and that they also upregulate BMP2 protein production.

Conflicts of interest

The authors had no conflicts of interest to declare in relation to this article.

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