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Prostaglandin E_2 production and viability of cells cultured in contact with freshly mixed endodontic materials

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Abstract

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Aim To determine whether commonly used endodontic sealers could either induce or increase the release of prostaglandin E_2 (PGE_2) when in contact with cell types found in the periapical tissues.

Methodology Freshly mixed samples of Roth 801 sealer, Sealapex® and ProRoot® mineral trioxide aggregate (MTA) were placed in contact with cultured macrophages and fibroblasts for 24 h. The supernatant from the cultures was assayed for PGE_2 using enzyme-linked immunosorbent assay. Cell viability counts were made. As a positive control, similar cultures were also exposed to lipopolysaccharide and the supernatant analysed for PGE_2. Data were compared by ANOVA.

Results The three materials examined in these experiments did not stimulate increased PGE_2 release from either of the cell lines. In control cultures, lipolysaccharide increased PGE_2 release from macrophages but not from fibroblasts. Viability counts revealed that, whilst Roth 801 sealer caused some cell death in both fibroblasts and macrophages, Sealapex® led to cell death only in the macrophage cultures. ProRoot® MTA did not lead to statistically significant cell death in either culture.

Conclusions Under 24-h culture conditions, the three freshly mixed test materials did not increase directly either production or release of PGE_2 from either macrophages or gingival fibroblasts. Roth 801 decreased cell viability counts for both fibroblasts and macrophages. Sealapex® decreases macrophage viability. ProRoot® MTA did not affect viability in either cell line.

Keywords: endodontic sealer, enzyme-linked immunosorbent assay, fibroblasts, macrophages, prostaglandin, viability.

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Introduction

Following root canal treatment, discomfort occurs in a proportion of endodontic patients (Harrison *et al.* 1983, Genet *et al.* 1986). The painful response is inflammatory in origin either as the continuation of a preoperative condition and/or as a response to new irritation. One possible source of new or additional irritation is the endodontic sealer used to enhance the apical seal. The effect of these materials in their set condition has been examined *in vitro* (Willershausen *et al.* 2000, Pistorius *et al.* 2003), but there are few data on the effect of these materials when freshly mixed which may be more relevant to the immediate post-filling period.

The principal mediators of inflammatory pain are products of the arachadonic acid pathway most noticeably prostaglandin E_2 (PGE_2) (Juan 1978, Cohen *et al.* 1985, Samad *et al.* 2002). This has been found in inflamed periapical tissue (McNicholas *et al.* 1991, Takayama *et al.* 1996, Shimauchi *et al.* 1997) and is released from mouse macrophages when exposed to gutta-percha particles (Sjögren *et al.* 1998) and from

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fibroblasts when exposed to a mixture of gutta-percha and calcium hydroxide but not when exposed to Sealapex® (Willershausen *et al.* 2000). Mineral trioxide aggregate (MTA) has also been linked to elevated levels of PGE_2 synthesis by gingival fibroblasts (Pistorius *et al.* 2003). Components of the sealers or reaction products resulting from their interactions may directly induce PGE_2 release or effect it by local changes in pH (Willershausen *et al.* 2000). It seems reasonable to suggest that materials that induce more PGE_2 to be released are more likely to cause pain and contribute, should they come into contact with the periapical tissues, to post-filling discomfort.

The aim of the present study was to assess the release of PGE_2 from macrophages (the most prominent producers of arachadonic acid metabolites in the early stages of the immune response) during their exposure to freshly mixed endodontic sealers. The response of gingival fibroblasts as a nonmyeloid control for which some data are already available was also evaluated. Some endodontic sealers are cytotoxic (Willershausen *et al.* 2000, Huang *et al.* 2002, Schwarze *et al.* 2002). To avoid the possibility that cell death might be responsible for apparent differences in PGE_2 levels, cell viability was also estimated. Three materials, Roth 801 sealer (Roth International, Chicago, IL, USA), Sealapex® (Kerr Sybron, Orange, CA, USA) and tooth-colored ProRoot® MTA (Dentsply, Tulsa Dental, Tulsa, OK, USA) were examined.

Materials and methods

Cell lines

Two cell lines, macrophages (RAW 267.4; ATCC, Manassas, VA, USA) and gingival fibroblasts (donated by C. T. Hanks, University of Michigan) were used. Both were derived from mice.

Cell culture

Cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Gibco BRL), 250 μg mL^{-1} L-glutamine, 125 U mL^{-1} penicillin and 125 U mL^{-1} streptomycin, and placed in a humidified 5% CO_2 incubator at 37 °C.

Controls

A line of inflammatory cells, macrophages, and a line of connective tissue cells, gingival fibroblasts, were exposed to the root canal sealing materials. As a positive control, to confirm that the macrophages would form and release PGE_2, both cell lines were stimulated with *Escherichia coli* lipopolysaccharide (LPS, serotype O5:55; Sigma, St Louis, MO, USA). As a negative control, cultures of both cell lines were not exposed to the materials but otherwise maintained under the identical conditions.

Enzyme-linked immunosorbent assay

An enzyme-linked immunosorbent assay kit (ELISA) (R & D Systems, Minneapolis, MN, USA) was used to evaluate the concentration of PGE_2 in the supernatant collected from cell cultures maintained in contact with the materials.

Viability tests

The percentage of viable cells was calculated using the Trypan Blue Dye Exclusion Test McAteer & Davis 2002 on a small sample from each well using a haemocytometer after 24 h of exposure to the test materials.

Experimental procedure

Five × 10^4 cells were seeded into each well of 12-well culture plates and allowed to attach overnight. The cell culture medium in each well was changed after 24 h. After a further 24 h, 100 mg (sufficient to cover the floor of a Transwell insert) of the freshly mixed materials (prepared according to the manufacturer's guidelines) was placed on 12-mm diameter, 0.4 μm pore size Transwell inserts (Corning Costar Corporation; Corning, NY, USA). The inserts were placed in the wells in contact with the media and the experimental materials were allowed to diffuse into the media for 24 h. The supernatant was then collected and the concentration of PGE_2 was measured by ELISA. Each experiment was carried out in triplicate and three independent series of experiments were performed resulting in nine replications of each condition. Three wells with cells were not exposed to the test material.

Lipopolysaccharide stimulation

Twelve-well plates were seeded with the two cell lines as above. After 24 h incubation, the media were replaced with new media in which LPS had been dissolved in concentrations of 0, 0.1, 0.5 & 2.0 μg mL^{-1} and incubated for a further 24 h.

The supernatant was collected and analysed for PGE_2 production by ELISA in triplicates.

Statistical analyses

Data were analysed using GRAPHPAD software (GraphPad Software, Inc., San Diego, CA, USA) by one-way ANOVA followed by Bonferroni's *post hoc* test to determine where significant differences lay ($P < 0.05$).

Results

The data summary, as included in the Figs 1–6, reports the mean and SD for all readings combined from all repetitions. Macrophages did not increase PGE_2 release after 24-h exposure to the test materials (Fig. 1), but did increase release of PGE_2 after 24-h exposure to LPS. (Fig. 2) Fibroblasts did not increase PGE_2 release after exposure to the test materials (Fig. 3) nor did they increase PGE_2 release after exposure to LPS (Fig. 4). Macrophage viability was reduced by exposure to both Roth 801 and Sealapex but not by exposure to ProRoot® MTA (Table 5). Fibroblast viability was reduced by exposure to Roth 801 but not by exposure to either Sealapex® or ProRoot® MTA (Table 6).

Discussion

The three materials examined in these experiments did not stimulate increased PGE_2 release from either of the cell lines examined. In the control cultures, LPS induced increased PGE_2 release from the macrophages. Although the fibroblasts released PGE_2 with no stimulation, the release was not increased by LPS. Thus, both cell lines maintained their ability to synthesize PGE_2 under these culture conditions.

The findings on PGE_2 agree, at least in part, with earlier studies, which have used similar *in vitro* models with macrophages and fibroblasts in culture but which examined different combinations of materials, cells and mediators. Neither macrophages nor fibroblasts produced interleukins-β or -6 after exposure to MTA though they did after exposure to LPS (Haglund *et al.* 2003). One report describes no significant PGE_2 release from fibroblasts and epithelial cells exposed to Sealapex (Willershausen *et al.* 2000) though another recorded levels of PGE_2 higher than controls from fibroblasts exposed to MTA for 4 days (Pistorius *et al.* 2003). This differs from the results of the present study but the exposure period was much longer (96 h) and a similar elevation occurred following the exposure to titanium

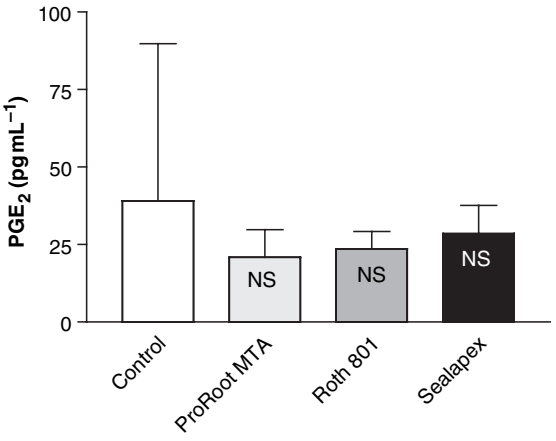

Figure 1 Prostaglandin E_2 production (pg mL^{-1}) by macrophages exposed to test materials. NS, not statistically significantly different from control.

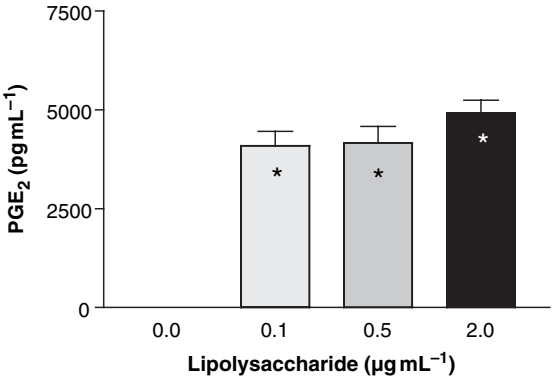

Figure 2 Prostaglandin E_2 production (pg mL^{-1}) by macrophages stimulated with lipopolysaccharide (LPS) (Control). *Statistically significantly different from control ($P < 0.05$).

(Pistorius *et al.* 2003). Gutta-percha in particulate form does induce macrophages to release PGE_2, but this too was seen after a longer exposure (72 h) than used in the presently reported experiments (Sjögren *et al.* 1998).

The present study establishes only that contact with freshly mixed materials does not directly induce PGE_2 release. *In vivo*, cytokines released from other stimulated cell types may act on macrophages and fibroblasts to release PGE_2. Osteoblasts exposed to MTA produce elevated levels of interleukins-1 and -6 (Koh *et al.* 1998). Both of these interleukins can cause the release of PGE_2 from fibroblasts (Richards & Rutherford 1988, Nichols & Maraj 1998, Lahiri *et al.* 2001).

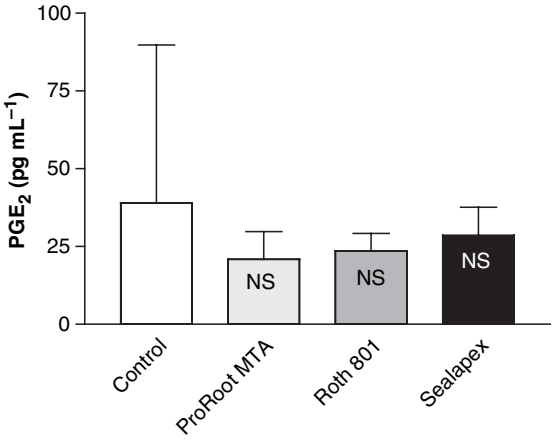

Figure 3 Prostaglandin E_2 production (pg mL^{-1}) by fibroblasts exposed to test materials. NS, not statistically significantly different from control.

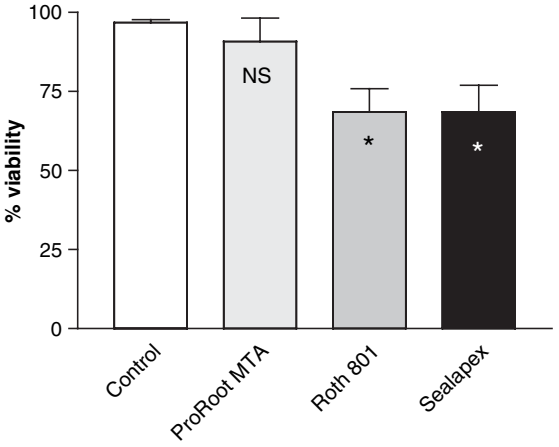

Figure 5 Macrophage viability (%). NS, not statistically significantly different from control. *Statistically significantly different from control ($P < 0.05$).

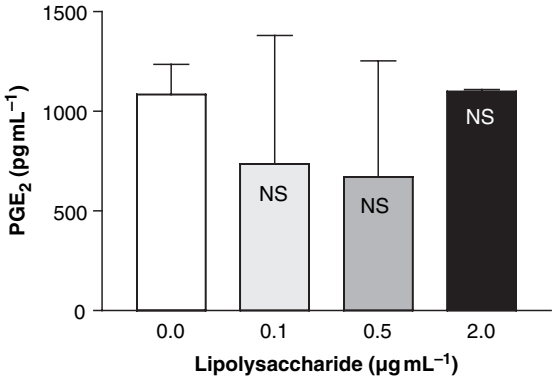

Figure 4 Prostaglandin E_2 production (pg mL^{-1}) by fibroblasts stimulated with LPS. NS, not statistically significantly different from control.

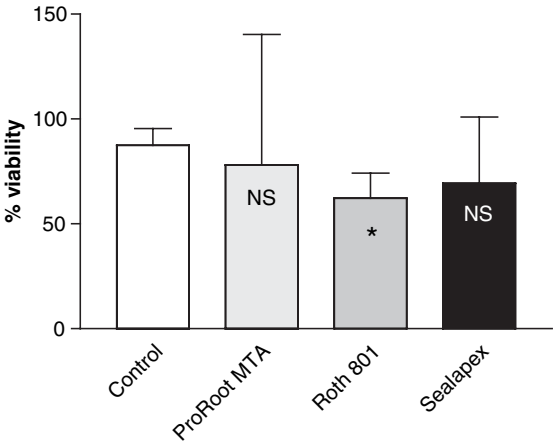

Figure 6 Fibroblast viability (%). NS, not statistically significantly different from control. *Statistically significantly different from control ($P < 0.05$).

The relatively high level of PGE_2 found in fibroblast cultures even in control conditions in our study confirms fibroblasts as high level producers of PGE_2 (Jordana *et al.* 1994). There was considerable variability in the values of PGE_2 concentration between different iterations of the experiments resulting in large SDs in the results as presented here. Viability counts were conducted largely to ensure that cell death did not obscure the response of the cells. When allowing for the reduced number of cells in each culture well after exposure to the materials, the differences in PGE_2 production between the materials were not statistically significant.

These findings on cell survival also confirm the work of others in that, whilst both the Roth 801 (ZOE-based sealer) and Sealapex (calcium hydroxide-based sealer) did induce some cell death (Beltes *et al.* 1995, Yoshimine *et al.* 2003), MTA did not (Pistorius *et al.* 2003, Saidon *et al.* 2003). These earlier studies used differing approaches to measure cell viability. The dye exclusion method adopted in this study should be interpreted with caution. Dye uptake marks cells that have disrupted membranes and may not detect other forms of injury that may later lead to cell death. Nor does this method account for cells that have fully lysed. Nonetheless, dye exclusion data are comparable between the different materials within the experiment and the consistency with other approaches is reassuring.

These findings are consistent with the overall impression that MTA is highly biocompatible whilst eugenol containing materials are seriously and calcium hydroxide-based materials somewhat cytotoxic (Hunag *et al.* 2001, Huang *et al.* 2005). The data presented here add only a very small increment to our understanding of the genesis of post-obturation discomfort. The pathway leading from tissue injury and toxicity to the activation of nociceptors is a complex one and many more steps require examination.

Conclusions

Under the culture conditions, none of the three freshly mixed test materials, Roth 801 sealer, Sealapex, and MTA, acts directly on either macrophages or gingival fibroblasts to increase the production of PGE_2. Gingival fibroblasts under the unstimulated conditions release significant amounts of PGE_2. MTA did not significantly reduce cell viability for either cell line. Roth 801 and Sealapex significantly decreased cell viability for macrophages, whilst only Roth 801 decreased fibroblast viability.

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