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Investigating endodontic sealers eugenol and hydrocortisone roles in modulating the initial steps of inflammation --Manuscript Draft--

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Abstract:	<p>Introduction</p> <p>Endodontic treatment success is achieved not only when the cement provides a hermetic seal but also when the 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 an hydrocortisone-containing sealer (Endomethasone N) modulatory effects on the initial steps of inflammation in vitro.</p> <p>Material and methods</p> <p>Hydrocortisone and eugenol leaching from the materials 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 and TGF-β1) secretion from cells were quantified by ELISA. Inflammatory cell (THP-1) adhesion to activated endothelial cells, their migration and activation were studied in vitro.</p> <p>Results</p> <p>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 all materials' extracts but was significantly lower with Endomethasone N than with PCS.</p>

	<p>Conclusion</p> <p>These results performed in vitro show that Endomethasone N has higher anti-inflammatory effects than PCS. Our results show that this effect is due to the presence of hydrocortisone in Endomethasone N</p> <p>Clinical Relevance</p> <p>It is the presence of hydrocortisone in Endomethasone N which is responsible for its local anti-inflammatory effects rather than eugenol.</p>
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Investigating endodontic sealers eugenol and hydrocortisone roles in modulating the initial steps of inflammation

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Keywords

Endodontic sealer, periodontal ligament inflammation, zinc oxide eugenol, hydrocortisone.

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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 a hydrocortisone-containing sealer (Endomethasone N) modulatory effects on 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. The presence of hydrocortisone in Endomethasone N is responsible for its local anti-inflammatory effects.

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 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) cell are known to secrete pro-inflammatory cytokines locally such as Interleukine 6 (IL-6) and Tumor necrosis factor (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. 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 [8]. 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 [9, 10].

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 reinfection, but also when the

periapical tissue is regenerated [11]. Following the root canal filling, an interaction frequently occurs between root canal sealer and periapical tissues including the periodontal ligament and potentially alveolar bone, leading to an initial inflammation and subsequent regeneration [12]. 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 [13]. Thus, the therapeutic success depends not only on the endodontic treatment, but also on the ability of the host to resolve the inflammatory response [14–16].

Zinc oxide eugenol sealers (ZOE) have been successfully used since decades for root canal filling in combinaison with Gutta Percha. These sealers are known to have good sealing properties and antimicrobial activity. But they have prolonged setting time, a high solubility [17] and cytotoxic effects to the periodontal cells [18]. ZOE sealers are also widely used for their reported anti-inflammatory effects due to eugenol. Yet, eugenol effects on dental tissues are controversial [19]. Furthermore, a localized inflammation with zinc oxide eugenol sealers has been observed, both in soft tissue and in the bone [20].

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 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 above mentioned sealers were applied on lipopolysaccharide (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 migration, adhesion, and pro-inflammatory activation of monocytes/macrophages.

MATERIALS AND METHODS

Reagent

Media, reagents and cell culture supplies were from Dutscher (Brumath, France).

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 [21]. The teeth were obtained from three different donors for each experiment (4 molars/donor). Cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, glutamine 2mM, penicillin 100UI/mL, streptomycin 100µm/mL, and amphotericin B 0.25µg/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 hours at 37°C to obtain unset materials 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 1mM in serum-free MEM and incubated at 37°C for 24 hours. Eugenol was solubilized in DMSO at 6M, diluted at 100 mM in serum-free MEM and incubated at 37°C for 24 hours. The four conditioned media were diluted in serum-free MEM to obtain the working concentration in the next steps of the experimental protocol.

Quantitative determination of hydrocortisone and eugenol concentrations in the materials extracts

Hydrocortisone concentration from samples of Endomethasone N and PCS extracts (20 mg/ml) was determined by enzyme immunoassay according to the 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 [22].

Material extract toxicity

hPDL cells were cultured at confluency in 96-well plates. The cells were stimulated with LPS (1 µg/mL) for 4h and incubated either with the materials 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 24h, 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 [23].

Cytokine secretion by hPDL cells

hPDL cells were cultured at confluency in 12-well plates. Cells were stimulated for 4h 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 24h the supernatants were used for pro-inflammatory (IL-6 and TNF-α) cytokines quantification by the 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 (HUVECs) and inflammatory THP-1 cells culture

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, 100µg/mL streptomycin, 0,25µg/mL amphotericin B, 2mM L-glutamine, 1mM sodium pyruvate), as described [13].

THP-1 cell adhesion on endothelial cells

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

Confluent HUVECs (80000 cells/cm²), in 12-well plates, were cultured with conditioned medium for 4 h at 37°C. THP-1 cells were first incubated with 0.1µM of BCECF acid (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein) for 1h. BCECF-labeled THP-1 cells (25000 cells/mL) were then added to the HUVECs monolayers for 30 min at 4 °C under gentle orbital rotation as described [24]. HUVECs were then washed four times with Phosphate Buffered Saline with calcium and magnesium (PBS) and THP-1 adherent cells were counted in 5 random fields using a fluorescent microscope (X100). Results are express 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 (1µg/ml) for 4 hours and then incubated with material extract (0.2 mg/mL), eugenol (0.1µM),

hydrocortisone (1 μ M) or serum-free MEM control media for 24h. THP-1 cells were activated with Phorbol Myristate Acetate (PMA 400ng/mL, 24h) and seeded (15000 cells /100 μ l) in the upper chambers. After migration for 24 hours, 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 (15min, cold ethanol 70%) and stained with eosin (20min). The number of migrating cells to the lower surface of the filter membrane was counted in 5 random fields using light microscopy (X100). Results are expressed as percentage of control.

THP-1 cell activation

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

THP-1 suspension (25000 cells/mL) was cultured with hPDL cells conditioned medium in 12 well plates for 24h. Then, the wells were washed four times with PBS to remove not activated and dead cells. Adherent cells were fixed (15min, cold ethanol 70%) and nuclei stained with DAPI (4',6-diamidino-2-phenylindole) (1 μ g/ml). The number of activated (adherent) cells was counted in 5 random fields using a fluorescent microscope (X100). Results are express as percentage of the control.

Statistical analysis

All experiments were done in triplicate and repeated three times. Data are expressed as means \pm 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 sealers extracts

After 24h of incubation of sealers 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 24h of incubation of sealers 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 24h of contact with the medium containing Endomethasone N and PCS extracts at 20mg/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 2mg/ml.

No statistically significant effect was observed on hPDL cells viability with 0.2 mg/ml sealers extract, 1 µM hydrocortisone and 0.1 µM eugenol (Fig. 1B) compared to the control. These non-toxic working concentrations were chosen for next step.

Endodontics sealers modified cytokine secretion

Applying hydrocortisone, eugenol and Endomethasone N for 24h, 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 control condition (Fig. 2).

Inflammatory cell recruitment modulation

Inflammatory THP-1 cell adhesion on HUVECs monolayer assay is illustrated with representative picture used for cell counts (Fig. 3A). THP-1 adhesion significantly decreased with hydrocortisone and Endomethasone N while no effect was observed with PCS and eugenol compared to control condition (Fig. 3B). THP-1 cell migration significantly decreased only with hydrocortisone and Endomethasone N extracts (Fig. 4). Inflammatory THP-1 cells activation assay is illustrated with representative pictures used for cell counts (Fig. 5A). This activation decreased with all materials 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 [25]. However, ZOE have some drawbacks such as cytotoxicity and harm to surrounding periodontal tissues [26].

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 sealers extracts (Endomethasone N and PCS). Our results confirmed a high toxicity of zinc-oxide eugenol sealers at 20mg/ml. This is consistent with previous works showing the toxicity of PCS [27, 28]. 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 anti-microbial, anti-inflammatory and anti-oxidant properties which have been evaluated in a variety of models [29, 30].

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

1 after leaching from the sealers. Our work show that Endomethasone N, hydrocortisone and
2 eugenol inhibited the secretion of pro-inflammatory cytokines (IL-6 and TNF- α) but not PCS.
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4 Thus our data confirm eugenol anti-inflammatory potential when used alone while these
5 effects are not observed in PCS extracts. This result is in agreement with a previous
6 investigation on ZOE sealers where Intermediate Restorative Material and Tubli-Seal sealers
7 extracts were compared to eugenol anti-inflammatory effects [31]. When eugenol was used
8 alone, it was found to inhibit inflammatory mRNA gene expression in LPS-pre-treated mouse
9 bone marrow monocytes while these effects were not observed in the sealers extracts. This
10 was also demonstrated with the use of the C3 cross immunoelectrophoretic technique where
11 complement system activation was stimulated with N2/RC2B root canal sealer and eugenol
12 [32]. This suggests that the anti-inflammatory effect of Endomethasone N observed in our
13 work can be attributed to hydrocortisone but not to eugenol.
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16 We further confirmed these results on cytokine secretion by investigation of the anti-
17 inflammatory effect of hydrocortisone containing material through adhesion of inflammatory
18 cells on the activated endothelium, their migration towards the stimulated cells, and their
19 activation. Even if neutrophils are strongly recruited *in vivo*, monocytes, such THP-1 have
20 been used to study of the inflammation *in vitro* [33]. Co-culture of THP-1 cells with HUVEC
21 model has been also used to study inflammatory cell adhesion [34, 35]. Incubating LPS-
22 stimulated hPDL cells with PCS extract and eugenol did not affect THP-1 adhesion on
23 HUVEC monolayer or their migration but significantly decreased their activation. However,
24 when hPDL cells were stimulated with LPS and incubated with hydrocortisone or
25 Endomethasone N, the number of migrated/activated THP-1 cells as well as THP-1 cell
26 adhesion on HUVEC monolayer drastically decreased. In our study, the anti-inflammatory
27 effect of eugenol on cytokine secretion was not observed while that of hydrocortisone was
28 confirmed at the cellular level. In line with these finding, the inflammatory effects of eugenol
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1 have been well demonstrated *in vivo* by investigating two ZOE sealers (Sargenti N2 and
2 Grossman sealer) that do not contain hydrocortisone. After intentional overfilling in monkeys
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4 over a 6-month period [36], both ZOE sealers led to a severe irritation over the full 6-month
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6 duration. Other *in vivo* studies have reported prolonged irritation effect of EndoFill, another
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8 hydrocortisone containing ZOE sealer [37]. On the other hand it has been shown that the
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10 subcutaneous tissue inflammation reactions to Endomethasone N decreased rapidly with time.
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12 This might be due to hydrocortisone local liberation [38, 39].
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17 Taken together, these results performed *in vitro* show that Endomethasone N has anti-
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19 inflammatory effects. Our results show that this effect is due to the presence of hydrocortisone
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21 rather than eugenol in Endomethasone N.
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Figure Legends

Figure 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 24h of incubation at 37°C, $85 \pm 9.9 \mu\text{mol/L}$ 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 ($10 \pm 1.6 \mu\text{mol/L}$) and PCS ($10 \pm 2.0 \mu\text{mol/L}$) extracts.

(B) Cell Viability after contact with the materials extracts.

A significant decrease in hPDL cell viability was observed after 24h 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).

Figure 2: Effects of sealers on cytokine secretion

A significant increase of IL-6 secretion by LPS stimulated hPDL cells was observed after 24h 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 pg/mL (*) corresponds to significant difference as compared to the control, (**) represents significant differences between two conditions (p-value < 0.05).

Figure 3: Inflammatory THP-1 adhesion on endothelial cells (HUVECs)

(A) Representative pictures on fluorescent microscopy of the adhesion assay of BCECF labelled THP-1 cells on endothelial cells for (a) control; (b) eugenol; (c) hydrocortisone; (d) Endomethasone N and (e) PCS. Scale bars: 50µm. (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).

Figure 4: THP-1 cell migration in Boyden chambers

(A) Schematic representation of Boyden chamber migration assay (B) Migration assay in Boyden chambers after 24 hours 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.

Figure 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 hours. (a) control; (b) eugenol; (c) hydrocortisone; (d) Endomethasone N and (e) PCS. Scale bars: 200µm. (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).

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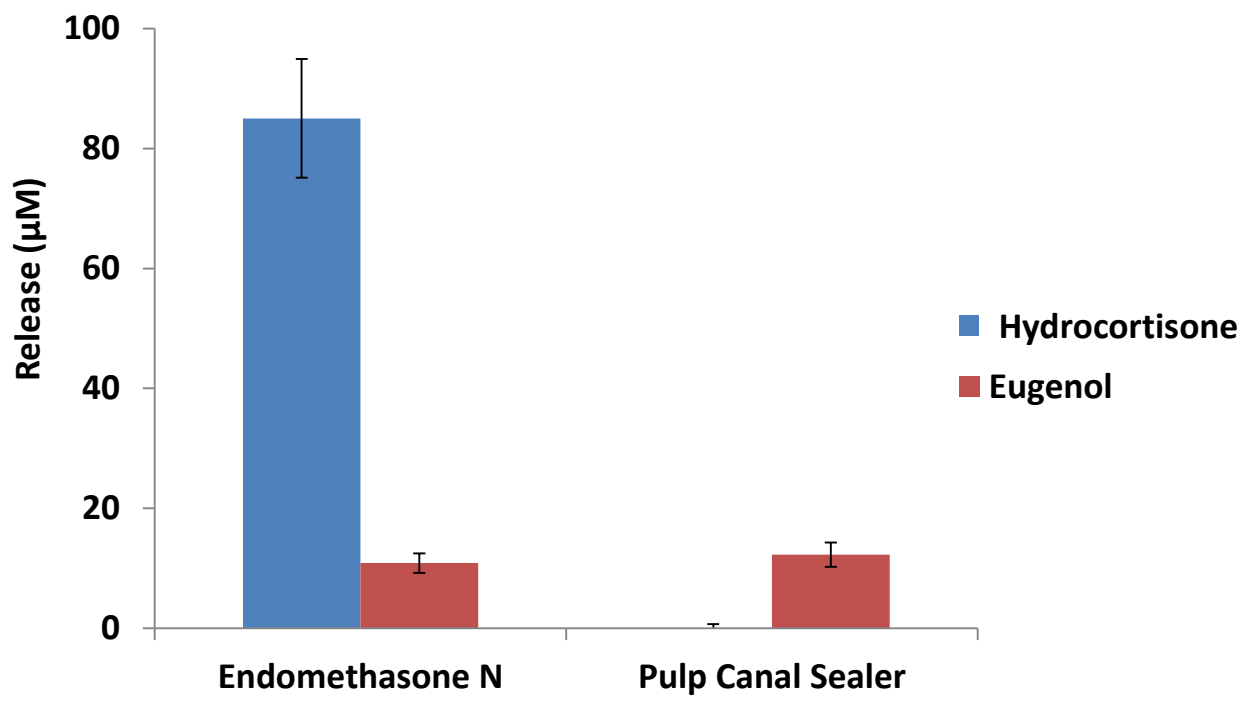
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Figure 1

A



B

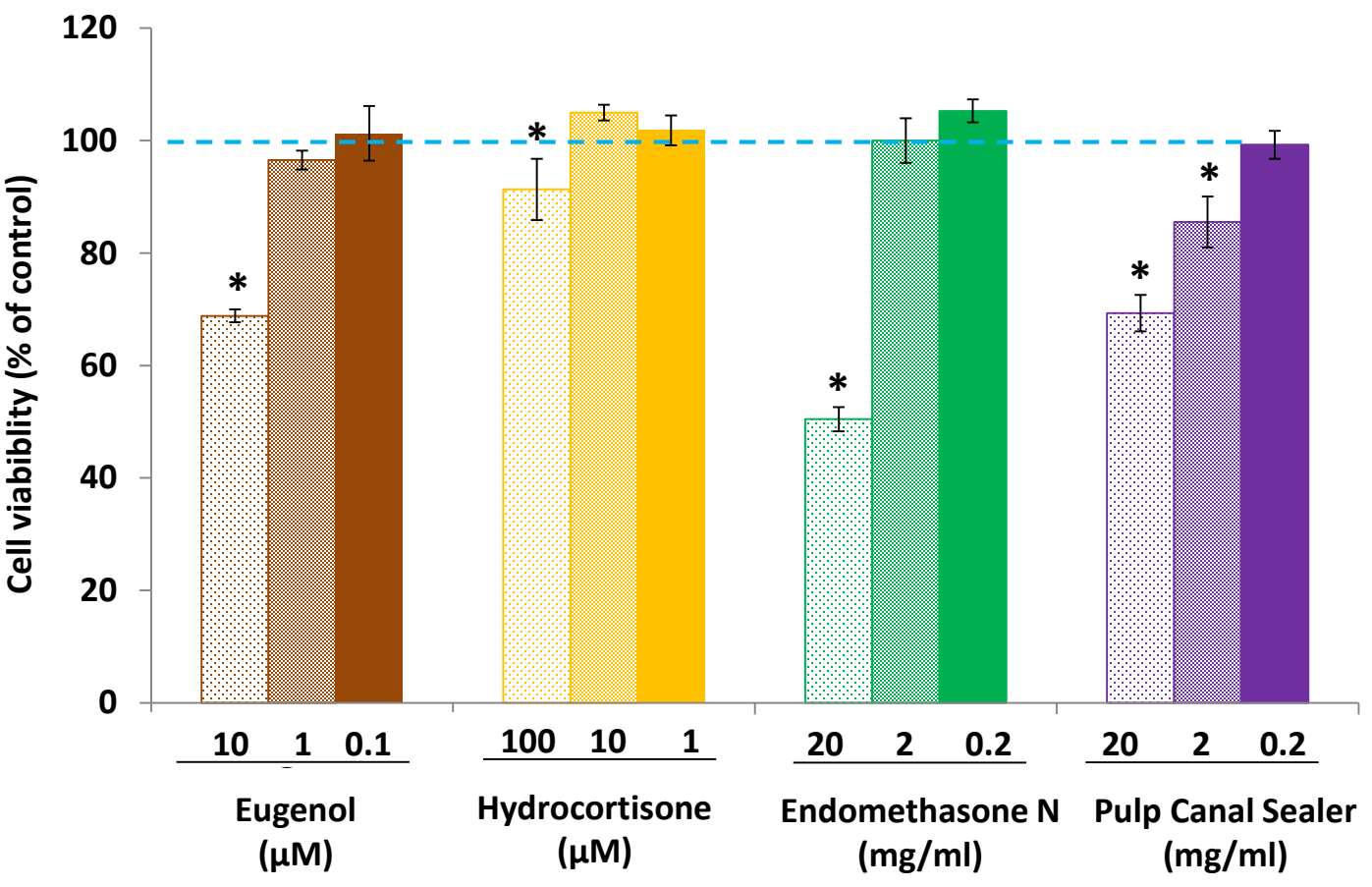
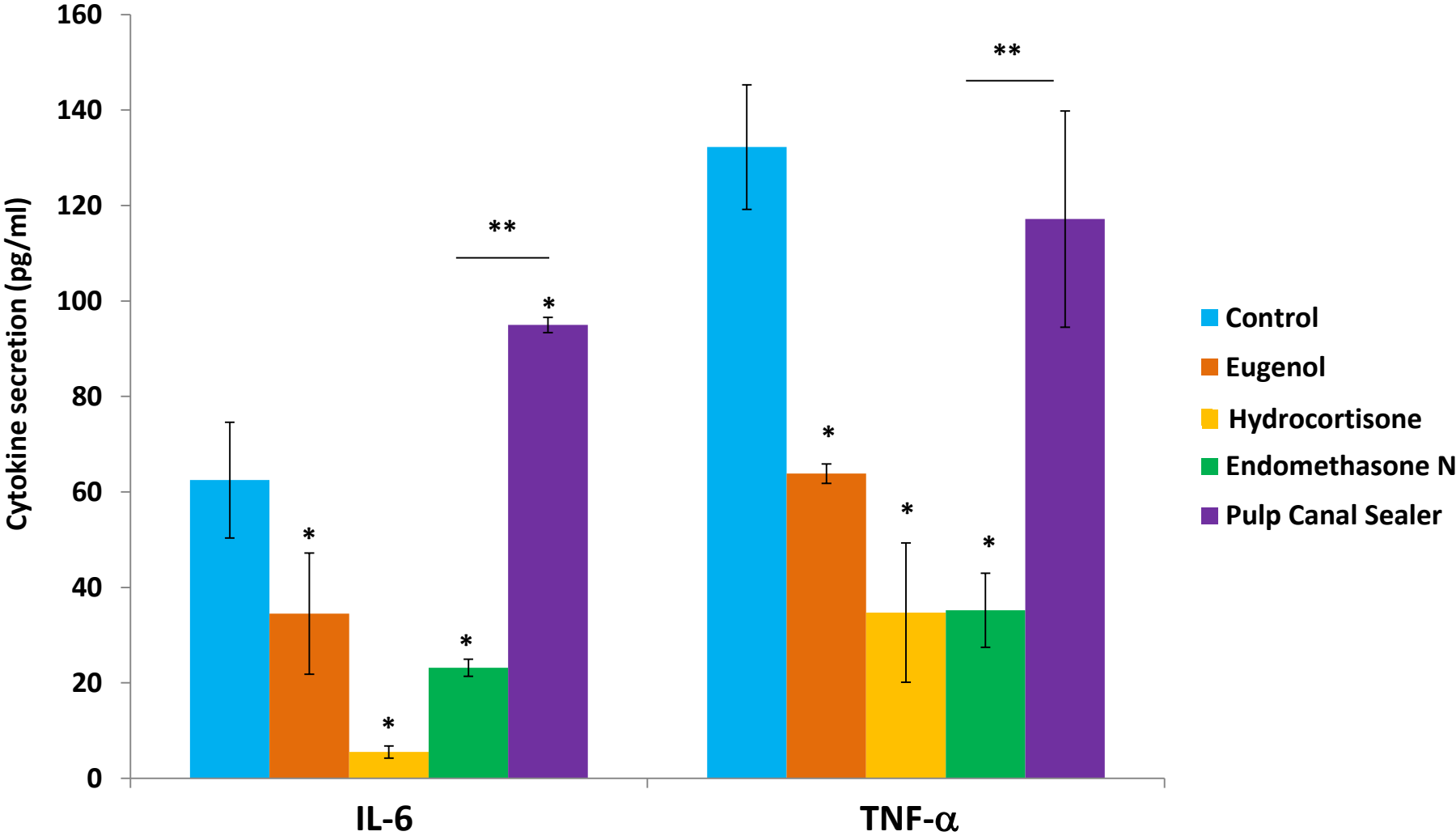
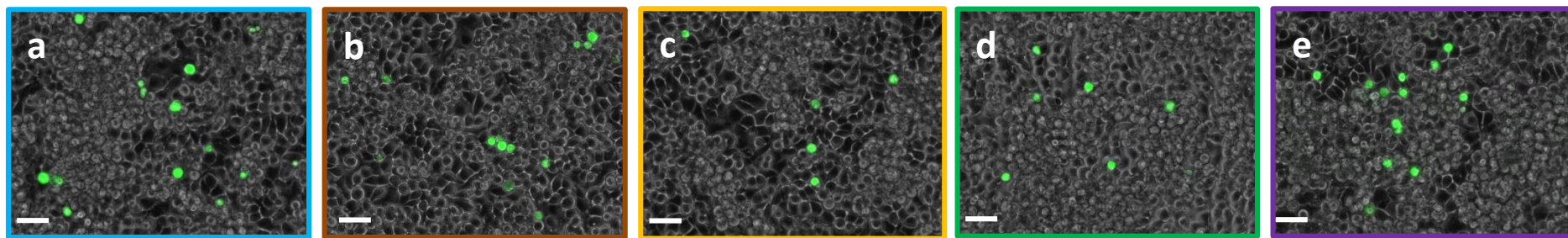


Figure2



A Figure 3



B

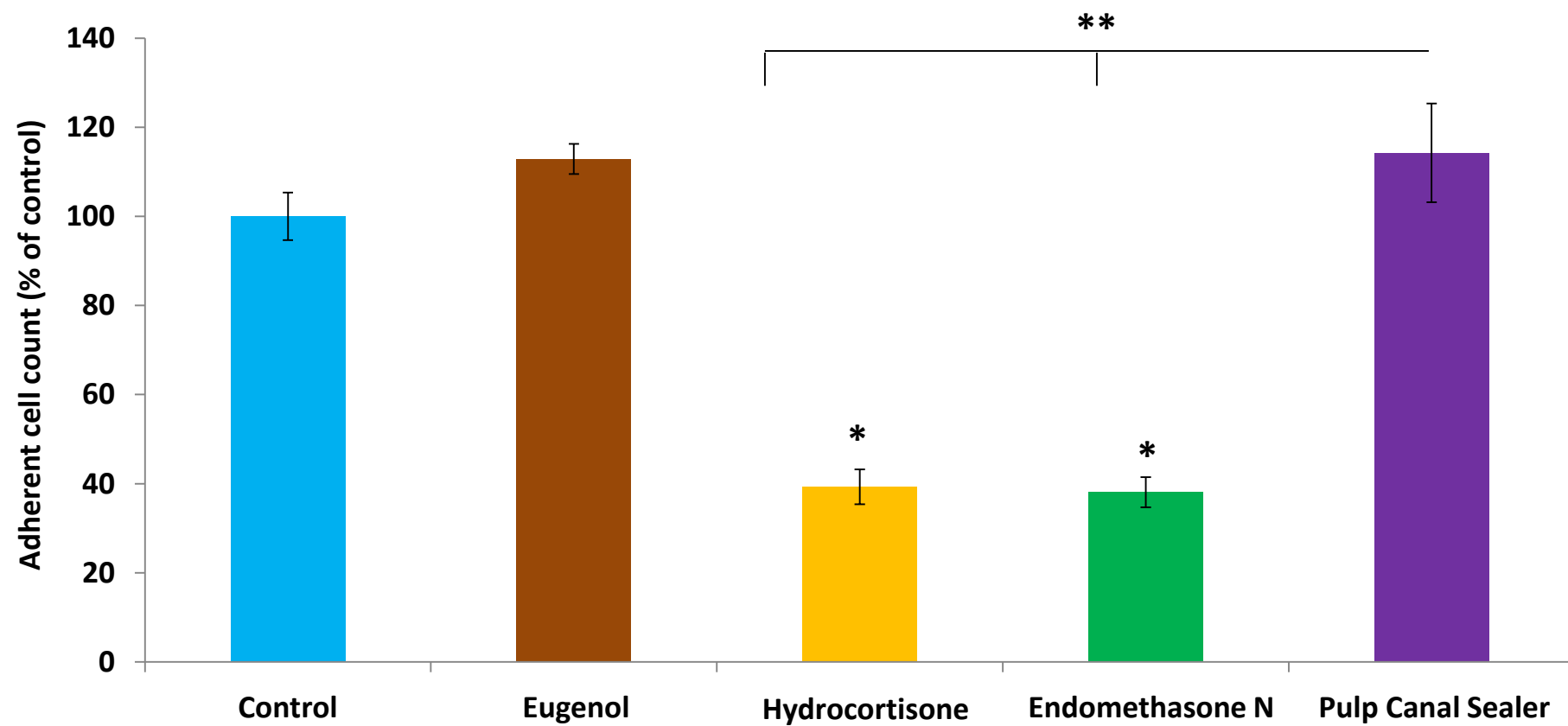
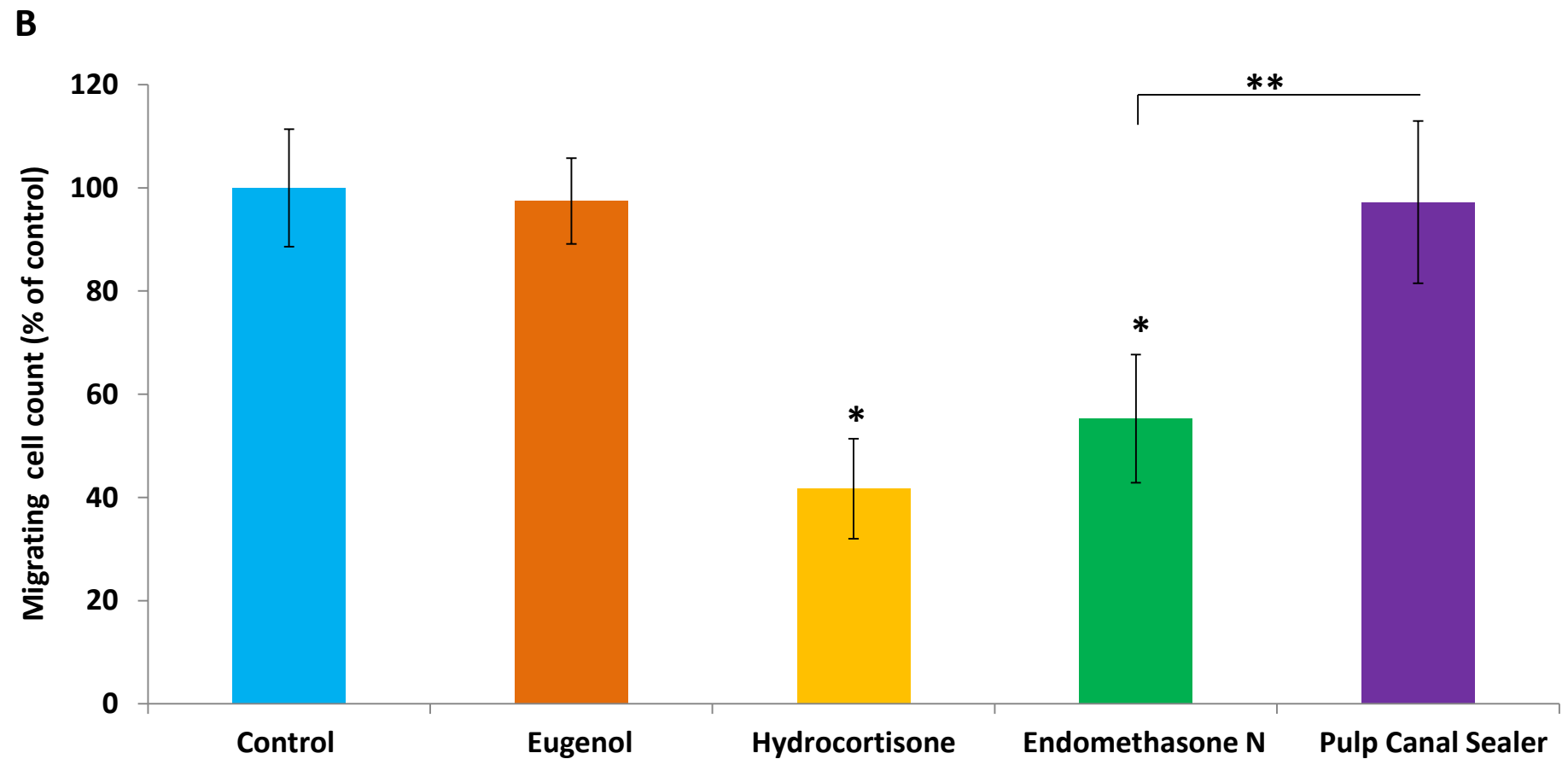
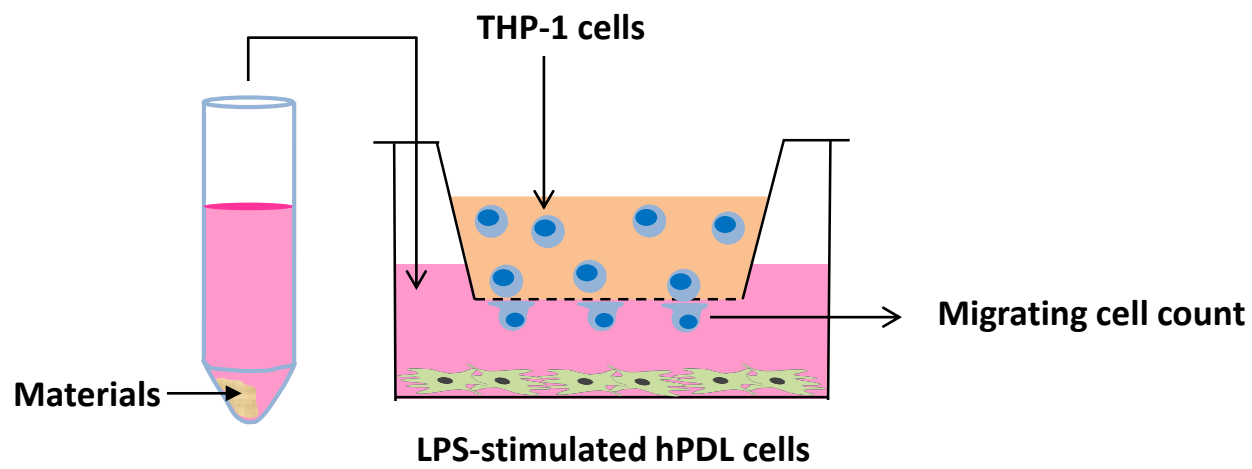
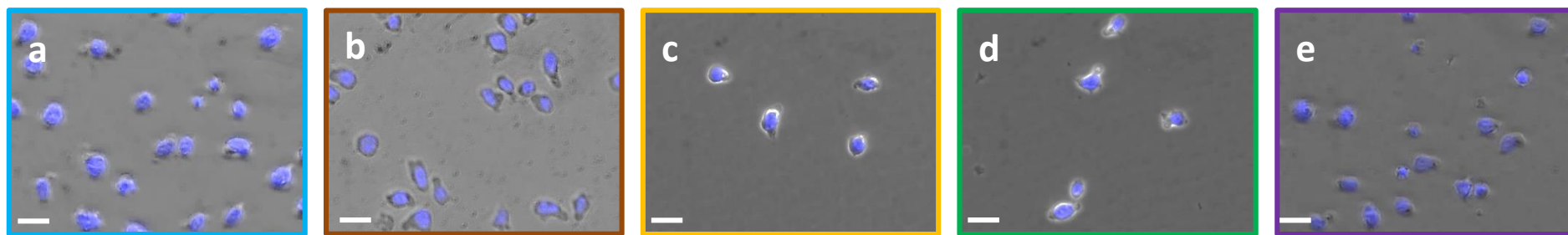
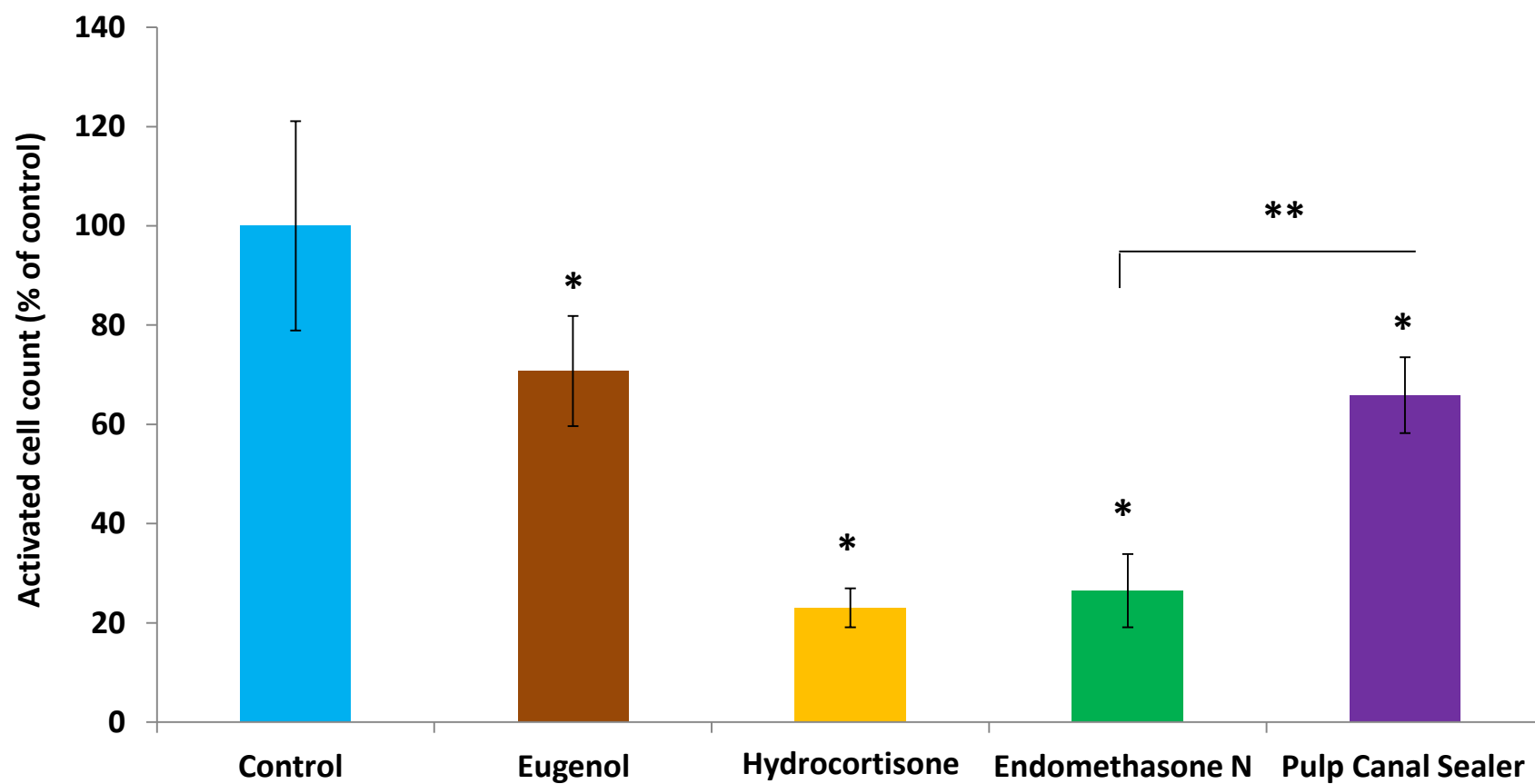


Figure 4





B



Endodontic sealer	Powder	Liquid
Endomethasone N	Hydrocortisone 1% Diiodothymol Barium sulfate 15% Zinc oxide Magnesium stearate	Eugenol 100%
Pulp Canal Sealer	Zinc Oxide 34-41 % Precipitated silver 25-30% Oleo resins 30-16% Thymol iodide 11-12%	Eugenol 78-80% Canada basalm 20-22%

TABLE 1: Composition of the sealers used