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Cytokine Release of Two Endodontic Biomaterials

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Abstract: This study evaluated the effect of two endodontic biomaterials on Transforming Growth Factor (TGF)-β1 and Bone Morphogenetic Protein (BMP)-2 levels produced by cultured human osteosarcoma cell line (Saos-2) and the cytotoxicity of these materials. Mineral Ttrioxide Aggregates (MTA) and Calcium Enriched Mixture (CEM) were prepared and extracted by immersing in 1 mL complete medium (RPMI-1640/10%FBS) for 24 or 72 h. Exactly ×0.25, 0.5 and 1.0 dilutions of each extract and control were added onto cultured Saos-2. Cell viability was determined using MTT assay and TGF-β1/BMP-2 secretion in the supernatant was measured by ELISA assay. After 24 h, all dilutions showed no cytotoxicity, however the 72 h extract showed cytotoxicity. The secreted BMP-2 concentration by both biomaterials at ×0.5 dilutions were higher than the control ($p<0.05$). Researchers can conclude the cytokine inducing properties of MTA and CEM were comparable as well as their cytotoxicity. Moreover, their extracts could possibly induce BMP-2 secretion. This phenomenon might allow a partial reversal of the condition to pre-disease status one of the main goals in medicine.

Key words: Bone Morphogenetic Proteins (BMP), Calcium Enriched Mixture (CEM) cement, cytotoxicity, Mineral Trioxide Aggregate (MTA), Transforming Growth Factor (TGF)

INTRODUCTION

Root-end filling materials, like many other dental materials should consist of various properties. These qualities have been extensively referred to by previous studies; dimensional stability, easy handling, radiopacity, moisture indifference, non-absorbability, non-cytotoxicity, non-corrosiveness, non-carcinogenicity, non-staining, impermeability, antibacterial and antifungal efficacy. Achieving a good apical seal of resected root-end as well as biocompatibility and bioregenerative ability is also essential (Bodrumlu, 2008).

It is thought that subsequent to periradicular surgery, mesenchymal cells can initiate the healing process by differentiating into mature cells (Osteoblasts, fibroblasts or cementoblasts). This allows the potential for osseous regeneration and apical attachment healing. Recent studies have suggested that root-end fillings should be able to stimulate hard/soft tissue healing/regeneration in periradicular tissues including cementogenesis over the filling material (Huang *et al*., 2005; Asgary *et al*., 2010). These qualities are also recommended for pulp capping and pulpotomy agents which also require low cytotoxicity, good biocompatibility and bioregenerative abilities.

Mineral Trioxide Aggregate (MTA) is a hydrophilic root-end filling material that has shown good sealing ability, radiopacity, minimal toxicity and biocompatibility (Parirokh and Torabinejad, 2010; Torabinejad and Parirokh, 2010), animal studies have revealed cementogenesis over MTA, furcally and apically (Samiee *et al*., 2010; Asgary *et al*., 2010). However, the drawbacks include difficult handling, long setting time and high cost. Many studies are concentrating on finding biomaterials with suitable properties. Calcium Enriched Mixture (CEM) cement is a tooth-colored biomaterial with improved handling properties, <1 h setting time and a reasonable price; CEM powder is composed of major components, calcium oxide, sulfite, phosphorus oxide and silica (Asgary *et al*., 2009a). Previous studies have shown that CEM cement possesses good sealing ability (Asgary *et al*., 2008), antibacterial effect (Asgary and Kamrani, 2008), minimal cytotoxicity (Mozayeni *et al*.,

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2010), cementogenic effect (Asgary *et al.*, 2010) and inducing hard tissue formation similar to MTA (Tabarsi *et al.*, 2010).

Bone Morphogenetic Protein (BMP) plays an important role in regeneration of the periodontium by regulating cellular responses, i.e., cell growth/differentiation. BMPs are members of the Transforming Growth Factors (TGF) super-family (Ripamonti and Reddi, 1997). BMPs have been shown to perform important functions in bone, cartilage and dentin formation as well as cementogenesis and periodontal ligament formation (Ripamonti and Reddi, 1997; Nakashima *et al.*, 1994), these proteins may have multiple functions. BMP-2, a known inducer of osteoblast maturation can induce the differentiation of a subpopulation of PDL fibroblastic progenitor cells into cementoblasts (Pitaru *et al.*, 2002). Transforming Growth Factor-β (TGF-β) is one of the most abundant cytokines found in the bone matrix. TGF-β1 is a multifunctional cytokine that regulates various cellular activities including proliferation, synthesis of extracellular matrix proteins and differentiation (Kingsley, 1994). TGF-β1 stimulation induces the proliferation of PDL cells. It seems to play an important role in inducing fibroblastic differentiation of PDL stem cells and in maintaining the PDL apparatus under physiological conditions (Fujii *et al.*, 2010).

The aim of this *ex vivo* study was to evaluate the cytotoxicity of MTA and CEM cement root-end fillings as well as compare their effects on TGF-β1 and BMP-2 levels produced by cultured human osteosarcoma cell line (Saos-2).

MATERIALS AND METHODS

The study protocol was approved by Cellular and Molecular Biology Research Center (CMBRC), Babol University of Medical Sciences and Iranian Center for Endodontics Research (ICER), Shahid Beheshti University of Medical Sciences as well as the related ethics committees.

Cell culture: The Saos-2 cell line was acquired from National Cell Bank (Pasteur Institute, Iran), cultured in RPMI-1640 supplemented with 2 mM L-Glutamine, 10% FBS and 1% PenStrep® (Sigma-Aldrich, UK) and incubated in 5% CO_2 humid incubator at 37°C. Working banks of the cells were made from the collection 3 and 24 h after seeding the cells in 24-wells plates, different concentrations of cements extracts were added to the medium.

Cements extraction and exposure to the cells: In this experiment tooth-colored Pro-Root MTA (Dentsply Tulsa Dental, OK, USA) and CEM cement (Bionique Dent, Tehran, Iran) were used as tested biomaterials. The cements were prepared according to the manufacturers' instruction. They were set in stainless steel casts under sterile condition and were subsequently incubated at 37°C. After 24 h, corresponding discs with diameter and thickness of 3×3 mm and approximate weight of 0.03 g were UV irradiated for 1 h. Each disk was then sunk in 1 mL complete medium using 24-wells plates. In addition, 3 different extract dilutions (×0.25, 0.5 and 1.0) in complete medium were added to the cells in 24-wells plates (1.5×10^5 cells with 1 mL final volume of medium per well). Each experiment was conducted three times and plain complete medium was used as the control group. After 24 h exposure, the supernatants of corresponding wells were taken and kept frozen at -20°C until cytokine measurements were made by the ELISA reader (Stat Fax 2100).

Cytotoxicity assay: To study the cytotoxicity of cement extracts on Saos-2 cell line, the MTT assay was performed in 24-well plates at 24 and 72 h. Briefly, after media (containing different extracts dilutions) were taken, cells were washed by D-PBSA then 200 μL of MTT (Sigma-Aldrich, UK) solution in PBS (5 mg mL^{-1}) was added to each well and incubated for 4 h at 37°C. Using 800 μL of acidic isopropyl alcohol (0.04 N) the purple/blue formazan precipitate was dissolved and the colored solution absorbance was read at 570 nm by a UV-Vis spectrophotometer.

Cytokines assays (ELISA): The concentrations of BMP-2 and TGF-β1 in culture supernatants were measured by predesigned ELISA kits (USCN, China and Bender MedSystem, Austria, respectively) in accordance with manufacturers' instructions. An ELISA reader apparatus was used to read the resulting colored reaction absorbance at 450 nm.

Statistical analysis: Corresponding Optical Density (OD) values from ELISA assays were normalized against MTT results according to the control percentage. The mean±SE for three replicates were calculated and reported. For statistical analysis one-way ANOVA with Tukey Post-hoc Test and t-test were used. The $p<0.05$ were considered significant.

RESULTS

Cytotoxicity: MTA and CEM cement undiluted extracts, regardless of the incubation period, showed significant cytotoxicity ($p<0.01$ and $p = 0.023$, respectively). Diluted extracts (×0.25 and 0.5) with 24 h extraction period did not show significant toxicity, however they showed

Table 1: Cell viability of MTA and CEM in various dilutions and time intervals

Group dilution	24 h CEM	MTA	Control	72 h CEM	MTA	Control
1.00	66	70	100	83	87	100
0.50	95	96	-	92	92	-
0.25	100	99	-	94	94	-

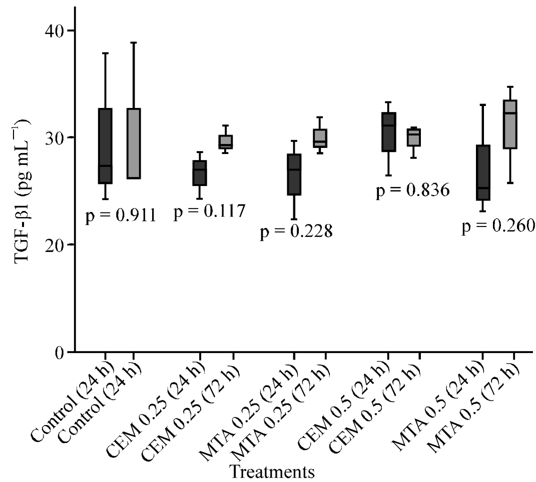

Fig. 1: Comparison of 24 and 72 h extracts of MTA and CEM (at ×0.25 and 0.5 dilutions) on TGF-β1 secretion by Saos-2 cell line

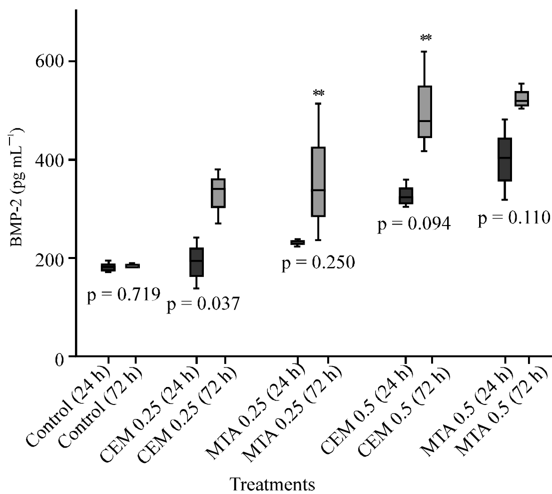

Fig. 2: Comparison of 24 and 72 h extracts of MTA and CEM (at ×0.25 and 0.5 dilutions) on BMP-2 secretion in Saos-2 cells; **Indicates significant difference with control group

significant cytotoxicity when the extraction period increased to 72 h ($p<0.05$; Table 1). There were no significant differences in cell survival percentage among corresponding MTA or CEM treated extracts.

Cytokine assay (TGF-β1 and BMP-2): Based on obtained cytotoxicity results, the concentration of TGF-β1 and BMP-2 in supernatant were measured only for 0.25 and 0.5× dilutions and normalized against MTT results to adjust any decreases in cell numbers. There were no significant differences in TGF-β1 concentrations between control, CEM and MTA groups ($p>0.5$; Fig. 1). On the other hand, BMP-2 concentration was increased by both CEM and MTA×0.5 dilutions. However, the differences in BMP-2 induction by CEM or MTA were not statistically significant in the corresponding groups ($p>0.05$; Fig. 2).

DISCUSSION

Biomaterials which induce cytokine synthesis in blastic cells have great impact on replacement of lost body tissues. This phenomenon might allow a partial reversal of the condition to pre-disease status which is one of the main goals in medicine. The results of this *ex vivo* study demonstrated that the cytotoxicity of MTA and CEM root-end filling biomaterials and their potential ability for stimulating TGF-β1/BMP-2 release in Saos-2 cell lines were similar.

As a common endodontic biomaterial, MTA has some advantages including great compatibility with cavity walls, water insolubility, radiopacity, antifungal properties, minimal toxicity, tissues biocompatibility and hard tissue induction (Parirokh and Torabinejad, 2010; Torabinejad and Parirokh, 2010; Asgary *et al*., 2006; Eghbal *et al*., 2009). Naturally, MTA has been presented as the gold standard for endodontic materials. CEM possesses similar clinical applications as MTA but its chemical composition is different (Asgary *et al*., 2009b). *In vitro* studies showed identical sealing ability for CEM cement and MTA as root-end filling biomaterials (Asgary *et al*., 2008). *In vivo* studies have demonstrated that like MTA, PDL regeneration and cementogenesis occurs adjacent to CEM cement (Asgary *et al*., 2010; Samiee *et al*., 2010). In addition, similar dentinogenesis induction of CEM and MTA has been shown in DPCs (Tabarsi *et al*., 2010). The molecular basis of these cementogenesis/dentinogenesis process is unclear however the production of TGF-β1 and BMP-2 may play a central role.

The TGF-β1 and other members of this growth factor family are involved in tooth development and dental tissue repair. TGF-βs and their receptors have been identified in teeth odontoblast (Sloan *et al*., 2000). Thus, the role of this cytokine in the mechanisms involved in the repair of bone tissue and teeth is not far-fetched. Additionally, BMP-2 has been recognized as a stimulator of osteoblast maturation and initiator of PDL osteoblasts

differentiation (Fujii *et al.*, 2010). It has been proved that when hard tissue cells produce BMP-2, the reconstruction and regeneration of bone, cementum and cartilage increase significantly (Ripamonti and Reddi, 1997; Nakashima *et al.*, 1994; Maeda *et al.*, 2010). Researchers have shown BMP-2 expression in human PDL cells by MTA which might be assumed as one of MTA's good characteristics (Maeda *et al.*, 2010). The data from the ELISA analysis indicates that while BMP-2 secretion is stimulated by ×0.5 dilutions of CEM and MTA extracts compared to the control group, there is no difference between these two test biomaterials. This finding is in accordance with previous results (Guven *et al.*, 2007) if sufficient concentrations of MTA derivatives extracts exist in the medium, cell lines might be induced to produce and secret BMP-2. On the other hand, the results showed no significant differences for TGF-β1 between control, CEM or MTA treated groups, contradicting the previous study. This may be because of different cell line used (HGF) and/or different extraction method. Since, *ex vivo* experiments have superior predictability than *in vitro* studies, the BMP-II induction by CEM should be tested *in vivo* as well.

Cell culture-based methods rely on contact between tested material and cells. MTA cytotoxicity has been evaluated through various methods such as morphologic observation, alkaline phosphatase activity, SEM observation, fluorescent measurements and cell viability tests (Torabinejad and Parirokh, 2010). Several cell lines have been used for biological response to MTA in the current study, researchers used a Saos-2 osteosarcoma cells which is one of the best cell lines to investigate the bone-producing potential of tested substances; it has been proven that Saos-2 cells express BMP -1, -2, -3, -4, -5, -6 and TGF-β1 mRNA (Sloan *et al.*, 2000). The Saos-2 is an adherent cell line with human osteosarcoma origin and seems to be an appropriate model for extrapolating biocompatibility results to human dental and periapical tissues. However, cell culture-based methods still ignore the influence of upstream events such as vascularisation and subsequent stem cell recruitment. Using formazan colour formation rate by biochemically alive cells (MTT assay) is an indicator of mitochondrial dehydrogenase enzyme activity because the reaction takes place only in cells with active mitochondrias (Mosmann, 1983). Since, MTA and CEM are hydrophilic endodontic biomaterials which are willing to release ions (Asgary *et al.*, 2009b), they are more prone to interfere with intracellular enzymes and MTT test seems to be a reliable cytotoxicity assay. The surface area in contact with the medium is an important factor which affects the extent of toxic materials extraction from the solidified cement and it should be the same in the control/experimental groups. Researchers used standard disc with a certain size and similar surface area which were in contact with a fixed volume culture media. The dilution method reduces the likelihood of false positive toxicity compared with when the cement material is placed directly onto the cells. Also, the extraction period (length) might affect the quantity and quality of toxic substances extracted into medium so using two different times of extraction (24 h/72 h) makes it possible to observe changes in cytotoxicity with different extraction periods. In the study undiluted extracts of MTA and CEM showed similar cytotoxicity on Saos-2 cells. Similar cytotoxicity results have been observed with CEM and MTA with L929 cell line (Mozayeni *et al.*, 2010; Ghoddusi *et al.*, 2008).

CONCLUSION

CEM and MTA showed similar cytotoxicity in Saos-2 cell line and their diluted extracts could upregulate BMP-2 production/secretion however in this study, there were no detectable changes in TGF-β1 secretion into the medium. TGF-β1 and BMP-2 are thought to play a central role in cementogenesis and dentinogenesis. Further studies are required to show the relationship of biomaterials CEM and MTA with these cytokines.

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