The effect of CaO-SiO$_2$-P$_2$O$_5$-B$_2$O$_3$ Glass-Ceramics (BGS-7) on human mesenchymal stem cells differentiation

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ABSTRACT INTRODUCTION:
The bioactive glass-ceramic has outstanding mechanical strength and excellent bioactivity compared to hydroxyapatite and has been used clinically including Cerabone-AW. The authors of this study will compare between the clinically used hydroxyapatite, which possesses previously established bioactivity and osteoconductivity, and plate (coverslip for the cell culture media) and BGS-7, which is CaO-SiO$_2$-P$_2$O$_5$-B$_2$O$_3$ glass-ceramic, through in vitro study in order to produce BGS-7 and to test its bioactivity and possibility as bone graft extender.

METHODS:
CaO-SiO$_2$-P$_2$O$_5$-B$_2$O$_3$ based glass-ceramic, named BGS-7, was prepared by the following method: A powder mixture of the nominal composition CaO 41.8, SiO$_2$ 35.8, P$_2$O$_5$ 13.9, B$_2$O$_3$ 0.5, CaF$_2$ 2.0, MgO 6.0 (wt%) was prepared and this mixture was melted at 1550°C for 2 hours in a furnace to form a glass plate. The glass powder was granulated with 10% deionized water and isostatically pressed into pellets that are 15 mm in diameter and 3 mm thick at 1000kg/cm$^2$. The pellets were sintered for 2 hours at 1050°C. The pellets were machined to be disks of 12mm in diameter and 2mm in thickness. The machined disks were rinsed in ultrasonic cleaner and dried at 100°C. The disks were sterilized by EO-gas.

As a control, EO-gas sterilized hydroxyapatite disks of same dimensions were prepared by medical grade hydroxyapatite and also showed a porosity of less than 1%. Also, coverslip (BD Biosciences, NJ, USA), a cell culture medium, was used as a positive control.

Cell Culture and Materials
Human mesenchymal stem cells (Combrex, Cat. No. PT-2501) that have been separately cultured from normal human bone marrow were purchased and used in the experiment, and used MSCGM Kit (Combrex, PT-3001, 3238) as the culture media. Also, Osteogenic Differentiation Kit (Combrex, PT-3002) was used as the differentiation media.

Cell Proliferation by MTS assay
Two test samples of plate (coverslip), HA, and BGS-7 each are inserted into 24-well plate, and hMSC(P3) (total cell number: 2X10$^4$ cells/well) were seeded (1X10$^3$ cells/well and 4X10$^3$ cells/well were seeded. After culturing these plates for one, four, seven and fifteen day period, MTS solution of Celltiter 96 Aqueous Assay (Promega Corp., WI, USA) was added at the concentration 200uL/mL. Finally, the measurement was taken at the wavelength of 490nm using ELISA Reader after 4 hours of culturing at 37°C.

ALP Assay
ALP and MTS values were measured by transplanting hMSC in the amount of 2X10$^3$ cells/well and 4X10$^3$ cells/well on plate (coverslip), HA, and BGS-7, and the ALP value was measured after culturing two of each hMSC(P3) for the period of one, two, three and four weeks.

Initial Cell Attachment Test
For the evaluation of cell attachment, each sample (plate, HA, BGS-7) was autoclaved for 30 minutes at 121°C, then hMSC-01(P4) were seeded (1X10$^3$cells/mL). And then, four spots of each stained sample were photographed using a fluorescent microscope and the number of attached cells was measured.

Calcium Assay
After culturing on plate, HA and BGS-7 using hMSC for 4 weeks, 300ul 0.5% HeLa was added to the eluted solution and extracted Ca$^{2+}$ from the cells by shaking the tubes on an orbital shaker for 3-24 hours at 4°C. The sample tube was centrifuged and the supernatant extracted calcium was collected without disrupting the pellet. The pellets were transferred to a new tube and incubated for 1 minute at room temperature. And then, the calcium level was analyzed by measuring at OD 650.

RT-PCR
To verify osteoblastic differentiation, we investigated the expression of mRNA of ALP, osteocalcin, osteopontin, runx2 by RT-PCR after 1 and 3 weeks in culture. We also performed RT-PCR for GAPDH independently as an internal control.

DISCUSSION:
Western Blot
Western blotting and ELISA were used to evaluate celluar osteocalcin and runx2 production after 3 weeks in culture.

Statistical Analysis
Statistical analysis was performed using SPSS statistical package and the values were considered significant at p<0.05. Data were compared by One-Way Analysis of Variance (ANOVA) using Kruskal-Wallis followed by Fisher’s Exact Test.

RESULTS SECTION:

ALP assay
In the 15-day result, the ALP value for the group that transplanted on BGS-7 was higher than the other two test samples for the entire period. Observing the continuous change in the ALP values on three types of test samples, BGS-7 was significantly higher compared to plate and HA (p=0.0016 and 0.0042 respectively) during Week 1, and BGS-7 was again higher compared to plate and HA (p=0.0111 and 0.0133 respectively) during Week 2 as well. Also during Week 3, the ALP value of BGS-7 was statistically significantly higher than the values of plate and HA (p=0.01 and 0.0054 respectively), and BGS-7 was again significantly higher than plate and HA (p=0.0034 and 0.0003 respectively) during Week 4. The ALP value of plate was higher during Week 2, 3 and 4 than Week 1 but there was no statistical significance, and the ALP value of HA was statistically significantly higher (p=0.0127) during Week 2 than Week 1 but showed no difference during the remaining weeks. The ALP value of BGS-7 also showed no significant difference from one week to another. The amount of total protein for BGS-7 was significantly higher than plate during week 1 through 4, and was even higher than HA during week 1. The ratio of ALP value on total protein didn’t present any significant difference among the three groups.

MTS assay
In the cell proliferation assay measured using the hMSC, the MTS value measured at 2X10$^4$ and 4X10$^4$ cells showed no difference between the group transplanted on BGS-7 and the other two groups during the initial stage of transplant, but on Day 15, showed a lower value compared to the group transplanted on HA. The ALP value of HA was statistically significantly higher than the group transplanted on HA and showed a similar pattern in the remaining weeks. The ALP value of BGS-7 also showed no significant difference from one week to another. The amount of total protein for BGS-7 was significantly higher than plate during week 1 through 4, and even higher than HA during week 1. The ratio of ALP value on total protein didn’t present any significant difference among the three groups.

Initial Cell Attachment Test
According to the Initial cell attachment, the cell count on BGS-7 (p=0.0058) after 6 hours was significantly greater than the cell count on plate, and the cell count on BGS-7 (p=0.0419) after 18 hours was significantly greater than the cell count on HA.

Calcium Assay
The calcium level was higher in both HA and BGS-7 than plate, and was especially high in BGS-7.

RT-PCR
At 1 week after implantation of mesenchymal stem cells with HA, BGS-7 and plate, the relative gene expression of ALP and osteopontin in BGS-7 was stronger than those of HA and plate. The gene expression of osteocalcin and runx2 at 3 weeks after implantation of mesenchymal stem cells in BGS-7 was also stronger than those of HA and plate. The gene expression of APL, osteopontin, osteocalcin and runx2 in HA was stronger than those of plate.

Western Blot
At 3 weeks after implantation of mesenchymal stem cells, the expression of osteocalcin and runx2 in BGS-7 was stronger than those of HA and plate and the expression of osteocalcin and runx2 in HA was also stronger than those of plate.

DISCUSSION: