Cytokine Production from Human Primary Teeth Pulp Fibroblasts Stimulated by Different Pulpotomy Agents

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ABSTRACT

Purpose: After exposing the pulp tissue, cytokines are produced that regulate the pulp inflammatory response. The dental literature, however, lacks information on the participation of primary tooth fibroblasts in this process. The purpose of this study was to verify the participation of human primary tooth pulp fibroblasts in the inflammatory process, evaluate the production of interleukin 1 beta (IL-1β) and interleukin 8 (IL-8) from these cells.

Methods: Pulpotomy agents were applied as conditioned media on cell cultures in the following groups: (1) negative control; (2) positive control (Lipopolysaccharide –LPS); (3) calcium hydroxide (powder); (4) mineral trioxide aggregate (MTA); (5) adhesive resin; and (6) formocresol. After 24 hours in contact with the cells, the conditioned media were removed, the proteins were extracted from the cells and IL-1β and IL-8 were quantified by ELISA (Enzyme linked immuno-sorbent assay).

Results: Data were analyzed by analysis of variance ($P<0.05$) and Tukey's test ($P<0.05$). It was observed that calcium hydroxide has stimulated the production of IL-1β, without stimulating IL-8. Conversely, the adhesive resin and formocresol stimulated the production of IL-8, and did not stimulate IL-1β. MTA stimulated both cytokines in an intermediate level when compared to the other materials.

Conclusion: Primary tooth fibroblasts can respond immunologically, and different pulp capping materials can help in this process.

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The preservation of the dental pulp after a tooth injury is one of the most important steps in a pediatric clinical practice. This procedure depends on the presence of infection, patient's age, and maintenance of tooth space in the arch. It should also not impair the root resorption during the exfoliation process. In this case, the pulpotomy is the elected treatment, since it preserves the root pulp tissue, thus allowing the coronal restoration.

Some materials can be used for this purpose. For many years, diluted formocresol has been used and accepted for deciduous teeth pulpotomy.[1-5] Another option for this procedure is calcium hydroxide, which is widely used for direct pulp capping with good performance, since it stimulates tissue repair through its high pH and the formation of a dentin bridge.[6,7] In the past decade, some authors had suggested the use of bonding systems for pulp capping.[8-10] Studies are demonstrating poor results for treatment of the exposed pulp, however, since it is not the original purpose of adhesive resins.[11-14] Recently, mineral trioxide aggregate (MTA) has been suggested for pulpotomy and pulp capping due to its physical and chemical properties, cavity sealing efficacy, and biocompatibility.[15-18]

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The production of cytokines can be used to analyze the inflammatory potential of different materials. Some dental pulp cells produce interleukin 1 beta (**IL-1β**) and interleukin 8 (**IL-8**), after tissue injury. IL-1 is a proinflammatory cytokine, related to the acute and chronic phases of the inflammatory process, that modulates inflammation and repair. IL-1β, meanwhile, is an active predominant form of IL-1, with an important role in the progression of inflammation and healing.[19-22] IL-8 is an important inflammatory chemokine, with a potent inductive chemotactic capacity for neutrophils. This cytokine can start and increase the inflammatory process in pulp injury and is responsible for the recruiting of immune cells to the affected area.[24,25]

The purpose of this study was to evaluate the participation of human dental pulp fibroblasts from deciduous teeth in the inflammatory process, analyzing the production of interleukin 1 beta and interleukin 8 when stimulated by different materials suggested for pulpotomy.

METHODS

We analyzed the production of cytokines by human pulp fibroblasts stimulated by different materials suggested for pulpotomy. This work was approved by the Institutional Review Board of the Ibirapuera University (process no. 278/07).

EXPERIMENTAL GROUPS

The following groups were established, according to the conditioned media applied on the cell cultures:

1. negative control (fresh culture media);
2. positive control (culture media with Escherichia coli lipopolysaccharide [LPS], L4391, Sigma, St. Louis, Mo);
3. calcium hydroxide–powder (Fórmula e Ação, Sao Paulo, S.P, Brazil);
4. mineral trioxide aggregate–MTA (MTA Bio, Angelus, Londrina, Paranã, Brazil);
5. adhesive resin (Prime & Bond 2.1, Dentsply, Petropolis, Rio de Janeiro, Brazil);
6. Diluted formocresol (Fórmula e Ação, Sao Paulo, S.P, Brazil).

SUBSTANCE PREPARATION AND MEDIA CONDITIONING

In the positive control group, 10 ng/ml of *E coli* LPS was added to the culture media to induce the production of cytokines by the cells.

The calcium hydroxide powder was placed into a 15-ml centrifuge tube with a no. 24 sterile spatula. The MTA was manipulated according to the manufacturer's instructions with a sterile no. 24 spatula and glass plate and placed into the bottom of a 15-ml centrifuge tube. The bonding system and the formocresol were also placed into 15-ml centrifuge tubes. To obtain conditioned media (eg, media containing the dissolved substances from calcium hydroxide or adhesive resin), the test tubes containing the capping materials were filled with Dulbecco's Modified Eagle Medium (**DMEM**; Sigma). Conditioning was carried out for 1 hour at 37°C using 0.2 g of each substance per milliliter of fresh medium.[5] In the adhesive group, light polymerization was performed after filling the tube with fresh medium, allowing dissolution of substances before and during this procedure. The stock conditioned media obtained during this step were diluted with fresh media (1%) and further applied into the cell cultures.[11]

CELL CULTURE

Human pulp fibroblasts from primary teeth were collected from explants of pulps of extracted primary teeth. The tissues were placed immediately into Petri dishes containing DMEM and supplemented with 10% fetal bovine serum (Cultilab, Campinas, São Paulo, Brazil) and 1% antimycotic-antibiotic solution (10,000 units of penicillin, 10 mg of streptomycin, and 25 μg of amphotericin B per mL in 0.9% sodium chloride; Sigma). The cells were kept in an incubator at 37°C and a humidified 5% CO_2 atmosphere. The tissues were repeatedly sliced into very small pieces to properly obtain the explants. Cultures were supplied with fresh medium every other day. Cells between the fifth and 10^{th} passages were used in all experimental procedures.

After cell culture, the cells were counted and plated on 6 well plates ($1x10^6$ cells per well) for a total of 3 wells per group. After 24 hours, the DMEM was replaced by the conditioned culture media, according to each group previously described. All experiments were made in triplicate to confirm data and reduce variation among samples.

PROTEIN EXTRACTION

After 24 hours of contact between the conditioned media and cells, the media were removed and the cells were washed with cooled (4°C) phosphate buffered solution. During the extraction of proteins, the plates were always kept on ice to avoid the denaturation of cytokines. Each well was filled with 100 μl of RadioImmuno Precipitation Assay buffer (Pierce Biotechnology, Rockford, Ill), supplemented by 1 μl of protease inhibitor (Sigma) and kept in contact with the cells for 15 seconds. Next, the cells were vigorously scrapped from the bottom of the wells and the contents of each well were transferred into a microcentrifuge tube. These tubes were centrifuged at 20,000 *g* for 15 minutes at 4°C. The supernatant was collected and diluted by a 1:4 ratio for the ELISA assay.

ELISA

The protein quantification was carried out with ELISA kits for IL-1β (RPN-5971, Biotrak, Amersham Pharmacia Biotech, Buckinghamshire, UK) and IL-8 (RPN-5969, Biotrak) following the manufacturers' instructions. Data obtained from each well (three independent wells for each sample) were submitted to Levene's statistical test, which

confirmed their normal distribution. Data were statistically analyzed by analysis of variance (ANOVA; $P<0.05$) and complemented by Tukey's posthoc test ($P<0.05$).

RESULTS

IL-1B

The production of IL-1β is illustrated in Figure 1. ANOVA found differences among the tested materials ($P<0.01$), and Tukey's test (Table 1) presented these differences. Calcium hydroxide showed a higher IL-1β production level, similar to the positive control. MTA demonstrated an intermediary level, similar to both controls. The adhesive resin and formocresol did not induce the production of IL-1β, similar to the negative control.

IL-8

The analysis of the production of IL-8 is illustrated in Figure 2. ANOVA found differences among the tested materials ($P<0.01$). In this case, Tukey's test (Table 2) has shown that the calcium hydroxide had the lowest IL-8 production level, similar to the negative control. Again, MTA was at an intermediary level, but in this case with differences when compared to both controls. The adhesive resin and formocresol-induced levels of IL-8 were as high as those observed with MTA.

DISCUSSION

The results of the present study show that calcium hydroxide can induce IL-1β, which can be explained by this cytokine's multifunctional character.[19-22] Thus, the increase of this cytokine production after calcium hydroxide stimulation can be related to the fact that the IL-1β has different functions according to its dose and time of action, being participative in different phases of inflammation, including inflammation control.[19] On the other hand, the low production of IL-8 induced by calcium hydroxide is probably related to the low inflammatory potential of this material, since IL-8 is a potent chemo attractive cytokine for neutrophils.[19,23,24]

The use of bonding systems for pulp capping is controversial. Although some authors have suggested this procedure,[8-10] many studies have demonstrated high cytotoxic effects of this material when used on pulp exposures, deep cavities, and cell cultures.[11-14] These cytotoxic effects are mainly related to the dissolution of nonpolymerized components in the tissue.[11,14] In this study, it was observed that the bonding system induced the IL-8 production by pulp fibroblasts, which agrees with the inflammatory potential observed in some *in vivo* studies.[12,13] The same results were observed for the formocresol, since this material and bonding systems have similar performance in previous in vivo studies.[1,2,5] Formocresol is recommended for pulpotomy in primary teeth since the literature has information that calcium hydroxide can induce internal tooth resorption when used for this purpose.[3-5]

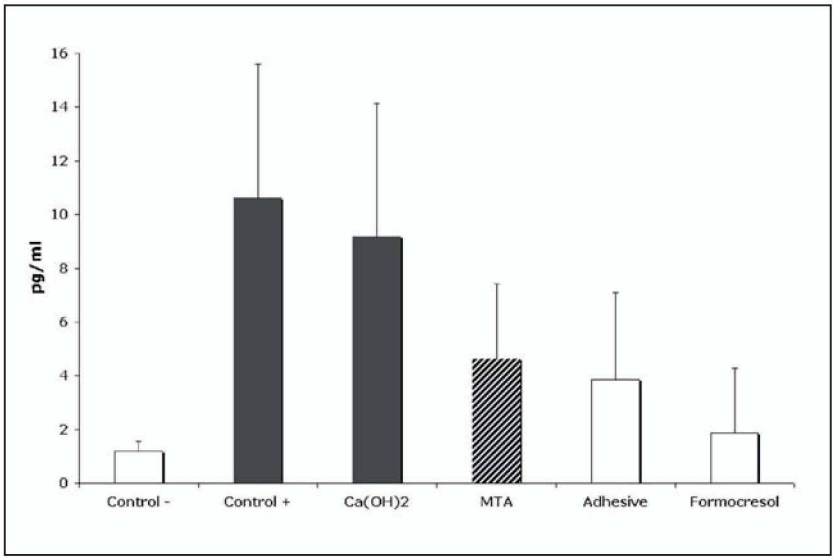

Figure 1. Average IL-1β production by human pulp fibroblasts from primary teeth, according to the tested groups. Bars indicate the standard deviation. Different colors indicate statistical differences. The MTA column has stripes of both colors.

Table 1. Differences between Means for the IL-1β Analysis

	Control -	Control +	$Ca(OH)_2$	MTA	Resin	Formocresol
Control -	-	-	-	-	-	-
Control +	9.409*	-	-	-	-	-
Ca(OH)2	7.993*	1.415	-	-	-	-
MTA	3.415	5.993	4.578	-	-	-
Resin	2.658	6.750*	5.335	0.757	-	-
Formocresol	0.661	8.747*	7.332*	2.754	1.997	-

**** Statistical differences (Tukey's critical value=6.152).***

MTA has been used for pulp capping due to its biocompatibility and performance, which is similar or better than calcium hydroxide.[15-18] This valuable performance is extended to direct pulp capping and pulpotomy of primary teeth[1,2]. In the present study, MTA has induced both cytokines at an intermediate level. This could indicate that MTA has the inflammatory potential (IL-8) and the property of inducing some levels of IL-1β, with a performance close to that obtained by calcium hydroxide.[23]

The production of IL-1β and IL-8 was assessed by ELISA.[20-24] In the present study, LPS induced both IL-1β and IL-8 by human primary teeth pulp fibroblasts. This production was not detected in the negative control, indicating good experimental standardization. This standardization is based in the fact that the literature has information on the ability of LPS to be a positive control, since it stimulates human pulp fibroblasts to produce different cytokines.[21,24,25]

Obviously, a laboratory result should be carefully extrapolated to the clinical practice, since it is difficult (if not impossible) to simulate all natural conditions present in living tissue (the three-dimensional environment, different cell types, presence of blood flow, and turnover of cell populations). Once many conditions are standardized and some important factors insulated, however, this method is reliable in offering guidance in the use of dental materials. In this context, the results presented here can indicate that the clinical use of formocresol and bonding systems is not suitable regarding the production of IL-1β and IL-8. This statement comes from the fact that high IL-8 levels are directly related to acute inflammation, which, if not stopped by other body mechanisms, can be harmful to the pulp tissue.

The dental literature has information of the inflammatory potential of these substances,[1,2,11,14] particularly the bonding systems, which are not biocompatible.[12,13] MTA also stimulated IL-8 production, but it is only active during its initial setting times (up to 72 hours). The MTA would probably not induce these IL-8 levels in a long-term study, which was impossible to check with the methods used, due to the short half-life of cytokines.

The high levels of IL-1β observed in the presence of calcium hydroxide can be related to this cytokine's multifunctional aspects. This means that IL-1β, although proinflammatory, is related to other steps of the inflammatory events, such as the induction of macrophages to clean the injured site.[19] Thus, according to this study's results, both calcium hydroxide and MTA could have this property, making them suitable for clinical use. On the other hand, the low levels of IL-1β stimulated by formocresol and a bonding system could indicate that these substances induce acute inflammation (high IL-8 levels), but not other factors responsible for the remission of inflammation. Consequently, these substances can harmfully affect the tissue, making them less suitable for clinical use regarding IL-1β and IL-8 production.

Considering the data presented here, this study can be extended to analyze in more depth the pulp response from primary teeth pulp fibroblasts. Further studies should consider membrane receptors, signaling pathways,

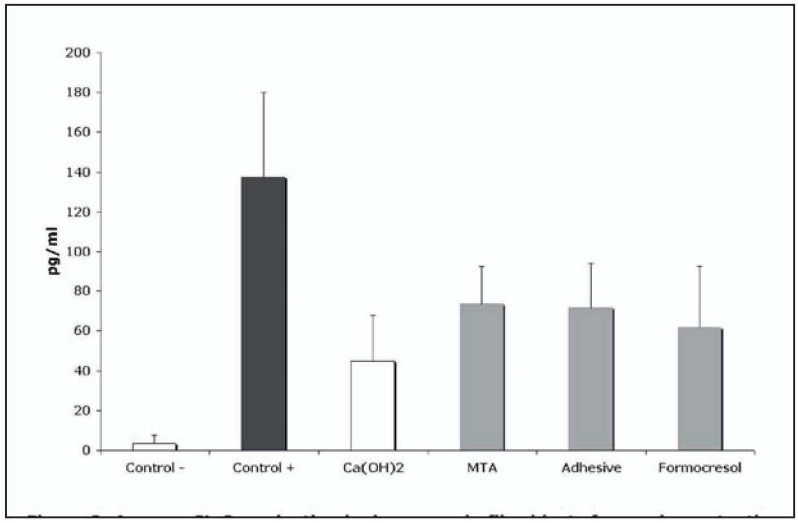

Figure 2. Average IL-8 production by human pulp fibroblasts from primary teeth, according to the tested groups. Bars indicate the standard deviation. Different colors indicate statistical differences.

Table 2. Differences between Means for the IL-8 Analysis

	Control -	Control +	Ca(OH)2	MTA	Resin	Formocresol
Control -	-	-	-	-	-	-
Control +	133.855*	-	-	-	-	-
Ca(OH)2	41.110	92.746*	-	-	-	-
MTA	69.864*	63.991*	28.754	-	-	-
Resin	68.011*	65.844*	26.901	1.853	-	-
Formocresol	58.094*	75.762*	16.984	11.771	9.918	-

**** Statistical differences (Tukey's critical value=46.108).***

and genes involved in the inflammatory process to better understand this and provide improved clinical assessment of primary teeth pulp pathology.

CONCLUSIONS

Based on this study's results, the following conclusions can be made:

1. Primary tooth fibroblasts can respond immunologically, and different pulp capping materials can help in the modulation of this process.
2. Calcium hydroxide stimulates the production of IL-1β without stimulating IL-8.
3. Adhesive resin and formocresol, meanwhile, stimulate the production of IL-8 and do not stimulate the production of IL-1β.
4. Mineral trioxide aggregate stimulates both cytokines, but at an intermediate level compared to the other materials.

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