Effect of CPoint, EndoSequence BC, and Gutta-percha Points on Viability and Gene Expression of Periodontal Ligament Fibroblasts

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ABSTRACT

Objective: This study aimed to investigate the cytotoxic and biomodulatory potential of conventional gutta-percha (CGP) points, gutta-percha points containing bioceramics (BC), and CPoint polymer (CP) points on periodontal ligament (PDL) cells *in vitro*.

Methods: PDL fibroblasts were cultured and stimulated with extracts of CGP, BC, and CP in serial dilutions to evaluate cell viability using MTT assay. Next, the 1:5 dilution was used to stimulate the cells for 72 h to assess the gene expression of type I collagen (COL-1) and cement protein 1 (CEMP-1), by reverse transcription followed by quantitative PCR. Data were statistically analyzed using one-way analysis of variance (ANOVA) ($P<0.05$).

Results: Pure extracts of CGP and CP were found to be cytotoxic for PDL ($P<0.01$). Once diluted to 1:5, only CP showed cytotoxicity. BC did not affect cell viability in any extract sample. No extract significantly altered the gene expression of COL-1. For CEMP-1, a significant increase in gene expression was observed only for CGP ($P<0.05$).

Conclusion: CP was found to be more cytotoxic than CGP, while BC demonstrated no cytotoxicity. The tested cones did not affect COL-1 gene expression, while CGP upregulated CEMP-1. Our results suggest that obturation point components may affect the biological responses of PDL fibroblasts.

Keywords: Bioceramic point, CPoint, cytotoxicity, gutta-percha point, periodontal ligament fibroblasts

HIGHLIGHTS

- Obturation points used for root canal filling can be cytotoxic depending on their composition.
- Obturation points can affect the expression of healing factors in periodontal ligament cells.

INTRODUCTION

Root canal treatment aims to eliminate dental pulp inflammation and control root canal infection, which allows recovery of the apical tissues. Materials used for root filling must possess important properties, such as biocompatibility, antimicrobial activity, sealing ability, and ability to allow or induce healing (1). Most of these materials inhibit or alter the growth of various cell types (2). Since cell cycle progression strictly regulates cell growth, interference in this cycle by toxic materials can lead to its inhibition, cytotoxicity, and even apoptosis (3). Therefore, the choice of material used for root canal obturation should be made based on its chemical and physical properties as well as its biological profile, including the possibility of the material's contact with periapical tissues (4).

Histologically, endodontic healing occurs by closure of the apical foramen by the deposition of a hard tissue barrier. Therefore, root filling materials should create an environment conducive to healing of the periapical tissues that were previously affected by pulp and periapical disease (5). Periodontal ligament (PDL) fibroblasts are the main cells involved in the cellular response to endodontic materials, since they are typically present around the apical area (6). Therefore, obturation materials should provide a favourable environment for PDL fibroblast adhesion, as well as for the growth of PDL fibroblasts and cementoblasts (7, 8).

Gutta-percha points, associated with an endodontic sealer, are widely used as filling material for root canal filling (9). In contrast to endodontic sealers, obturation points comprise a solid material, and their cytotoxicity and ability to affect surrounding tissues have not been thoroughly investigated. Although some studies confirm the acceptable biocompatibility of gutta-percha (10),

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others state that extruded gutta-percha is associated with delayed healing and maintenance of a persistent periapical radiolucency, after root canal treatment (11).

To improve their biocompatibility and ability to adhere to the dentin walls, obturation points have undergone important changes in composition. EndoSequence BC points (Brasseler USA, Savannah, GA) (BC) are gutta-percha cones impregnated and coated with bioceramic nanoparticles known as bioactive substances (12). According to the manufacturer, the bioceramic particles found in the EndoSequence Sealer bind to the bioceramic particles in the BC to form a gap-free filling. The CPoint (EndoTechnologies, LLC, Shrewsbury, MA) (CP) is a hydrophilic polymer cone that does not contain gutta-percha. Instead, it consists of an inner core containing two distinct nylon polymers, Trogamid T and Trogamid CX, which radially expand as they absorb water from the canal and dentinal tubules. The CP also contains an outer polymer coating, consisting of a cross-linked copolymer of acrylonitrile and vinylpyrrolidone (13).

Since questions have been raised concerning the neutrality of gutta-percha (14), this study aimed to investigate the cytotoxicity of obturation points used in contemporary endodontics, as well as their ability to modulate the functions of PDL fibroblasts.

The null hypothesis was that the obturation points tested would not influence cell viability of fibroblasts or alter the expression of genes related to mineralization and repair.

MATERIALS AND METHODS
The Human Ethics Committee of the School of Dentistry of University of São Paulo (1.774.956) approved the study protocol.

Preparation of the PDL fibroblast cells
A human PDL fibroblast lineage, previously established and characterized, was used for this study (15). The cells were thawed in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco), supplemented with 15% fetal bovine serum (FBS) (Gibco), 2 mM L-Glutamine (Invitrogen, Life Technologies, Carlsbad, CA), 100 U/mL penicillin (Invitrogen), and 100 µg/mL streptomycin (Invitrogen) at 37°C in 5% CO_2 atmosphere. After reaching 90% confluency, the cells were detached using 0.25% trypsin solution and 0.05% ethylenediaminetetraacetic acid (EDTA) (Invitrogen) (15) for subcultivation. Cells at passage 4 were used in this study.

Preparation of the obturation point extracts
Conventional gutta-percha points (CGP) (VDW GmbH, Munich, Germany), 28 mm in length, BC, and CP points with an apical diameter of #35 and taper 0.06 were cut at 20 mm. Each cone was inserted into 1 mL of 10% FBS/DMEM and maintained at 37°C for 7 days. The supernatant was then collected and sterilized by 0.22 µm membrane filtration and stored at −20°C. Prior to use, the extracts were submitted to a serial dilution of 1:5.

Cell stimulation with obturation point extracts
Cells were seeded at 1.25×10^4 and 5×10^4 cells/well in 96-and 24-well plates, respectively, and held for 24 h to allow for adhesion. The medium was replaced by 10% FBS/DMEM, with and without extracts, and analysis was performed 72 h later. The experiments were performed in triplicate.

Cell viability by MTT assay
The cytotoxicity of the extracts to the fibroblasts was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Molecular Probes, Waltham, MA). After the experimental period of 72 h, succinate dehydrogenase activity was determined by adding 20 µL of 5 mg/mL MTT solution in PBS to each well, followed by 180 µL of 10% DMEM. Fibroblasts were then incubated at 37°C for 4 h, protected from light. After incubation, the solution was discarded, and the resultant formazan was solubilized in 100 µL of isopropyl alcohol. The plates were incubated on a shaker table for 30 min to completely dissolve the formazan crystals. The optical density value was measured by spectrophotometer (Synergy H1, BioTek, Winooski, VT) at 570 nm (15). The data obtained by each group were normalized based on cells with culture medium.

Detection of Collagen type 1 (COL-1) and Cementum protein 1 (CEMP-1) gene expression by quantitative polymerase chain reaction (qPCR)
Once the conditions that rendered at least 75% cell viability were determined, the experiment was conducted in 24-well plates for 72 h. The cells were collected using TRIzol® (Invitrogen, Carlsbad, CA) for RNA isolation, followed by reverse transcription and qPCR.

Total RNA isolation
The guanidinium thiocyanate-phenol-chloroform method was used as described in the literature (15). The RNA was extracted in Trizol, isolated by chloroform, precipitated by isopropyl alcohol, and washed in 75% alcohol in water with 0.1% diethylpyrocarbonate (DEPC). A resuspension of the total RNA was performed with 15 µL of 0.1% DEPC water and stored at −80°C until reverse transcription was performed.

Quantification of total RNA and reverse transcription
The concentration of total RNA in the samples was determined using spectrophotometer readings at 280 and 260 nm wavelengths (Synergy H1, BioTek, Winooski, VT). Reverse transcription of the RNA samples into complementary DNA (cDNA) was performed using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), following the manufacturer's instructions.

qPCR
The quantitative expression of the chosen genes was performed using Taqman Universal Mastermix II (Life Technologies) and Taqman assays (Life Technologies) as follows: Collagen type I (COL1A1–Hs00164004_m1) and cementum protein 1 (CEMP-1–Hs03004478_s1) using GAPDH (Hs02786624_g1) as the housekeeping gene. Amplification was performed using StepOne Plus equipment (Life Technologies) following the standard parameters. The results were analyzed based on the value of the threshold cycle (Ct), which corresponds to the number of cycles of amplification that allowed for a quantitative analysis of the expression of

the evaluated target. The means of the Ct values of the duplicate specimens were used to calculate the expression of the target gene with normalization based on the internal control (GAPDH). These values were then compared to those of an internal target control (an unstimulated sample), thus allowing for calculation of the increase in expression, using the formula 2-ΔΔCT.

Statistical analysis

Statistical analysis was performed by one-way analysis of variance followed by Tukey post-test for comparisons among dilutions, materials, and control. The criterion for statistical significance was defined as P<0.05 using the GraphPad Prism 5.0 software (GraphPad Software, Inc, San Diego, CA).

RESULTS

Cell viability

Cell viability of the PDL fibroblasts in contact with the obturation point extracts was analyzed at the experimental time of 72 h (Fig. 1). Cell viability significantly decreased in the pure extract of CGP compared to that of the medium only (0). However, diluted extracts (1:5 and 1:25) did not show cytotoxicity. BC did not affect cell viability in any extract sample. While with CP, significant cytotoxicity was found in the pure extract (1) and also at the 1:5 dilution. Both CGP and CP were significantly more cytotoxic than BC in the pure extracts (P<0.05).

Gene expression of COL-I and CEMP-1

Since the dilution of 1:5 allowed for a cell survival rate of at least 75% (Fig. 1), this dilution was chosen for the assay. The gene expression of proteins involved with tissue repair was detected by RT-qPCR (Fig. 2). Any of the point extracts at the 1:5 dilution did not significantly alter the constitutive expression (medium) of COL1 (Fig. 2a). Conversely, CGP extract significantly increased the expression of CEMP-1 compared to those of the control and BC extracts (Fig. 2b). BC and CP did not affect the physiological gene expression of CEMP-1.
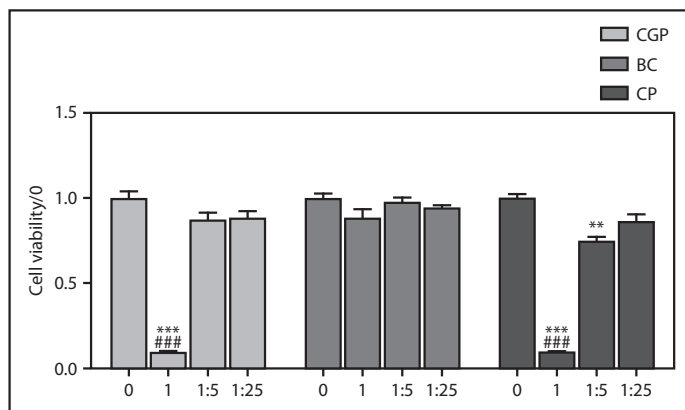
Figure 1. Periodontal ligament fibroblasts in contact with obturation point extracts from conventional gutta-percha (CGP), Bioceramic (BC), and CPoint, serially diluted in 10% FBS/DMEM. Viability assay was performed using MTT after 72 h in culture. Bars representatives of mean±standard error (n=6). Statistical significance represented by *** when P<0.001 compared to 0 (medium only) and ### for P<0.001 compared to BC at the same conditions
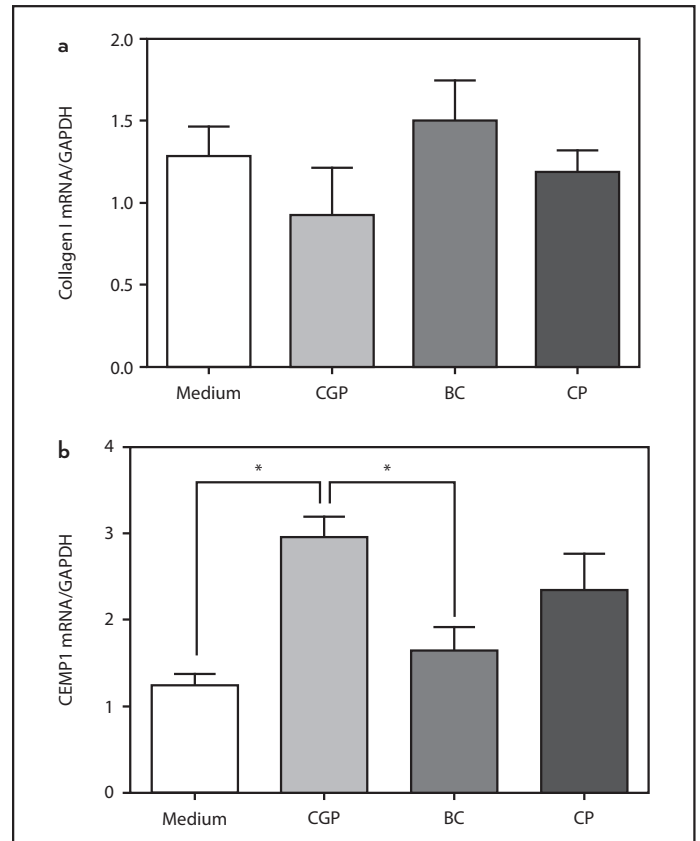
Figure 2. Periodontal ligament fibroblasts in contact with obturation point extracts. Cells kept in culture with extracts from conventional gutta-percha (CGP), Bioceramic, and CPoint, at a 1:5 dilution for 72 h, were assessed for detection of COL1 (a) and CEMP1 (b) by means of RT-qPCR. Bars representative of mean±standard error (n=3). Statistical significance represented by * for P<0.05 compared to indicated conditions

DISCUSSION

A wide variety of root canal filling materials is commercially available. Varying amounts of the soluble components may come in contact, either directly or indirectly, with the periradicular tissue (16). The biocompatibility of the materials proposed for obturation points is important to be evaluated. In this study, extracts of different obturation points were evaluated in primary cell cultures. PDL fibroblasts were chosen to closely simulate the clinical microenvironment (8). The advantages of using extracts are that they can be sterilized by filtration, facilitating the evaluation of their effects on the cells, and they simulate a clinical situation, in which the substances leach and diffuse into the periapical tissue (17). Ions release by obturation points, such as phosphorus, calcium, sodium, and zinc, vary significantly based on material formulation (9); and we may speculate a concentration-dependent cellular/tissue response that in turn is suitable for evaluation using this model *in vitro*.

In this study, we evaluated the biocompatibility of three different types of cones based on their effects on cell viability and gene expression of molecules involved in mineralization and repair. Undiluted extracts of CGP and CP showed significant cytotoxicity to PDL fibroblasts, yielding a survival rate of approximately 10% (Fig. 1). A cytotoxic effect was also

observed at the 1:5 dilution of CP, though to a lower extent (75% of survival rate). Corroborating our study, other authors have shown that CP is significantly more cytotoxic than CGP to odontoblast-like cells during the first experimental week (13). However, after this period, the biocompatibility of both is similar, and the authors suggested that the presence of an incompletely polymerized air inhibition component on the CP surface is responsible for this toxic effect (13). Our study showed that CGP leached toxic components, evidenced by the cytotoxic assay of the pure extract. This finding may be attributed to the leaching of zinc ions from zinc oxide fillers, as suggested by Pascon & Spangberg (14). Conversely, BC showed no cytotoxicity and no statistical difference compared to the control results (medium only). This can be explained by the presence of bioceramics, which are often used as root repair materials and known to provide regenerative properties to PDL cells (18).

The specific behaviour of PDL fibroblasts to each of the tested materials may result from the distinct composition of the obturation points chosen for our study. Based on available data (9), BC points were shown to release phosphorus at similar levels to ProTaper points, but only traces of Zn were found in both. On the other hand, Na and Ca released by BC was significantly higher than the amounts leached by ProTaper points an Obtura pellets (9). Further studies may elucidate the precise mechanisms and other ions potentially involved with the toxicity of obturation points. Also, the role of the concentration of each of these ions on cellular behavior deserves attention.

When investigating the effects of dental materials on living tissues, it is essential to consider their effect on the functions of surviving cells. In this study, the gene expression of COL-1 and CEMP-1 was investigated in PDL fibroblasts in contact with elements from the obturation points. COL-1 is the most common form of collagen in the PDL (19), and it is an essential component of the extracellular matrix in mature bone (20). COL-1 has been proposed to induce osteoblast differentiation and facilitate mineralization (21). Within the repair process, collagen is essential for the formation of the extracellular matrix that serves as a substrate for cell adhesion and confers stability to neoformed connective tissue. In contrast, excessive production of collagen may indicate fibrosis and scar tissue formation instead (22). In our study, the extracts did not significantly alter the constitutive expression of COL-1, which suggests that the substances released by the materials do not disturb the constitutive gene expression of COL-1 in surviving cells. Therefore, it is believed that these materials would not affect the physiological production of collagen during the repair process. In contrast with our study, Fayazi et al. (23) reported an upregulation of COL1 in human PDL fibroblasts exposed to extracts of two bioceramics, ProRoot MTA and Portland cement. Similarly, an upregulation of COL1 was observed in human dental pulp cells (hDPCs) treated with the bioceramic iRoot BP when compared with MTA-treated hDPCs after a 72-h time period (24). Despite the presence of bioceramics in the obturation points, chemicals released by cements are likely more significant than those of solid core obturation materials, like the ones evaluated in our study.

The expression of CEMP-1 is related to the formation of bone tissue and cementum and has been previously reported to promote osteoblastic and/or cementoblastic differentiation in human gingival fibroblasts in vitro (25). Its expression is restricted to cementoblasts and progenitor cells of the human periodontium, and it regulates cell attachment, differentiation, deposition rate, composition, and morphology of the hydroxyapatite crystals formed by these cells (26). In our study, CGP induced significant upregulation of CEMP-1 when compared with the control, BC, and CP extracts. In the context of tissue regeneration, cement production is essential for the formation of replacement tissue, also known as a biological seal, considered the "ideal scenario" for endodontic treatment repair (27). Thus, the induction of cement deposition by endodontic materials is considered a beneficial process. In our study, CGP upregulated the gene expression of CEMP-1, while BC and CP did not, despite numerous studies that have demonstrated the repair potential stimulated by bioceramic-containing materials (28). The absence of CEMP-1 inhibition by any of the materials in this study is also relevant, since obturation materials should not disrupt or delay the natural repair of periodontal tissue (29). Collectively, results of the gene expression analysis in this study may be considered positive from a cytotoxicity context.

In addition, this study suggests that the perception of gutta-percha and other filling points as inert core materials for root canal obturation may be incorrect. Our data demonstrated the potential cytotoxicity in vitro of the soluble components, based on cell viability, but not by gene expression assays, thus partially rejecting the null hypothesis.

CONCLUSION

Except for BC, CGP and CP may exhibit cytotoxic effects on PDL fibroblasts. Considering cellular function, none of the tested points affected COL-1 gene expression, and CGP upregulated CEMP-1. In conclusion, obturation points may exert different biological responses on PDL fibroblasts.

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