Biological Behavior of Mineral Trioxide Aggregate and a Calcium Phosphate-Based Compound (A Comparative *in vitro* Study)

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Abstract

The aim of the present study was to compare the biological behavior of MTA and a calcium phosphate based compound (Bonitmatrix®) on human pulp-derived fibroblast-like cells extirpated from freshly-extracted, sound lower third molars. The cells were exposed to 5-days extracts of the materials for testing cell viability and number, and were put in direct contact with discs of the materials for assessment of the cell morphology after 5days. Cell viability was assessed using the MTT assay. The crystal violet assay was used to assess cell number. All assays were made in triplicate and repeated to assure reproducibility. The cell morphology was examined using the inverted, phase-contrast microscope. Cells incubated without materials extracts served as control. Results showed that there was no statistically significant difference between the two materials as to their effect on cell viability and cell number ($p>0.05$). Three zones could be identified around MTA discs:

zone of lysis, cell-free zone and zone of normal cell morphology. Bonitmatrix discs tended to fragment and the cells showed wider intercellular spaces than the control and the zone of normal cell morphology of the MTA group. It could be concluded that both materials showed favorable *in vitro* biological behavior and Bonitmatrix might have a potential as a pulp capping agent.

Introduction:

Mineral trioxide aggregate (MTA) is a relatively new material that has a wide range of applications, including pulp capping (1, 2), root-end filling (3), and root perforation repair (4). Direct pulp-capping experiments have reported that MTA induced the formation of dentin bridges with little or no inflammation (1, 2). MTA also has the ability to stimulate cytokine release from bone cells indicating that it actively promotes hard tissue formation (5).

MTA has been examined on several cell lines of pulpal origin. It induced proliferation rather than apoptosis of mouse odontoblast-like cells (MDPC-23) and undifferentiated pulp cells (6). The elution components of MTA produced higher proliferation of human dental pulp cells in comparison to Dycal (7). Compared to SuperEBA and Vitrebond, ProRoot MTA and MTA Angelus exerted milder suppression of the cellular mitochondrial activity of rat pulp cells (RPC-C2A) and human lung fibroblasts (MRC-5) (8).

The synthetic calcium phosphate biomaterials, hydroxyapatite and tricalcium phosphate ceramic, have been extensively employed in bone repair because of their biocompatibility and their ability to promote new bone formation (9) and their usage was extended as pulp-capping agents (10-12). Reparative dentine bridge formation was always observed

without initial necrosis except when bacterial infection occurred. These *in vivo* investigations showed that new hard tissue was deposited directly on the calcium phosphate biomaterial, in contrast with the characteristic necrotic area formed under calcium hydroxide (13). The effects of such synthetic materials on pulpal cell metabolism, however, have not been extensively investigated (14).

Bonitmatrix® consists of a mixture of the two calcium phosphates hydroxyaptite (HA) and β-tricalcium phosphate (β -TCP) in the clinically proven ratio of 60/40. In contrast to conventional HA and β-TCP-based ceramics and bioglasses, Bonitmatrix® is manufactured in a sol-gel-procedure where nanocrystalline calcium phosphates are embedded in a biologically-active silicon dioxide-matrix. It is well-known that silicon has regulating and stimulating influence on the natural mineralization process (15, 16). An *in vitro* study showed that Bonitmatrix® is particularly effective in supporting the growth and the functional performance of developing bone cells (osteoblasts and osteoclasts) (17), however, it has not been examined on pulp cells. Thus, the aim of the present study was to assess the *in vitro* biological effects of Bonitmatrix in comparison to MTA regarding cell viability, cell number and cell morphology.

Materials and Methods

Human pulp cell culture:

Freshly-extracted, impacted, lower, third molars were obtained from 23- and 24-years-aged female patients. The teeth were grooved using a diamond stone at nearly the level of cement-enamel junction and split to obtain the pulp tissue under sterile conditions. The extirpated pulp was then minced into small pieces nearly 1 mm^3 in size using sterile scissors

and surgical blades and washed twice in culture medium. The minced pieces were then placed in sterile, labeled, 15-ml cell culture tubes containing 1 % trypsin (BioWhittaker^{TM} Europe, Cambrex Co., Belgium) and kept in the incubator at 37˚C in a humidified atmosphere of 5% CO_2-95% air for 10 min. The large particles in each tube were discarded while the culture media containing the detached cells of each tube were then centrifuged at 3000 xg for 10 min then were resuspended in complete culture medium containing culture medium (α-MEM with L-glutamine, Gibco, Invitrogen Life technologies, USA) supplemented with 10% fetal bovine serum (Gibco, Invitrogen Life technologies, USA), antibiotics (penicillin G, 100 U/ml; streptomycin, 100µg/ml), and an antimycotic agent (Fungizone, 0.25 µg/ml) in sterile 25-cm^2, polystyrene, filter-cap cell culture flasks labeled by cell type and date and incubated in the previously mentioned conditions until fibroblast-like cells grew. Monolayers of cells grown in the flasks were daily observed using the inverted phase-contrast microscope for adhesion and multiplication. The culture medium was changed as needed (every 3 to 4 days). Cells were then continuously passed at a ratio of 1:3 when confluent. Cells were subcultured twice per week. Cell cultures from the passages between the 3rd to 7th passages were used for the experiments. All previous procedures were conducted under aseptic conditions in an air-filtered laminar-flow cabinet using sterile instruments.

Preparation of the materials' extracts:

Mineral trioxide aggregate was mixed following the manufacturer's instructions with a final water-to-powder ratio of approximately 0.3. The Bonitmatrix® was mixed with sterile saline to create a putty-like mixture. The freshly-mixed materials were placed at the bottom of flat-

bottomed wells in 24-well culture plates to achieve a thickness of approximately 1 to 2 mm. The surface area of the test materials exposed was approximately 190 mm^2. Extracts of each of both materials were prepared as follows: 600 µl of culture medium was placed in each well containing the freshly-mixed materials (ratio of surface area exposed to volume of extract vehicle = 190 mm^2/600 µl = 316 mm^2/ml). The plates were incubated at 37˚C and 100% relative humidity. Extraction media of both materials were collected after 5days with a change of medium during this period. The extraction media of the wells containing the same material were then collected into single sterile syringes, centrifuged to remove any particulate matter, sterile-filtered using 0.22 µm filters, preserved in sterile, sealed 15 ml tubes labeled with the type of material and the date and stored at -20˚C until required. Before testing, the media samples were brought to room temperature. Untreated controls were cells cultured with media free of the materials' components.

The MTT viability assay:

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) colorimetric assay was used for the cell viability assessment of the tested materials. Approximately 2 $x10^4$ cells were seeded per well in 96-well flat-bottomed microtitre plates in 100 µl of complete culture medium and were incubated for 24 h in the humidified CO_2 incubator at 37˚C to allow for adhesion of the cells to the plate bottom. Then, culture medium from each well was replaced with 100 µl of the extraction medium of each test material in triplicate. Cells incubated with complete culture medium without materials' extracts representing 100% viability were included as a negative control. The microtitre plate was then

incubated for another 24 h in the incubator. The extracts and the media were then removed from the test and control wells, replaced by MTT solution (Sigma, St.Louis, MO, USA) and incubated for 4 h at 37˚C, then sodium dodethyl sulfate (SDS) was added. The spectrophotometric absorbance of the reduced MTT was then measured in a dual-beam, microtitre plate reader at a wavelength of 595nm obtaining an optical density (OD) value for each well. Original optical density (OD) values of the test cultures exposed to the extracts of the materials were related to values of untreated cell culture and expressed as the *cell viability ratio* which is the ratio of the optical density obtained for each well to the optical density obtained for the control after the subtraction of the background value. The assay was made in triplicate and repeated at least thrice to ensure reproducibility.

The crystal violet proliferation assay:

The crystal violet (CV) assay is based on the observation that, in fixed cells, crystal violet binds to nuclear proteins and the amount of the bound chemical correlates in a linear relationship to the cell number in culture.

At approximately 60 to 70% confluence, the cells were seeded at a number of approximately 2 x 10^4 in each well of a 96-well plate. After 24h, the culture medium was aspirated and 100 µl of 5-days extracts for each material, were placed on the cells in each well and incubated for 72h. Complete culture medium only was placed on other wells to serve as controls. The cells were then fixed by the addition of methanol then the fixation solution was removed by inverting and tapping. The cells were stained by solution of crystal violet and incubated for 30 minutes. Unbound dye was then removed by extensive washing with double-

distilled water and the plates were allowed to air dry. The procedure resulted in cells that exhibited stained nuclei, whereas the cytoplasm remained clear. Finally the bound dye was solubilized in methanol reading absorbance using an ELISA reader device at test wavelength of 595 nm. Optical density as measured in the CV assay was used as a surrogate for cell number. Original optical density (OD) values of the test cultures exposed to the extracts of the materials were related to values of untreated cell cultures and were expressed as the *cell number ratio* which is the ratio of the optical density obtained for each well of test culture to the optical density obtained for the control after subtraction of the background values. Three replicates were assessed per extract or control. The experiment was repeated at least twice.

Cell morphology:

The morphology of the human, pulp-derived, fibroblast-like cells was examined. The cells were grown in 25 cm^3 flasks according to the methods previously mentioned. Circular discs of the tested materials, 3 mm in diameter and 1mm in thickness, were prepared in a plastic template to be incubated with the cells. At approximately 60 to 70% confluence of cells, the cells were trypsinized, counted using a heamocytometer then seeded at a density of 1×10^4 in 3 ml in each well of a 6-well plate, 35 mm in inner diameter, which contained one disc of either of the tested materials; three wells were assigned for each material. Wells without discs of the tested materials were used as controls. The cell culture plates were kept in an incubator at 37˚C with 5% CO_2 in 95% air. Cells were daily observed to check for vitality and growth by observation under an inverted, phase-contrast microscope. After 5days, the cells were photographed.

Statistical analysis:

Student's *t*-test was used for comparing the means of the cell viability and the cell number ratios for the two experimental groups. The data were analyzed by the SPSS version 16.0 statistics software package. A significance level of 0.05 was used throughout all statistical tests within this study.

Results

The MTT viability assay:

The recorded mean and standard deviation values of the cell viability ratio for MTA and Bonitmatrix are shown in table (1) and represented in figure (1); each column in the figure represents the mean of twelve readings. The mean and standard deviation values of the cell viability ratio in the MTA group were 1.59 (0.29), while, in the Bonitmatrix group, they were 2.21 (1.07). Student's *t*-test showed no statistically significant difference between the tested materials (p=0.07, p> 0.05).

The crystal violet proliferation assay:

The recorded mean and standard deviation values of the cell number ratio for MTA and Bonitmatrix are shown in table (2) and represented in figure (2); each column in the figure represents the mean of nine readings. The mean and standard deviation values of the cell number ratio in the MTA group were 1.14 (0.39), while, in the Bonitmatrix group, they were 1.98 (1.43). Student's t-test showed that there was no statistically significant difference between the two materials (p=0.11, p> 0.05).

Cell morphology:

The cell morphology and cell culture architecture of human, pulp-derived, fibroblast-like cells were examined against discs of the tested

materials (MTA and Bonitmatrix) in comparison to controls after 5 days in culture.

In the ***control*** group, the cells showed appeared spindle-shaped, adherent to the bottom of the well plate giving the signs of normal cell morphology of fibroblast-like cells. Cells were subconfluent with some intercellular spaces (Fig. 3).

In the ***MTA*** group, the disc remained intact throughout the test period. Three zones could be identified around the disc, namely: zone of lysis, just adjacent to the disc where cells were lysed and culture proteins denatured; a cell-free zone, devoid of cells; and a zone of normal cell growth, with spindle-shaped cells, adherent to the bottom of the well. Cells were subconfluent with wider intercellular spaces than in the control group (Fig.4).

In the ***Bonitmatrix*** group, the disc was fragmented into particles. Cells appeared spindle-shaped, fibroblast-like and adherent to the well as well as the material particles. The intercellular spaces were much wider than in the MTA and control groups (Fig.5).

Discussion

The protective and reparative function of dental pulp is well known. Odontoblasts are responsible for this function, and produce physiological secondary dentin. When odontoblasts are irreversibly damaged, they are replaced by a second generation of newly differentiated odontoblasts that give rise to a reparative dentin matrix under the correct conditions e.g. a suitable pulp capping material. Mineral trioxide aggregate has proved to be effective pulp capping due to its biocompatibility and its ability to induce hard-tissue formation. Mineral trioxide aggregate has proved to be effective in many dental

applications including pulp capping due to its biocompatibility and its ability to induce hard-tissue formation. Calcium phosphate compounds have been previously used as pulp capping agents and enhanced hard-tissue formation. Bonitmatrix is a recently-introduced calcium phosphate-based material that was found to enhance bone regeneration. Thus, it was of interest to compare its biological behavior with MTA *in vitro* on human, pulp-derived, fibroblast-like cells.

Primary cell lines provide good simulation of the *in vivo* situation, thus being indicated for specific scientific problems as recommended by ISO standards (18). Although permanent cell lines have the advantage of providing phenotypically-consistent and stable populations, large enough for biochemical analysis, yet, it is not always possible to obtain results from them as immortalization can result in altered cellular behavior (19).

The ratio of the surface area of the test material to the volume of the extraction vehicle between 50 and 600mm^2/ml is in accordance with ISO standard 10993-5:4.2.3.5. In the present study, the surface area-to-volume ratio used for extract preparation was 316mm^2/ml in order to conform to the ISO standards allowing for comparison of results among different studies (20).

Bonitmatrix showed higher mean cell viability ratio than MTA although without statistical significance (Table 1). The mean cell viability ratio of MTA and Bonitmatrix appeared to be between one and half to two folds the control respectively. A previous study recorded higher mean cell viability for 3-days extracts of white MTA than the control with human osteosarcoma cells (21).This is partially in accordance with another study (22) where, although the cell viability of ProRoot was 45% of the

control on human ECV 304 endothelial cells after 24 h of direct contact with the material, cell viability increased after 48 h and 72h where it reached the control level. The differences could be attributed to differences in the study designs.

As for the cell number, results showed that the mean cell number ratio was 1.14 for MTA and 1.98 for Bonitmatrix, however, with no statistically significant difference. In the spite of the relative higher concentration of the extracts of the present study, the results are partially in accordance with previous studies (21, 23) where MTA did not cause enhancement in cell proliferation at the 3-days interval in comparison to the control. This also agreed with another study (24) where cell number of L929 fibroblasts after a 3day incubation with freshly –mixed ProRoot MTA was significantly lower than the control in spite of the diluted concentration of material extracts used compared to this study; this was supported by another study on rat bone marrow cells (25). This is, however, in contrast to previous studies (6, 7, 23) where it was reported that some enhancement of cell proliferation of cells with MTA, however this could be attributed to the extremely low concentrations of the material used in the two studies as well as the use of preset materials. There seemed to be enhancement in the cell number with Bonitmatrix which was in accordance with previous studies on β-TCP scaffolds (26) and Bonitmatrix-collagen scaffolds (27).

Three distinct zones could be identified with MTA. The zones were: *a zone of lysis*, with lysed cells and denatured proteins of the culture medium just adjacent to the discs, and probably caused by the high pH expected to exist close to MTA as a result of the high amount of calcium hydroxide release as the main hydration product of MTA; *a cell free*

zone, adjacent to the first zone where the pH partially buffered by the culture medium, but still not optimum enough to the presence of cells; and, finally, *a zone of normal cell morphology and culture architecture*, where cells appeared adherent, spindle-shaped, fibroblast-like. The results of the present study were partially in accordance to those described by a previous study (24) of the effect of MTA on the cellular morphology of L929 mouse fibroblasts with the existence of denatured medium proteins and dead cells in the area just beneath the inserts with the materials, around which a zone of lysed cells could be observed, and, finally, at a distance from the materials, a zone of normally-growing cells could be seen. The results of this study were, however, not in accordance with the morphological findings of a study of the effect of calcium hydroxide (considered the main byproduct of MTA) on the cellular activity of human pulp fibroblasts *in vitro*, where no zones of reaction could be explained on the base of the difference could be identified by phase contrast observations (14). This, however, could be explained on the basis of the method of material application in the culture assembly being in a powder form, as a suspension at a final concentration of 0.8 mg/ml (14), and the differences in physicochemical properties. With Bonitmatrix, the cells showed normal cell growth and morphology throughout the whole experimental time which is in partial accordance with ***Alliot-Licht et al. (14)*** where human pulp fibroblasts cells in contact with hydroxyapatite powder showed normal growth similar to the control.

Taken together, from the results of this study, it seems that Bonitmatrix might be considered as a potential pulp capping material that needs to be further examined in animal *in vivo* studies.

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Table (1): The cell viability ratio of the 5-days extracts of MTA and Bonitmatrix (Bon).

MTA Mean (SD)	**Bon** Mean (SD)	*p-value*
1.59 (0.29)	2.21(1.07)	0.07

SD = standard deviation

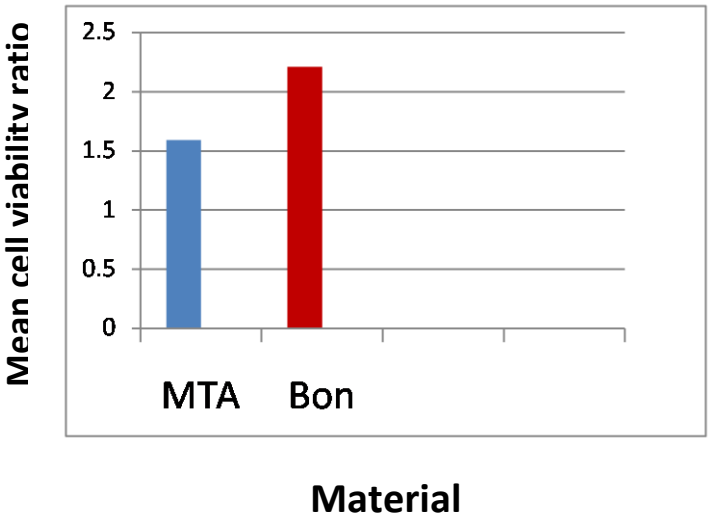

Fig. (1): Column chart showing the cell viability ratio of the 5-days extracts of MTA and Bonitmatrix (Bon).

Table (2): The cell number ratio of the 5-days extracts of MTA and Bonitmatrix (Bon).

MTA	Bon	
Mean (SD)	Mean (SD)	*p-value*
1.14 (0.39)	1.98 (1.43)	0.11

SD = standard deviation

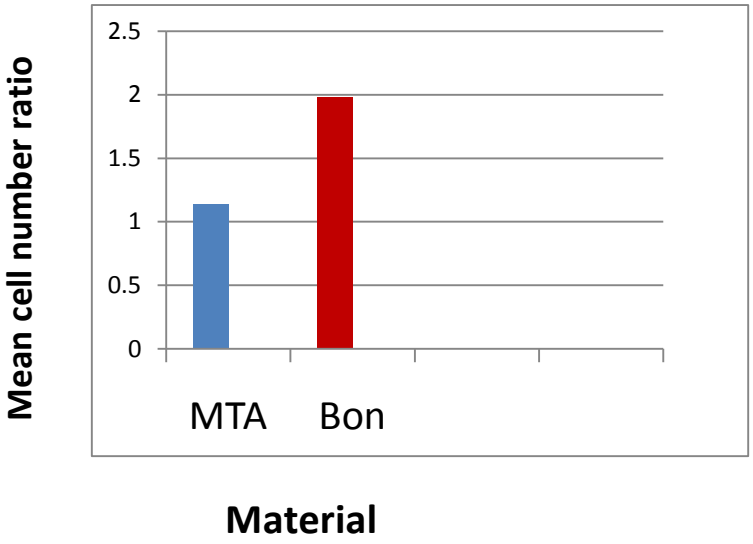

Fig. (2): Column chart showing the cell number ratio of the 5-days extracts of MTA and Bonitmatrix (Bon).

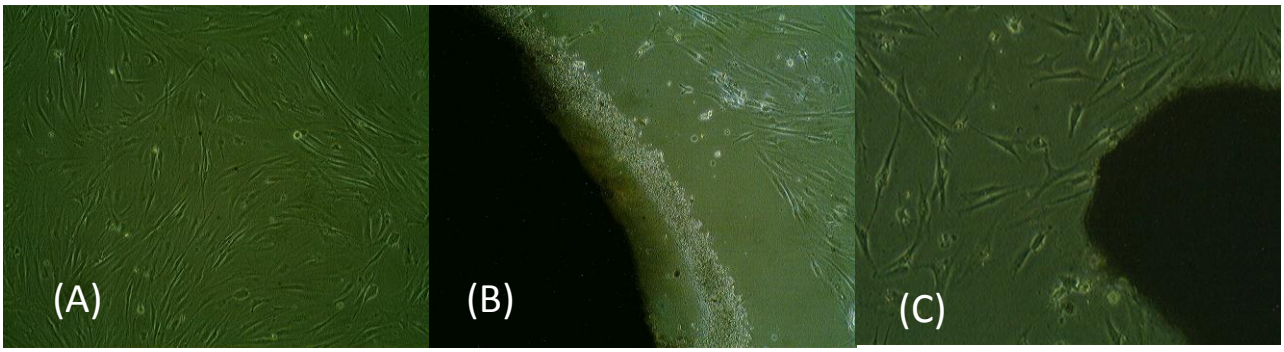

Fig. 3: Inverted photomicrograph showing (HP) cells after 5 days (A) untreated (control) human pulp-derived. Adherent, spindle-shaped cells are seen,(B) cells' reaction to MTA disc. Three zones could be observed: a zone of lysis, a cell-free zone; and a zone of normal cell morphology and (C) cells' reaction to Bonitmatrix. Normal cell morphology with wider intercellular spaces than in the control (original magnification 40X).