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ORIGINAL ARTICLE

Investigating unset endodontic sealers' eugenol and hydrocortisone roles in modulating the initial steps of inflammation

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Abstract

Introduction Endodontic treatment success is achieved not only when the cement provides a hermetic seal but also when the injured periapical tissue is regenerated. However, an exaggerated inflammatory reaction hinders tissue regeneration and it has been shown that dental materials affect the inflammatory response through modulation of cytokine secretion. This work was set to investigate the effects of the presence of hydrocortisone in zinc oxide eugenol sealers (Endomethasone N) on modulating the initial steps of inflammation in vitro.

Material and methods Hydrocortisone and eugenol leaching from Endomethasone N and Pulp Canal Sealer (PCS) were quantified by ELISA and spectrofluorometry, respectively. The effects of Endomethasone N and Pulp Canal Sealer were studied on lipopolysaccharides (LPS)-stimulated human periodontal ligament (hPDL) cells. Cytokine (IL-6, TNF-α) secretion from cells was quantified by ELISA. Inflammatory cell (THP-1) adhesion to activated endothelial cells, their migration and activation were studied in vitro.

Results Endomethasone N decreased secretion of IL-6 and TNF-α from hPDL cells. THP-1 adhesion to activated endothelial cells (HUVECs) and migration significantly decreased with Endomethasone N while no effect was observed with PCS. Activation of THP-1 decreased with both materials' extracts but was significantly lower with Endomethasone N than with PCS.

Conclusion These results performed in vitro show that Endomethasone N anti-inflammatory effects are due to the presence of hydrocortisone.

Clinical relevance Endomethasone N has potential local anti-inflammatory effects which appear to be due to its hydrocortisone rather than eugenol content. Decreasing the inflammatory response is a pre-requisite to initiate the periapical healing.

Keywords Endodontic sealer · Periodontal ligament inflammation · Zinc oxide eugenol · Hydrocortisone

Introduction

Localized periapical lesions consecutive to bacterial infection of the root canal system are frequently reported [1]. Initiating the host inflammatory reaction is essential to prevent bacterial proliferation in the periapical tissues [2]. The cellular response of inflammation implies immune cell recruitment. Cells such as neutrophils and monocytes adhere to the activated vascular endothelium and, following a chemotaxis gradient, migrate to the injury site where it can be activated into macrophage-like cells [3]. After activation, they carry out phagocytosis of dead cell debris and pathogens.

Many cytokines such as interleukins and growth factors are involved in the inflammatory events. Human periodontal ligament (hPDL) cells are known to secrete pro-inflammatory cytokines locally such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) [4–6]. IL-6 trans-signaling leads to the immune response activation by inducing monocytes recruitment to the inflamed area, inhibition of T-cells apoptosis, and differentiation [7]. TNF-α is considered as a major inflammatory mediator. It is synthesized in response to the presence of a variety of bacteria and bacterial products, especially lipopolysaccharides (LPS). LPS is a Gram-negative bacteria component [8]. The involvement of Gram-negative bacteria in root canal infection has been well demonstrated

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[9], and LPS presence was correlated to endodontics symptoms in necrotic teeth [10]. In addition, LPS stimulation led to periapical tissue inflammation and destruction [11, 12]. Additionally, previous works shown that hPDL cell stimulation with LPS induced expression and/or secretion of pro-inflammatory cytokines such as IL-6, IL-8, or monocyte chemoattractant protein-1 (MCP-1) [13, 14]. Furthermore, the local cellular effects of TNF-α include the capacity to induce vasodilatation through the superoxide burst to allow polymorphonuclear leukocytes to bind to vascular endothelium and activate phagocytosis [15]. It should be reminded however that the inflammatory reaction has some tissue deleterious effects as it may induce the destruction of periodontal tissues. Indeed, a large number of molecules, involved in the inflammatory reaction such as prostaglandins, bacterial endotoxins, and complement molecules or pro-inflammatory cytokines, has been reported to activate bone resorption by osteoclasts [16, 17].

The root canal treatment is considered as the most effective and conservative method for treating periapical lesions. Endodontic treatment is achieved not only after canal shaping, disinfection and canal hermetic sealing to prevent the canal re-infection, but also when the periapical tissue is regenerated [18]. Following the root canal filling, an interaction frequently occurs between the root canal sealer and periapical tissues including the periodontal ligament and potentially alveolar bone, leading to an initial inflammation and subsequent regeneration [19]. These processes are highly dependent on the inflammation arrest. Moreover, it has been shown that dental materials can modulate the inflammatory reaction through modulation of cytokine secretion [20]. Thus, the therapeutic success depends not only on the endodontic treatment, but also on the ability of the host to resolve the inflammatory response [21–23].

Zinc oxide eugenol sealers (ZOE) have been successfully used since decades for root canal filling in combination with gutta-percha. These sealers are known to have good sealing properties and antimicrobial activity. But they have prolonged setting time, a high solubility [24], and cytotoxic effects to the periodontal cells [25]. ZOE sealers are also widely used for their reported anti-inflammatory effects due to eugenol. Yet, eugenol effects on dental tissues are controversial [26]. Furthermore, a localized inflammation with zinc oxide eugenol sealers has been observed, both in soft tissue and in the bone [27].

Pulp Canal Sealer (PCS) (SybronEndo, Orange, CA, USA) and Endomethasone N (Septodont, Saint-Maur-des-Fossés, France) are zinc oxide eugenol sealers. However, Endomethasone N also contains hydrocortisone (Table 1). This raises questions about the local effects of hydrocortisone in this sealer's composition. This work was designed to determine the influence of these sealers: (1) on human periodontal ligament cell pro-inflammatory cytokine production, (2) on the initial steps of inflammation, and (3) to investigate the effect of the presence of hydrocortisone on these events. Extracts of the abovementioned sealers were applied on LPS-stimulated hPDL cells. The inflammatory response of hPDL cells was investigated through the secretion of pro-inflammatory cytokines and by investigating their effects on inflammatory cell adhesion, migration, and pro-inflammatory activation of monocytes/macrophages.

Materials and methods

Reagent

Media, reagents, and cell culture supplies were from Dutscher (Brumath, France). LPS (*Escherichia coli*) was from InvivoGen (San Diego, CA, USA).

Primary periodontal ligament cell cultures

hPDL cells were prepared from immature third molars, freshly extracted for orthodontics reasons in compliance with French legislation (informed patient consent and institutional review board approval of the protocol used), by the explant outgrowth method [28]. The teeth were obtained from three different donors for each experiment (4 M/donor). Cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, glutamine 2 mM, penicillin 100UI/mL, streptomycin 100 μm/mL, and amphotericin B 0.25 μg/mL, at 37 °C in a 95% air 5% CO2 atmosphere. The same culture medium was used for further experimentation but without serum.

Material extract preparation

Samples of Endomethasone N and Pulp Canal Sealer were prepared according to the manufacturers' instructions. Each sample was incubated in serum-free MEM 24 h at 37 °C to obtain unset material extracts (20 mg/mL). The resulting material extracts were filtered on 0.22 μm filters. Hydrocortisone was solubilized in DMSO at 100 mM, diluted to 1 mM in serum-free MEM and incubated at 37 °C for 24 h. Eugenol was solubilized in DMSO at 6 M, diluted at 100 mM in serum-free MEM and incubated at 37 °C for 24 h. The four conditioned media were diluted in serum-free MEM to obtain the working concentration in the next steps of the experimental protocol. At these final concentrations of hydrocortisone and eugenol used in our experiments, DMSO content was lower than or equal to 0.001%. This has been reported to be a nontoxic concentration [29].

Quantitative determination of hydrocortisone and eugenol concentrations in the material extracts

Hydrocortisone concentration from samples of Endomethasone N and PCS extracts (20 mg/ml) was determined by enzyme immunoassay according to the

Table 1 Composition of the sealers used

Endodontic sealer	Powder	Liquid
Endomethasone N	Hydrocortisone 1%	Eugenol 50–100%
	Iodothymol 10–25%	Anethol 2.5–10%
	Barium sulfate 15%	Menthol 2.5%
	Zinc oxide 25–50%	Dipentene 1%
Pulp canal sealer	Zinc oxide 34–41%	Eugenol 78–80%
	Precipitated silver 25–30%	Canada balsam 20–22%
	Oleo resins 30–16%	
	Thymol iodide 11–12%	

manufacturer's instructions (Parameter Hydrocortisone Assay; R&D Systems).

Eugenol concentration from samples of Endomethasone N and PCS extracts (20 mg/ml) was determined by spectrofluorimetry as described [30].

Material extract toxicity

hPDL cells were cultured at confluency in 96-well plates. The cells were stimulated with LPS (1 μg/mL) for 4 h and incubated either with the material extracts (20/2/0.2 mg/mL), eugenol (10/1/0.1 μM), hydrocortisone (100/10/1 μM), or serum-free MEM control media. After 24 h, the supernatants were removed and MTT test (Sigma-Aldrich, St Louis, MO) was performed with the MTT substrate (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) as described [31].

Cytokine secretion by hPDL cells

hPDL cells were cultured at confluency in 12-well plates. Cells were stimulated for 4 h with LPS (1 μg/ml) and incubated with material extract (0.2 mg/mL), eugenol (0.1 μM), hydrocortisone (1 μM), or serum-free MEM control media. After 24 h, the supernatants were used for pro-inflammatory (IL-6 and TNF-α) cytokines quantification by enzyme-linked immunosorbent assay (ELISA) using Duoset kits (R&D Systems) according to the manufacturer's instructions.

THP-1 cell recruitment sequence

Human umbilical vein endothelial cells and inflammatory THP-1 cell culture

Human umbilical vein endothelial cells (HUVECs) (PromoCell, Heidelberg, Germany) were cultured in Endothelial Cell Growth Medium 2 (ECGM 2, PromoCell). THP-1 cells, a human monocytic cell line (Sigma, St Quentin Fallavier, France), were cultured in RPMI medium supplemented (10% FBS, 100UI/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, 2 mM L-glutamine, 1 mM sodium pyruvate), as described [20].

THP-1 cell adhesion on endothelial cells

hPDL cells were cultured at confluency in 12-well plates. Cells were stimulated for 4 h with LPS (1 μg/ml) and incubated with material extract (0.2 mg/mL), eugenol (0.1 μM), hydrocortisone (1 μM), or serum-free MEM control media. After 24 h, the supernatant was recovered and called conditioned medium.

Confluent HUVECs (80,000 cells/cm^2), in 12-well plates, were cultured with conditioned medium for 4 h at 37 °C. THP-1 cells were first incubated with 0.1 μM of BCECF acid (2′,7′-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein) for 1 h. BCECF-labeled THP-1 cells (25,000 cells/mL) were then added to the HUVECs monolayers for 30 min at 4 °C under gentle orbital rotation as described [32]. HUVECs were then washed four times with phosphate-buffered saline with calcium and magnesium (PBS), and THP-1 adherent cells were counted in five random fields using a fluorescent microscope (× 100). Results are expressed as percentage of the control.

THP-1 cell migration

Cell migration was assayed with Boyden chambers (8 μm pore size) in 12-well plates. Confluent hPDL cells, cultured in the lower chambers, were stimulated with LPS (1 μg/ml) for 4 h and then incubated with material extract (0.2 mg/mL), eugenol (0.1 μM), hydrocortisone (1 μM), or serum-free MEM control media for 24 h. THP-1 cells were activated with phorbol myristate acetate (PMA 400 ng/mL, 24 h) and seeded (15,000 cells/100 μl) in the upper chambers. After migration for 24 h, THP-1 cells on the top side of the filter were wiped off using a cotton bud, and cells that migrated to the lower surface of the filter were fixed (15 min, cold ethanol 70%) and stained with eosin (20 min). The number of migrating cells to the lower surface of the filter membrane was counted in five

random fields using light microscopy (× 100). Results are expressed as percentage of control.

THP-1 cell activation

hPDL cells were cultured as under "THP-1 cell adhesion on endothelial cells" to obtain the conditioned medium.

THP-1 suspension (25,000 cells/mL) was cultured with hPDL cells conditioned medium in 12-well plates for 24 h. Then, the wells were washed four times with PBS to remove not activated and dead cells. Adherent cells were fixed (15 min, cold ethanol 70%) and nuclei stained with DAPI (4′,6-diamidino-2-phenylindole) (1 μg/ml). The number of activated (adherent) cells was counted in five random fields using a fluorescent microscope (× 100). Results are expressed as percentage of the control.

Statistical analysis

All experiments were done in triplicate and repeated three times. Data are expressed as means ± standard error of mean. Student *t* test was used to determine statistical significance (p value < 0.05).

Results

Quantitative determination of hydrocortisone and eugenol concentration in the sealer extracts

After 24 h of incubation of sealer samples at 37 °C, 85 ± 9.9 μM of hydrocortisone was released from Endomethasone N (20 mg/ml) in serum-free MEM medium (Fig. 1a). No hydrocortisone was detected with PCS.

After 24 h of incubation of sealer samples at 37 °C, 10 ± 1.6 μM of eugenol was released from Endomethasone N (20 mg/ml) and 10 ± 2.0 μM from PCS (20 mg/ml) in serum-free MEM medium (Fig. 1a).

Endodontics sealer extracts toxicity

LPS-stimulated hPDL cell viability decreased significantly after 24 h of contact with the medium containing Endomethasone N and PCS extracts at 20 mg/ml, 100 μM of hydrocortisone, and 10 μM of eugenol as compared to the control medium (Fig. 1b). PCS also induce a significant decrease in cell viability at 2 mg/ml.

No statistically significant effect was observed on hPDL cell viability with 0.2 mg/ml sealer extract, 1 μM hydrocortisone, and 0.1 μM eugenol (Fig. 1b) compared to the control. These nontoxic working concentrations were chosen for the next step.

Endodontics sealers modified cytokine secretion

Applying hydrocortisone, eugenol, and Endomethasone N for 24 h led to a significant decrease of the pro-inflammatory IL-6 and TNF-α secretion. PCS induced a significantly higher secretion of IL-6 but had no effect on TNF-α compared to the control condition (Fig. 2).

Inflammatory cell recruitment modulation

Inflammatory THP-1 cell adhesion on HUVECs monolayer assay is illustrated with representative pictures used for cell counts (Fig. 3Aa-e). THP-1 adhesion significantly decreased with hydrocortisone and Endomethasone N while no effect was observed with PCS and eugenol compared to the control condition (Fig. 3B). THP-1 cell migration significantly decreased only with hydrocortisone and Endomethasone N extracts (Fig. 4). Inflammatory THP-1 cell activation assay is illustrated with representative pictures used for cell counts (Fig. 5Aa-e). This activation decreased with all material extracts but was significantly lower with hydrocortisone and Endomethasone N as compared to PCS and eugenol (Fig. 5B).

Discussion

This work shows that, after simulating hPDL cell bacterial infection, applying endodontics sealers on these cells affects the local inflammatory response. It demonstrates the efficiency of hydrocortisone-containing root canal sealer (Endomethasone N) in modulating the initial steps of inflammation.

ZOE sealers have been widely used in root canal treatment because of their handling properties, reasonable cost, excellent sealing ability, and therapeutic effects including sedative, antibacterial, and anti-inflammatory effects [33]. However, ZOE have some drawbacks such as cytotoxicity and harm to surrounding periodontal tissues [34].

During endodontic obturation with gutta-percha, the unset endodontic sealer may exceed the root apex and release substances that could reach and interact with inflamed periodontal tissues. To simulate, in vitro, this clinical situation, hPDL cells were stimulated with LPS and then cultured in contact with endodontics sealer extracts (Endomethasone N and PCS). Our results confirmed a high toxicity of zinc oxide eugenol sealers at 20 mg/ml. This is consistent with previous works showing the toxicity of PCS [35, 36]. However, none of the two root canal sealers or eugenol used alone had cytotoxic effects at the lowest concentration on stimulated hPDL cells. After hydrolysis in water, these sealers release zinc hydroxide and eugenol. Eugenol is derived from essential oil which is known for its antimicrobial, anti-inflammatory, and anti-oxidant properties which have been evaluated in a variety of models [37, 38].

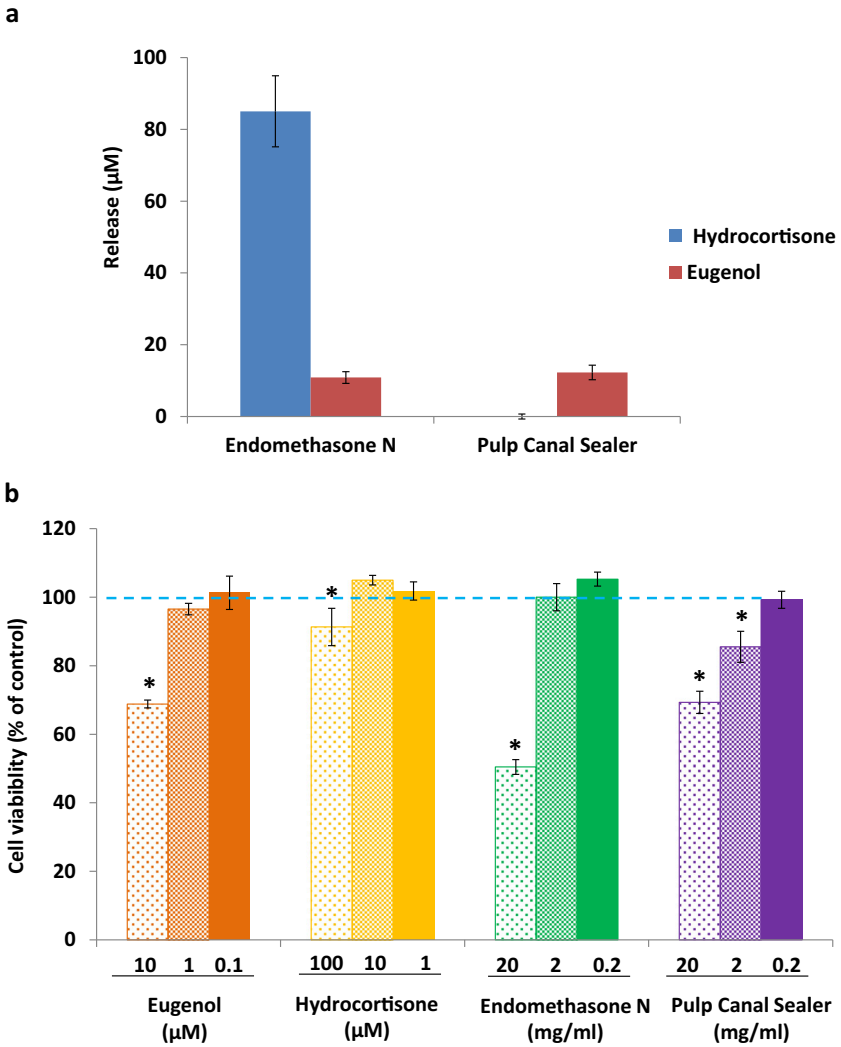

Fig. 1 **a** Quantification of hydrocortisone and eugenol concentrations in the materials extracts. The quantification of hydrocortisone was performed using the ELISA assay and eugenol by spectrophotometry. After 24 h of incubation at 37 °C, 85 ± 9.9 μmol/L of hydrocortisone was released from Endomethasone N (20 mg/ml) in serum-free MEM media. No hydrocortisone was detected in PCS extracts or in the control condition. Eugenol was detected in Endomethasone N (10 ± 1.6 μmol/L) and PCS (10 ± 2.0 μmol/L) extracts. **b** Cell viability after contact with the materials extracts. A significant decrease in hPDL cell viability was observed after 24 h of contact with the media containing Endomethasone N and PCS extracts (20 mg/ml), hydrocortisone (100 μM), and eugenol (10 μM) as compared to the control medium. A significant decrease in cell viability was observed with PCS extracts at 2 mg/ml. No statistically significant effect was observed in hPDL cell viability with both sealer extracts at 0.2 mg/ml, 1 μM hydrocortisone nor 0.1 μM of eugenol as compared to the control. Results are expressed in percentage of the control. (*) corresponds to significant differences as compared to the control (p value < 0.05)

To investigate the effect of Endomethasone N and PCS on modulating the inflammatory response of hPDL cells, we first quantified eugenol and hydrocortisone release from the sealers. Our results show that PCS and Endomethasone N released the same concentration of eugenol. But hydrocortisone was released only from Endomethasone N. Then, we used equivalent concentrations of eugenol and hydrocortisone to link our findings to those obtained after leaching from the sealers. Our work show that Endomethasone N, hydrocortisone, and eugenol inhibited the secretion of pro-inflammatory cytokines (IL-6 and TNF-α) but not PCS. Thus, our data confirm eugenol anti-inflammatory potential when used alone while these effects are not observed in PCS extracts. This result is in agreement with a previous investigation on ZOE sealers where Intermediate Restorative Material and Tubli-Seal sealer extracts were compared to eugenol anti-inflammatory effects [39]. When eugenol was used alone, it was found to inhibit inflammatory mRNA gene expression in LPS-pre-treated mouse bone marrow monocytes while these effects were not observed in the sealer extracts. This was also demonstrated with the use of the C3 cross immunoelectrophoretic technique where complement system activation was stimulated with N2/RC2B root canal sealer and eugenol [40]. This suggests that the anti-inflammatory effect of

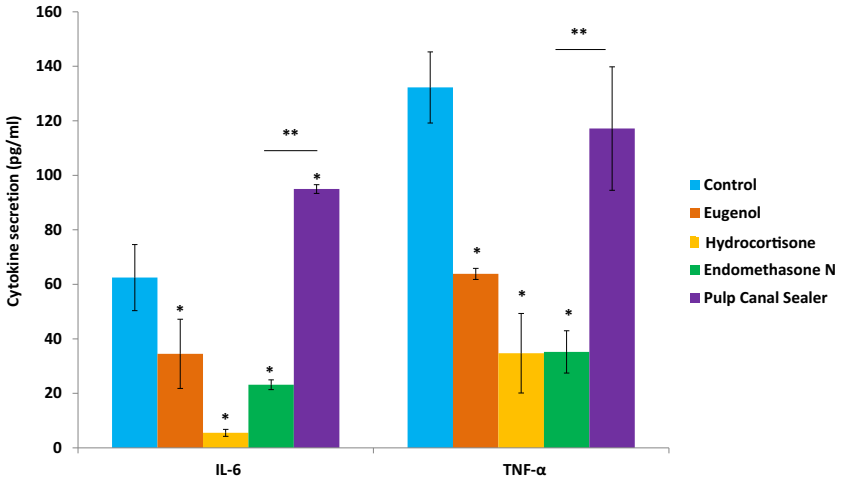

Fig. 2 Effects of sealers on cytokine secretion. A significant increase of IL-6 secretion by LPS-stimulated hPDL cells was observed after 24 h of incubation with PCS. This secretion significantly decreased with hydrocortisone, eugenol, and Endomethasone N. A significant decrease in TNF-α secretion was also observed with hydrocortisone and Endomethasone N as compared to the control and PCS. Results are expressed in picograms per milliliter. (*) corresponds to significant difference as compared to the control, (**) represents significant differences between two conditions (p value < 0.05).

Endomethasone N observed in our work can be attributed to hydrocortisone but not to eugenol.

We further confirmed these results on cytokine secretion by investigation of the anti-inflammatory effect of hydrocortisone-containing material through adhesion of inflammatory cells on the activated endothelium, their migration towards the stimulated cells, and their activation. Even if neutrophils are strongly recruited in vivo, monocytes, such THP-1, have been used to study the inflammation in vitro [41]. Co-culture of THP-1 cells with the HUVEC model has been also used to study inflammatory cell adhesion [42, 43]. Incubating LPS-stimulated hPDL cells with PCS extract and eugenol did not affect THP-1 adhesion on the HUVEC monolayer or their migration but significantly decreased their activation. However, when hPDL cells were stimulated with LPS and incubated with hydrocortisone or Endomethasone N, the number of migrated/activated THP-1 cells as well as THP-1 cell adhesion on HUVEC monolayer drastically decreased. In our

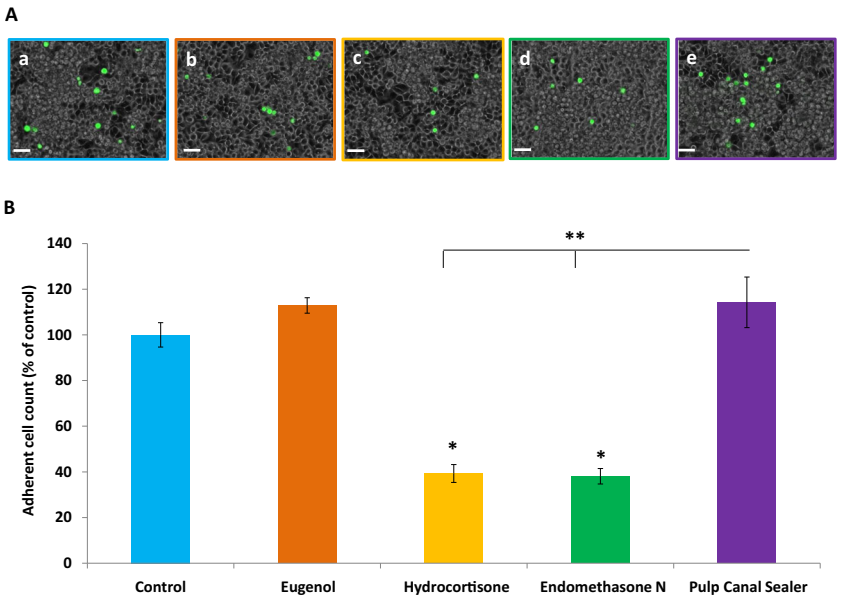

Fig. 3 Inflammatory THP-1 adhesion on endothelial cells (HUVECs). **A** Representative pictures on fluorescent microscopy of the adhesion assay of BCECF labeled THP-1 cells on endothelial cells for *a* control, *b* eugenol, *c* hydrocortisone, *d* Endomethasone N, and *e* PCS. Scale bars 50 μm. **B** Quantification of THP-1 adhesion on endothelial cells. Hydrocortisone and Endomethasone N extract significantly decreased THP-1 cell adhesion on HUVECs compared to eugenol, PCS, and to the control. Results are expressed in percentage of the control. (*) corresponds to significant differences as compared to the control; (**) represents significant differences between two conditions (p value < 0.05)

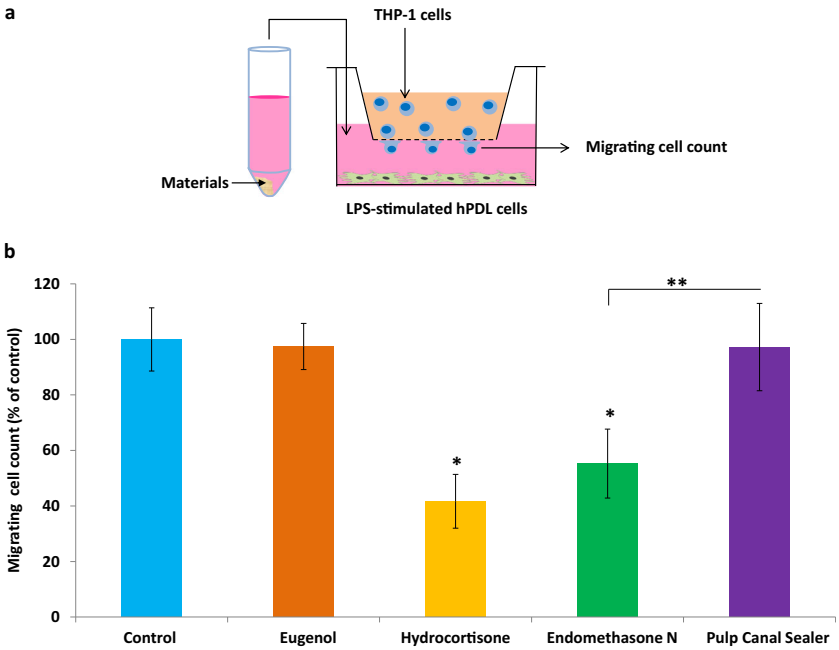

Fig. 4 THP-1 cell migration in Boyden chambers. **a** Schematic representation of Boyden chamber migration assay. **b** Migration assay in Boyden chambers after 24 h showed that hydrocortisone and Endomethasone N significantly reduced inflammatory THP-1 cell migration towards LPS-stimulated hPDL cells. This migration was not affected by PCS or eugenol. Results are expressed in percentage of control. (*) corresponds to significant difference as compared to the control; (**) represents significant differences between two conditions (p value < 0.05)

study, the anti-inflammatory effect of eugenol on cytokine secretion was not observed while that of hydrocortisone was confirmed at the cellular level. In line with these finding, the inflammatory effects of eugenol have been well demonstrated in vivo by investigating two ZOE sealers (Sargenti N2 and Grossman sealer) that do not contain hydrocortisone. After intentional overfilling in monkeys over a 6-month period [44], both ZOE sealers led to a severe irritation over the full 6-month duration. Other in vivo studies have reported a prolonged irritation effect of EndoFill, another hydrocortisone-containing ZOE sealer [45]. On the other hand, it has been shown that the subcutaneous tissue inflammation reactions to Endomethasone N decreased rapidly with time [46, 47]. While this protocol clearly demonstrates an anti-inflammatory effect of Endomethasone N, it does not

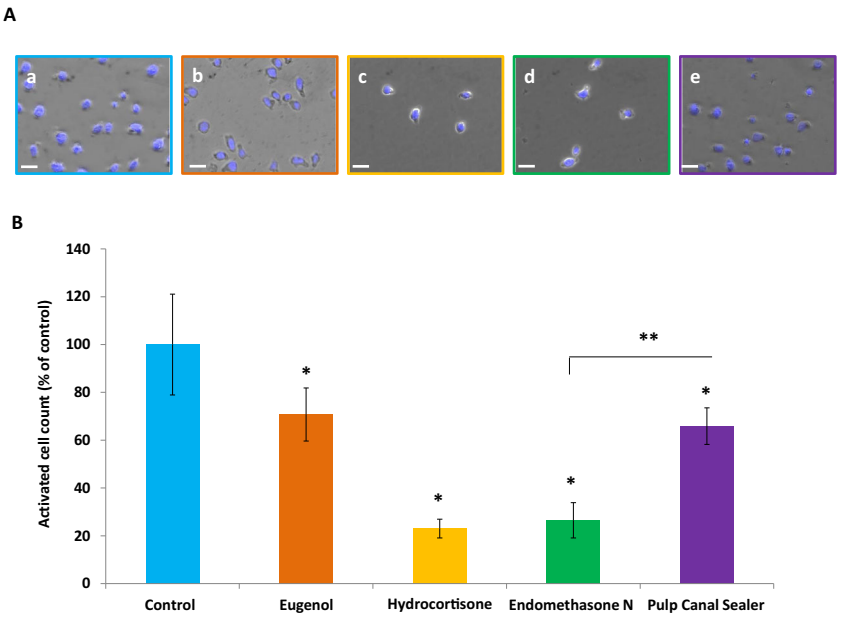

Fig. 5 THP-1 cell activation. **A** Representative pictures on fluorescent microscopy of THP-1 activation assay showing THP-1 activated cells after their incubation with material extracts for 24 h. *a* control, *b* eugenol, *c* hydrocortisone, *d* Endomethasone N, and *e* PCS. Scale bars 200 μm. **B** Quantification of THP-1 activation. THP-1 cell activation was significantly reduced by hydrocortisone and Endomethasone N and to a lesser extent by PCS and eugenol. Results are expressed in percentage of control. (*) corresponds to significant difference as compared to the control; (**) represents significant differences between two conditions (p value < 0.05)

demonstrate whether this effect is due to its eugenol or hydrocortisone local release.

Our investigation performed in vitro show that Endomethasone N has an anti-inflammatory potential. This is clearly demonstrated by the fact that these effects were not observed with eugenol alone neither with PCS which does not contain hydrocortisone. This anti-inflammatory effect may dampen the inflammatory reaction, enhance the subsequent periapical healing, and improve the clinical outcome. Whether such local anti-inflammatory effects can be observed using other sealers such as epoxy-resin-based or silicate-based sealers still remain to be investigated.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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