Effects of surface conditioning and ionic products of a novel bioactive glass-ceramic on *in vitro* osteogenic events

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Statement of Purpose: It has been demonstrated that a fully crystalline glass-ceramic in the Na_2O-CaO-SiO_2-P_2O_5 system (Biosilicate®) is highly bioactive and supports enhanced bone-like matrix formation compared to its parent glass and to Bioglass® 45S5 *in vitro*.[1] In addition, during the early interactions of osteogenic cells with such bioactive surfaces, changes in actin cytoskeleton organization have been detected by means of fluorescence labeling. Because in the *in vivo* environment biomaterial surfaces are immediately exposed to a series of serum and extracellular matrix proteins upon implantation, it would be important to address the impact of protein adsorption on osteogenic cell response to Biosilicate®. The present study aimed to evaluate the effects of Biosilicate® surface conditioning with supplemented culture medium and of its ionic dissolution products on early osteogenic events *in vitro*.

Methods: Biosilicate® discs, 2.5 mm thick and 12 mm in diameter, were produced by the Vitreous Materials Laboratory (Federal University of São Carlos, Brazil), stored in isopropyl alcohol, and sterilized by dry heat. Osteogenic cells were obtained by enzymatic digestion of newborn rat calvarial bone and plated on Biosilicate® discs conditioned with supplemented culture medium (Bio+SM) or with serum-free medium (Bio+M) for 3 days, on glass coverslips (Fisher Scientific) in the presence of the supplemented culture medium used for the Biosilicate® surface conditioning (Glass+SM), and on glass coverslips that were kept at a distance of 1 mm from Biosilicate® discs using a ring-shaped Teflon® device (Glass+Bio). Cells grown on Biosilicate® discs (Bio) and on glass coverslips (Glass) were used as controls. Cell viability/proliferation was evaluated by the MTT assay at days 1, 3, and 7 and data were expressed as absorbance. At the same time points, cells were fixed in 4% paraformaldehyde in phosphate buffer for 10 min and processed for immunofluorescence to detect the cytoskeletal proteins vimentin, tubulin, and vinculin, and the noncollagenous protein bone sialoprotein as an early marker of osteoblast differentiation. Phalloidin + Alexa Fluor and DAPI DNA stain were used to detect actin cytoskeleton and cell nuclei, respectively. Some samples were also evaluated by scanning electron microscopy (SEM). At 3 days, the proportion of cycling cells was determined by means of Ki-67 immunolabeling, which is strictly associated with proliferating cells. Total cell number was determined by hemacytometer cell counts at day 7. Where appropriate, comparisons were carried out using the non-parametric Kruskal Wallis test, for independent samples ($p \leq 0.05$).

Results: MTT assay revealed higher absorbance values for cultures grown on Glass, Bio+SM, and Bio+M at days 1 and 3, while at day 7 the highest values were only detected for Bio+SM and Bio+M. Although the proliferation rate was greater for Glass and Glass+SM compared with all other groups, at day 7 total cell number was significantly higher for Bio+SM. Data (mean ± SD, n=5) are summarized in Table 1.

Table 1. Cell viability (MTT), proliferation rate (Ki-67-positive cells) and total cell number in osteogenic cultures grown on different Biosilicate® and glass coverslips groups for periods of up to 7 days

	MTT assay (nm)			Ki-67 (%)	cell number ($x10^4$)
	Day 1	Day 3	Day 7	Day 3	Day 7
Bio	0.05±0.01	0.08±0.01	0.08±0.01	64.0±9.4	13.8±3.1
Bio+SM	0.08±0.01	0.14±0.0	0.23±0.01	66.8±13.3	22.2±3.2
Bio+M	0.08±0.01	0.16±0.01	0.22±0.01	64.6±7.1	18.2±3.3
Glass	0.08±0.01	0.16±0.01	0.17±0.01	86.3±4.8	9.4±2.5
Glass+Bio	0.04±0.01	0.05±0.0	0.1±0.02	72.5±6.5	6.7±1.9
Glass+SM	0.05±0.01	0.1±0.01	0.11±0.03	76.4±13.3	9.6±2.5

At days 1 and 3, epifluorescence showed that cells grown on Biosilicate® exhibited changes in the labeling pattern of all cytoskeleton proteins studied compared with those grown on glass coverslips. Actin labeling was significantly reduced on Biosilicate®, with stress fibers only focally detected. While cells on glass coverslips were immunoreactive for vinculin (perinuclearly and in focal adhesion sites), such protein was rarely detected in the peripheral cytoplasm on Biosilicate®. The typical filamentous pattern of vimentin and tubulin labeling was only conspicuously detected for the glass coverslips groups. The presence of roundish areas with no cytoskeleton labeling in cells grown on Biosilicate® correlated with spherical-shaped aggregates associated with the outer cell membrane as detected by SEM. Importantly, changes in cytoskeleton labeling appeared to be attenuated as a result of Biosilicate® surface conditioning. At day 7, BSP was localized in areas of initial cell multilayering on Biosilicate® surfaces; BSP labeling was rarely detected in cells grown on coverslips.

Conclusions: Biosilicate® conditioning with supplemented culture medium supports a significant increase in cell viability and in osteogenic cell number compared to the pristine material. While changes in fluorescence labeling of cytoskeletal proteins are noticed for Biosilicate®, such altered patterns may be attenuated by surface conditioning.

References: 1. Moura et al. J Biomed Mater Res A 2007;82:545-557.

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