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## **Biodentine™ Reduces TNF $\alpha$ -induced TRPA1 Expression in Odontoblast-like Cells**

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## **Biodentine™ Reduces TNF $\alpha$ -induced TRPA1 Expression in Odontoblast-like Cells**

### Abstract

### Introduction

The transient receptor potential (TRP) ion channels have emerged as important cellular sensors in both neuronal and non-neuronal cells, with TRPA1 playing a central role in nociception and neurogenic inflammation. The functionality of TRP channels has been shown to be modulated by inflammatory cytokines. The aim of this study was to investigate the effect of inflammation on odontoblast TRPA1 expression and to determine the effect of Biodentine™ on inflammatory-induced TRPA1 expression.

### Methods

Immunohistochemistry was used to study TRPA1 expression in pulp tissue from healthy and carious human teeth. Pulp cells were differentiated to odontoblast-like cells in the presence of 2mM  $\beta$ -glycerophosphate and these cells were used in qPCR, Western blotting, calcium imaging and patch clamp studies.

### Results

Immunofluorescent staining revealed TRPA1 expression in odontoblast cell bodies and in odontoblast processes which was more intense in carious versus healthy teeth. TRPA1 gene expression was induced in cultured odontoblast-like cells by TNF- $\alpha$  and this expression was significantly reduced in the presence of Biodentine™. The functionality of the TRPA1 channel was demonstrated by calcium microfluorimetry and patch clamp recording and our

results showed a significant reduction in TNF- $\alpha$  induced TRPA1 responses following Biodentine™ treatment.

### Conclusion

In conclusion, this study showed TRPA1 to be modulated by caries-induced inflammation and that Biodentine™ reduced TRPA1 expression and functional responses.

Keywords: Biodentine, Dental pulp; Human; Inflammation; Pulp capping, TRP channel.

## **Introduction**

Members of the transient receptor potential (TRP) family of ion channels have emerged as important cellular sensors in both neuronal and non-neuronal cells. The TRP channel family comprises more than thirty calcium permeable cation proteins that are activated by thermal, chemical and mechanical stimuli (1). The functionality of TRP channels has been shown to be modulated by inflammatory cytokines, such as tumour necrosis factor alpha (TNF- $\alpha$ ) (2), providing a potential mechanism for inflammatory hyperalgesia. Amongst the TRP channel family, TRPA1 plays a central role in nociception and neurogenic inflammation. It is a polymodal channel activated by chemical, mechanical and thermal stimuli (3-5) and is the principal target of many endogenous reactive molecules produced at sites of inflammation and tissue injury (6,7). A role for TRPA1 in mechanical and thermal hyperalgesia has been documented in a variety of inflammatory pain models (5,8,9).

In the dental pulp TRPA1 is expressed by dental afferents (10,11) and its expression is up-regulated in trigeminal neurons by nerve growth factor (NGF) (12) and following tooth injury (13). TRPA1 is also expressed in human odontoblasts (14,15) and its activation has been shown to release signalling molecules, such as ATP, that mediate sensory transduction in teeth *via* an odontoblast neuronal signalling axis (16), supporting an emerging role for odontoblasts as sensory cells (17). Lying on the periphery of the dental pulp odontoblasts also play important defence functions by regulating caries induced pulp inflammation and cytokine production including TNF- $\alpha$  (18). During restorative procedures, odontoblast function can also be influenced by the application of restorative dental materials, however the effect of these factors on odontoblast sensory receptors is not known.

Biodentine™ is calcium silicate cement with promising regenerative potential and is proposed for use in both direct and indirect pulp capping (19-21). Pulp capping agents in addition to their regenerative properties are also ideally suited to have additional sedative and pain relief properties to enhance their clinical efficacy. Investigations of the interactions of Biodentine™ with dentin have revealed that this material penetrates into the dentin, forming tag-like microstructures within the tubules. These structures obliterate the dentin tubules providing a hermetic seal and may be involved in reducing the post-operative hypersensitivity mechanically (22). In addition, promising clinical observations following the application of Biodentine™ as a restorative material have shown pain relief effects and absence of post-operative sensitivity in cases of symptomatic pulpitis (23). However, the potential cellular and molecular mechanism by which Biodentine™ induces such pain relief effects is not known. We hypothesised that Biodentine™ modulates the expression and functionality of TRPA1 in odontoblasts to reduce pain and postoperative sensitivity. The aim of this study was therefore to investigate the effect of inflammation on odontoblast TRPA1 expression and to determine the effect of Biodentine™ on inflammatory-induced TRPA1 expression.

## **Materials and Methods**

### *Cell culture and treatments*

Dental pulp cells were derived by explant culture as previously described (24). Immature permanent third molar teeth were obtained in accordance with French ethics legislation and cells were grown in minimal essential medium with L-glutamine supplemented with 10% fetal bovine serum (FBS), 100UI/ml penicillin and 100 µg/ml streptomycin. To differentiate pulp cells to odontoblast-like cells, the medium was supplemented with 2mM β-glycerophosphate as previously described (14). The odontoblast phenotype of the cells was confirmed by the expression of the odontoblast marker dentine sialophosphoprotein (DSP) as

previously shown (14). For experiments involving TNF- $\alpha$  and Biodentine™ treatments, odontoblast-like cells ( $3 \times 10^5$ ) were grown in 6 well plates to 50-70% confluence prior to treatment with cytokine in the presence or absence of Biodentine™ extracts. These were obtained following incubation of set Biodentine™ (0.05 mm<sup>2</sup>/mL) in tissue culture medium for 24 h as previously described (25). Cells were then maintained in an incubator at 37°C and 5% CO<sub>2</sub> throughout.

### *Immunohistochemistry*

Human immature third molars, freshly extracted for orthodontic reasons, and carious teeth were obtained in compliance with French legislation (informed patients' consent and institutional review board approval of the protocol used). Teeth were fixed and routinely processed as described (26). Before staining, tooth sections were rehydrated with decreasing ethanol concentrations. Antigen retrieval was performed at 98°C for 30 min in 1 mM Tris/0.1 mM EDTA/0.5% Tween and non-specific binding sites were blocked with Phosphate Balanced Saline (PBS) containing 1% Bovine Serum Albumin (BSA) for 30 min at room temperature. Tooth sections were incubated over night at 4°C with 20  $\mu$ g/mL rabbit anti-human TRPA1 IgG (LSBio, Seattle, WA) or control isotype. They were then incubated for 45 min at room temperature with 2 mg/mL secondary antibody Alexa Fluor 594 (red fluorescence) goat anti-rabbit (Life technology, Saint Aubin, France) and DAPI (1 mg/mL) counterstain (blue fluorescence). Some sections were hematoxylin-eosin-stained or stained using a tissue Brown and Brenn kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Images were captured with an Observer A1 light microscope (Carl Zeiss Microscopy, Jena, Germany).

### *qRT-PCR*

RNA was extracted from cultured odontoblast-like cells using the RNAeasy Plus kit (Qiagen, UK) according to the manufacturer's instructions. cDNA was prepared using the VILO Transcriptor First-strand cDNA Synthesis Kit (Invitrogen, UK). qPCR reactions were carried out using TaqMan® TRPA1 specific primers and probes along with TaqMan® Universal PCR Master Mix II (Invitrogen, UK). Changes in TRPA1 expression as a result of TNF- $\alpha$  and Biodentine™ treatments, as outlined above, were analysed using the Mx3005P qPCR System (Agilent Technologies, USA). Relative mRNA expression of TRPA1 was normalised against the house-keeping genes  $\beta$ -glucuronidase (GUSB) and beta-2-microglobulin (B2M) (Invitrogen, UK) (Supp data).

### *Western blotting*

Cultured odontoblast-like cells were lysed for 30 min on ice with 10 mM Tris-HCL buffer (pH 7.4) containing 1% SDS and 'Complete' protease inhibitor cocktail (Roche, UK). A total of 30  $\mu$ l of each cell lysate was fractioned by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose. The blots were blocked in 5% non-fat milk for 1 h prior to probing overnight with rabbit polyclonal TRPA1 primary antibody (1:500) (LS Bioscience, Seattle, WA, USA). Protein expression was normalised to GAPDH (1:500) (Santacruz, USA). Detection of bound primary antibody was achieved using appropriate anti-species antibody conjugates (Dako, Denmark) and chemiluminescent substrate. Immunoreactive bands were visualised and quantified using a G:BOX Chemi System and GeneSys software (Syngene, UK).



### *Ratiometric calcium imaging*

[Ca<sup>2+</sup>]<sub>i</sub> was estimated fluorometrically in cultured odontoblast-like cells treated as described above and loaded with the intracellular Ca<sup>2+</sup> probe fura-2 AM (5 μM) for 1 hr at 37°C. Coverslips bearing adherent cells were placed in a recording chamber mounted on the stage of an inverted microscope (Nikon Eclipse TE2000) and superfused with Hank's solution, (140 mM NaCl, 5 mM KCl, 5 mM D-glucose, 1.3 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 10 mM HEPES (free acid), pH 7.4 at 37 °C. Cells were stimulated with TRPA1 agonist cinnamaldehyde (100 μM) while alternating excitation wavelengths of 340 and 380 nm light were delivered from a dual monochromator (5 nm bandwidth) using a light chopper (Cairn Research Ltd., Faversham, UK). Emitted fluorescence was measured from the side port of the microscope via an adjustable rectangular window, a filter (510 nm) and a photon counting photomultiplier tube (PMT) in the light path. Fluorescence equipment was controlled by Acquisition Engine (Cairn) software (V1.1.5) which was also used for analysis of the fluorescence data. Changes in the ratio of fluorescence emitted at each excitation wavelength ( $R = F_{340}/F_{380}$ ) was used as a measure of changes in the cytoplasmic Ca<sup>2+</sup> concentration (27).

### *Whole-cell patch-clamp recording*

Whole-cell patch-clamp recording of TRPA1 channel activity was carried out using bath solution (140 mM NaCl, 5 mM KCl, 1.3 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 5 mM glucose and 10 mM HEPES, pH altered to 7.4 using NaOH) and pipette solution (120 mM CsCl, 1 mM MgCl<sub>2</sub>, 4 mM Na<sub>2</sub>ATP, 10 mM BAPTA and 10 mM HEPES, pH altered to 7.2 using CsOH with the addition of 0.39 mM Amphotericin B to induce membrane perforation and allow electrical access). 100 μM cinnamaldehyde and 10 μM HC030031 were applied in the bath solution using a rapid solution delivery device positioned adjacent to the cells. Experiments

were carried out at 37°C. Current-voltage relationships were elicited by a voltage ramp protocol (+80 to -80 mV over 1.6 s applied every 5 s from a holding potential of 0 mV) via an Axopatch 200B amplifier using PClamp version 10.2 software (Molecular Devices, Ca, USA).

#### *Cell viability assay*

A succinyl dehydrogenase assay (MTT) was performed to evaluate the effect of TNF- $\alpha$  and Biodentine™ on the viability of odontoblast-like cells. Cells were plated in 96 well plates and allowed to attach for 48 h in minimal essential medium with L-glutamine supplemented with 10% foetal bovine serum (FBS), 100 UI/ml penicillin, 100  $\mu$ m streptomycin and 2mM  $\beta$ -glycerophosphate. Cells were then incubated with Biodentine™ (0.05 mm<sup>2</sup>/mL) in the presence or absence of TNF- $\alpha$  (1, 10 and 100 ng/ml) for 24 h following which 100  $\mu$ l of a 0.5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to the culture media. After incubation for 2 h at 37 °C the supernatant was discarded and the formazan crystals were solubilized with 100  $\mu$ l per well of dimethylsulfoxide (DMSO). Absorbance at 570 nm was determined using an automatic microplate spectrophotometer (GENios, Tecan, Switzerland) and data were analysed using Prism Graph Pad software.

#### *Statistical Analysis*

Data were presented as mean  $\pm$  standard error (SEM) and analysed using Student *t* test and one way analysis of the variance (ANOVA) or the non-parametric Mann Whitney and Kruskal Wallis tests (where the data were not normally distributed). Post hoc tests were used as appropriate. The level of significance was set at  $p < 0.05$ .

## Results

### *TRPA1 expression in the odontoblast layer of intact and carious teeth*

TRPA1 expression in healthy and carious teeth was investigated with fluorescent immunohistochemistry as shown in Figure 1. In H&E staining of intact teeth, odontoblasts appear perfectly aligned at the pulp periphery (Fig. 1A, C). In carious teeth, bacteria infiltrating the dentine are visible and the dentine structure is irregular at the carious site. (Fig. 1B, D). This seems to be mainly due to cariogenic bacteria as seen in Brown and Brenn staining (Fig. 1J). Immunofluorescence reveals expression of TRPA1 (red fluorescence) in odontoblast cell bodies as well as in the odontoblastic processes of intact teeth (Fig. 1E). This expression is intense in the odontoblasts of carious teeth. Immunofluorescence labelling appears intense under the carious lesion (Fig. 1F) and elsewhere in the pulp at sites distant from the carious lesion (Fig. 1G, H). No immunostaining was seen in the negative control (Fig. 1I).

### *TNF- $\alpha$ modulates expression and function of TRPA1 in cultured odontoblast-like cells*

To investigate whether caries-induced TRPA1 expression was driven by inflammatory cytokines, odontoblast-like cells were treated with TNF- $\alpha$  for 24 h and gene and protein expression of TRPA1 were subsequently determined by qPCR and Western blotting. As shown in Figure 2A, TNF- $\alpha$  up-regulated TRPA1 gene expression in a concentration dependent manner. TRPA1 protein expression as shown by Western blot (Fig. 2B) was significantly increased following treatment with 10ng/ml TNF- $\alpha$  (Fig. 2C).

### *Biodentine™ reduces TNF- $\alpha$ induced expression and functional responses of TRPA1*

We next investigated whether TRPA1 expression induced by treatment with TNF- $\alpha$  could be modified following Biodentine™ treatment. Cells were treated with TNF- $\alpha$  at a concentration

of 10 ng/ml, in the presence or absence of Biodentine™, and TRPA1 gene and protein expression were subsequently determined by qRT-PCR and Western blotting. As shown in Figure 3A, TRPA1 gene expression induced by TNF- $\alpha$  was significantly reduced in the presence of Biodentine™ whereas Biodentine™ itself was shown to have no significant effect on basal TRPA1 expression (Fig. 3A). In line with the gene expression results, protein expression of TRPA1 which was induced by TNF- $\alpha$  was similarly reduced by Biodentine™ (Fig. 3B, C). The functionality of the TRPA1 channel, as shown by calcium microfluorimetry results further validate reduction of TNF- $\alpha$  induced TRPA1 responses following Biodentine™ treatment (Fig. 4A, B). TRPA1 is a calcium permeable channel that increases intracellular calcium ions upon activation. The increased TRPA1 protein expression following TNF $\alpha$  treatment and its reduction with Biodentine are reflected functionally in increased and decreased intracellular calcium levels respectively determined by calcium microfluorimetry. However, since calcium microfluorimetry cannot determine whether channel activity is associated with the plasma membrane, patch clamp recording was also undertaken to confirm the presence of functional TRPA1 on the plasma membrane of the odontoblast-like cells (Fig 4C). TNF- $\alpha$  (Fig. 4D) and Biodentine™ (Fig. 4E) had no significant effects on cell viability at the concentrations used in this study.

## **Discussion**

In the present investigation we show that human odontoblast TRPA1 expression is increased as a result of caries-induced pulpal inflammation and that this modulation of TRPA1 was mimicked in cultured odontoblast-like cells exposed to the inflammatory cytokine TNF- $\alpha$ . The effect of clinical application of restorative material on TRPA1 expression in inflamed pulp was simulated by incubating the TNF $\alpha$ -treated cells with Biodentine™ extracts. We show that TNF $\alpha$  -induced TRPA1 expression was attenuated by Biodentine™. The

expression of TRPA1 on the plasma membrane of the odontoblast-like cells, as shown by patch clamp experimentation, supports the role for the odontoblast as a sensory cell which could convey sensory information to the underlying pulpal nerves.

Caries induced pulpal inflammation often presents clinically as pain and exaggerated response to stimuli. At the molecular level, pulp inflammation is characterised by release of inflammatory cytokines such as TNF $\alpha$  within the dental pulp and odontoblast layer (18,28). TNF $\alpha$  is a pro-inflammatory cytokine that plays a critical role in development and maintenance of inflammatory pain *via* different signalling mechanisms including modulation of ion channel expression and function (29). Our results confirm the expression of TRPA1 within the odontoblast layer in intact teeth and also demonstrate increased TRPA1 expression in the odontoblastic layer of carious teeth. This increase in TRPA1 expression was visible all over the carious tooth pulp and may be due to increased TNF- $\alpha$  synthesis in the inflamed pulp. Indeed, our data clearly demonstrated an increased expression of TRPA1 in cultured odontoblast-like cells with increasing TNF- $\alpha$  concentrations, suggesting a role for this cytokine in the modulating TRPA1 in the dentin-pulp complex.

Biodentine™ is a calcium silicate cement with bioactive properties. The material was shown to induce TGF- $\beta$  release and mediate reparative dentine formation *in vitro* (25) and *in vivo* (30). The material was also reported to be biocompatible and induced no post-operative sensitivity in clinical applications (31). Previous data demonstrated the obliteration of dentin tubules by Biodentine™ and the formation of tag-like structures of Biodentine™ in the dentin tubules (22) which may explain the reduction of post-operative sensitivity at a mechanical level. At the molecular level, our results demonstrated that Biodentine™ reduced the TNF- $\alpha$ -induced expression of TRPA1, an ion channel that is considered the gate keeper of pain and

inflammation (32), thereby providing a possible explanation for the pain relieving effects of Biodentine™. Given the recently proposed role for TRPA1 in the transition from acute to chronic inflammation (33), then it is possible that early intervention with bioactive treatments such as Biodentine™ could be beneficial in halting the progression of inflammation within the dental pulp.

Whilst it is recognised that other tricalcium silicate-based restorative materials can be applied onto the dentin during restorative treatment, this study was focused only on Biodentine™. Therefore we cannot exclude the possibility that other restorative materials may have similar modulatory effects on TRPA1. Also, in this work, the isolated pulp cells were incubated with  $\beta$ -glycerophosphate in order to induce their differentiation into odontoblast-like cells. However, although the majority of these cells differentiate into odontoblast-like cells expressing DSP (14), the studied population may still include some fibroblasts which also express TRPA1 (34). Previous studies have also reported expression of TRPA1 in pulp nerves (15), and therefore the potential exists for modification of this channel in pulp nerves or any other cell type after dentin-pulp exposure to Biodentine™ *in vivo*. We chose only to explore the effects of Biodentine™ on TRPA1 functional modulation in odontoblasts since these are the cells that are exposed to Biodentine™ during a restorative procedure and have well described roles in caries-induced pulpal pain and inflammation (35,36).

The exact mechanism through which Biodentine™ could exert its effects on odontoblast TRPA1 channels remains to be determined. However, recent work by our research group (37) has shown that the inflammatory cytokine TNF $\alpha$ , known to be up-regulated in the odontoblast cell layer in human teeth in response to dental caries (36), can both sensitise TRPA1 channels in odontoblasts as well as upregulate odontoblast TRPA1 expression. The

enhanced channel activity and increased expression levels were shown to be mediated via the p38MAPK pathway (37) and thus it is possible that Biodentine™ could target p38MAPK signalling resulting in modulated TRPA1 responses in odontoblast-like cells.

### **Conclusion**

In conclusion, this study showed TRPA1 to be modulated by caries-induced inflammation and that Biodentine™ reduced TRPA1 expression and functional responses. This suggests a potential property for Biodentine™ in modulating nociceptive receptors in odontoblasts which may have implications in post-operative pain relief.

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## Figure Legends:

**Figure 1: Detection of TRPA1 in human intact and carious teeth sections.** In intact teeth, odontoblasts (o) appear perfectly aligned at the pulp periphery (A, C). Bacteria infiltrating the dentin (d) are visible in carious tissue sections (arrowheads). At the carious site dentin is irregular and has an altered morphology while inflammatory cell infiltration (dotted line) is visible in the underlying pulp (p) (B, D). This seems to be mainly due to cariogenic bacteria (red arrows) as seen in Brown and Brenn staining (J).

Immunofluorescence reveals expression of TRPA1 (red fluorescence) in odontoblast cell bodies, as well as in the odontoblastic processes of intact teeth (E). This expression is intense in odontoblasts of carious teeth. Immunofluorescence labelling appears intense under the carious lesion (F) and elsewhere in the pulp at sites distant from the carious lesion (G, H). No immunostaining was seen in the negative control (I).

A-D: H&E staining; J: Brown and Brenn staining; I: isotype control. E-I: immunofluorescence of TRPA1 (red) and DAPI counterstain of nuclei (blue). Scale bars: (A, B, J)=500  $\mu\text{m}$ ; (C, D, E, F, G, H, I)= 20  $\mu\text{m}$ .

**Figure 2: TRPA1 gene and protein expression is up-regulated in odontoblast-like cells following treatment with TNF- $\alpha$  for 24 h.** TNF- $\alpha$  up-regulates TRPA1 gene expression in a dose dependent manner (A). Representative western blot of TRPA1 protein expression (at predicted molecular weight) normalised to GAPDH (B). Bar chart summary of densitometry quantification for TRPA1 protein expression following TNF- $\alpha$  treatment, normalised to GAPDH. TRPA1 showed a modest but significant increase in protein expression following treatment with 10 ng/ml TNF- $\alpha$  (C). Error bars indicate SEM, ANOVA, \* $p \leq 0.05$ , \*\* $p \leq 0.01$  after post-hoc correction for multiple testing. Results of three independent experiments.

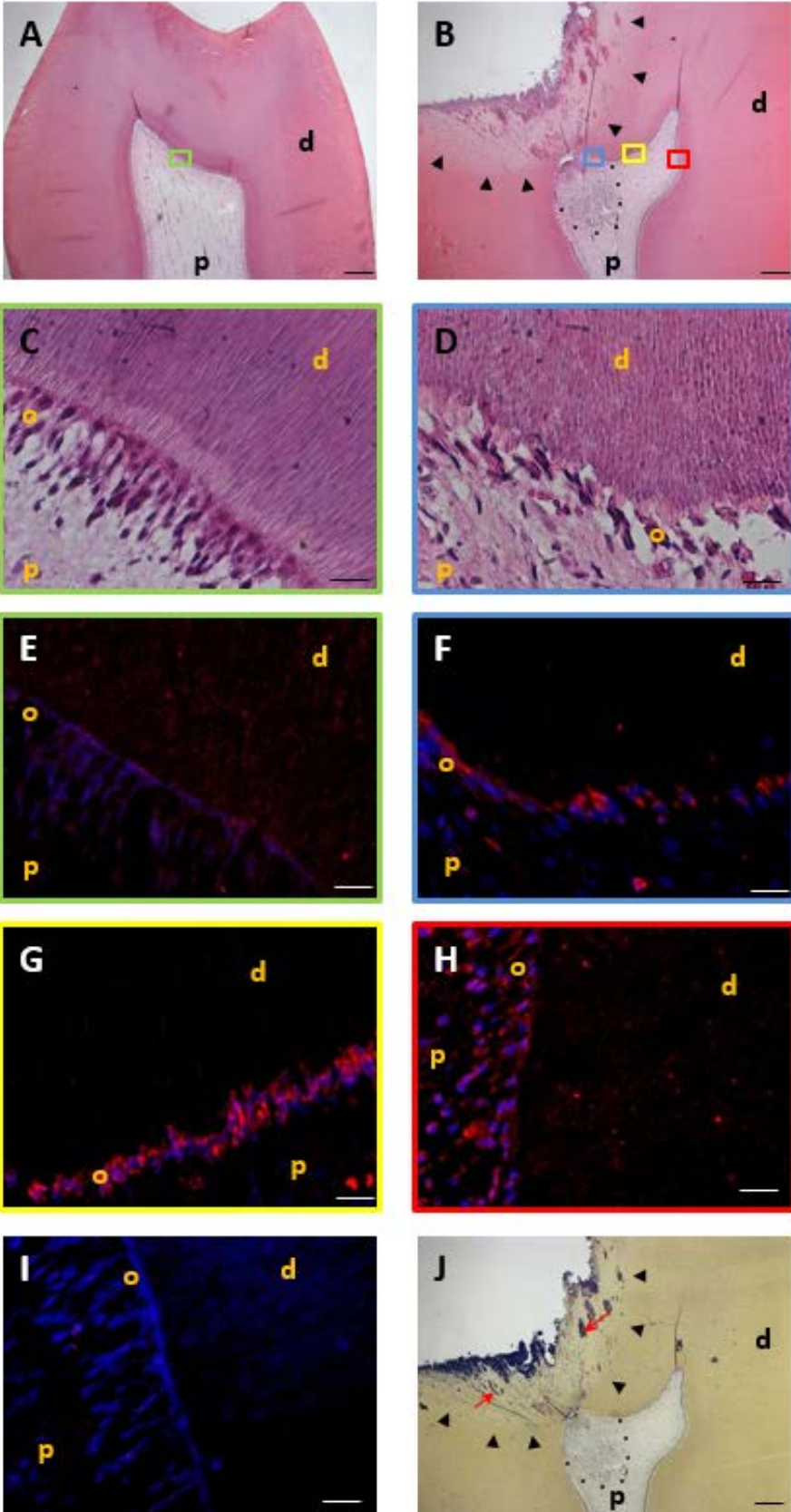
**Figure 3: Biodentine™ down regulates TNF- $\alpha$  induced TRPA1 expression.** Bar chart showing TNF- $\alpha$  induced upregulation of TRPA1 gene expression was down-regulated significantly in the presence of Biodentine™ (A). TRPA1 protein expression as shown by Western blotting (B) and bar chart densitometry summary, with TRPA1 expression normalised to GAPDH (C), was significantly reduced by Biodentine™ treatment. Error bars

indicate SEM, ANOVA,  $*p \leq 0.05$ ,  $**p \leq 0.01$  after post-hoc correction for multiple testing. Results of three independent experiments.

**Figure 4: Biodentine™ down regulates TNF- $\alpha$  induced TRPA1 function.**

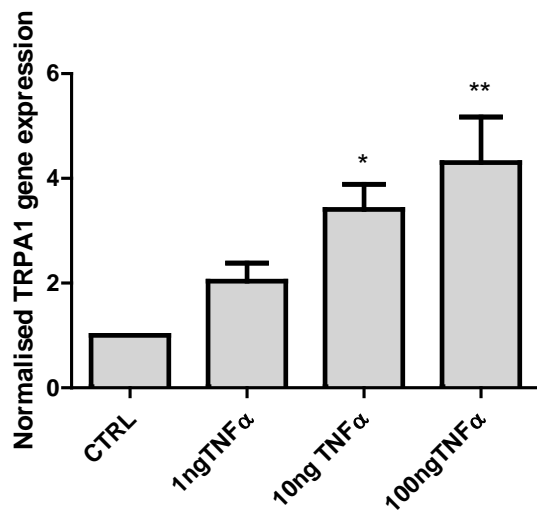
Representative traces of calcium imaging showing TRPA1 responses to its agonist cinnamaldehyde (CA) in control, TNF- $\alpha$  and combined TNF- $\alpha$  and Biodentine™ treated cells (A). Bar chart summary for mean changes in calcium responses following stimulation of cells with CA (B). Error bars indicate SEM, ANOVA,  $*p \leq 0.05$ ,  $**p \leq 0.01$  after post-hoc correction for multiple testing. Patch clamp data: *Upper panel*, current activation in the presence of CA is inhibited by addition of 10  $\mu$ M HC030031 indicating TRPA1 channels are expressed on the cell membrane and thus may constitute a calcium influx pathway in odontoblast-like cells; *lower panel*, voltage ramp protocol used to elicit TRPA1 currents (C). TNF- $\alpha$  or Biodentine™ treatments have no effects on odontoblast-like cell viability. Treatment of odontoblast-like cells with TNF- $\alpha$ , up to 100 ng/mL (D) and with Biodentine™, up to 50 mm<sup>2</sup>/mL (E) produced no significant effects on cell viability.

Figure 1

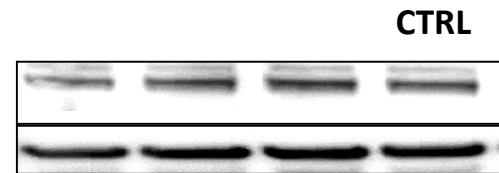


**Figure 2**

(A)



(B)



(C)

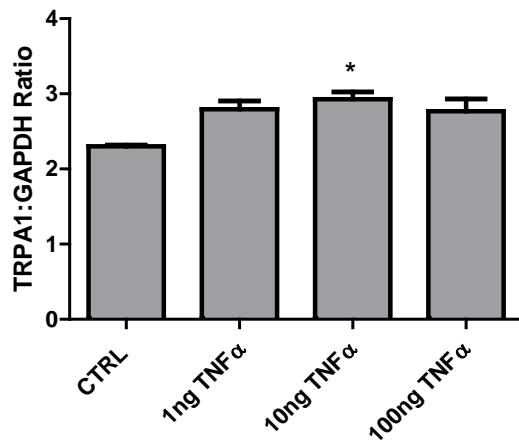
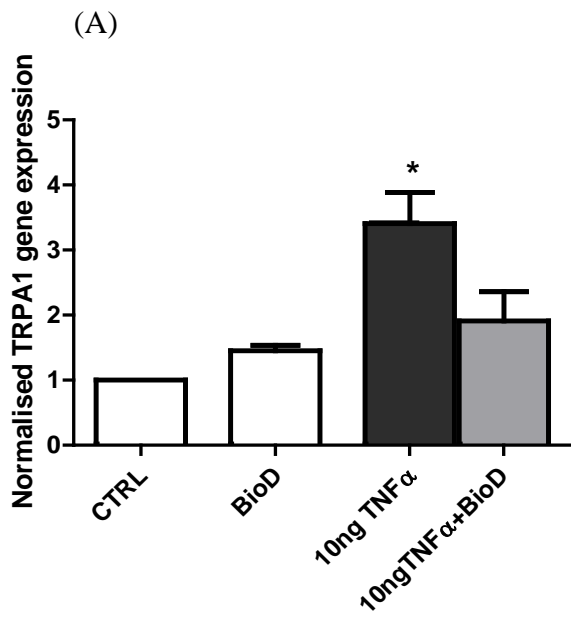
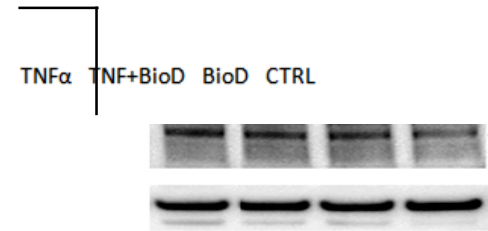


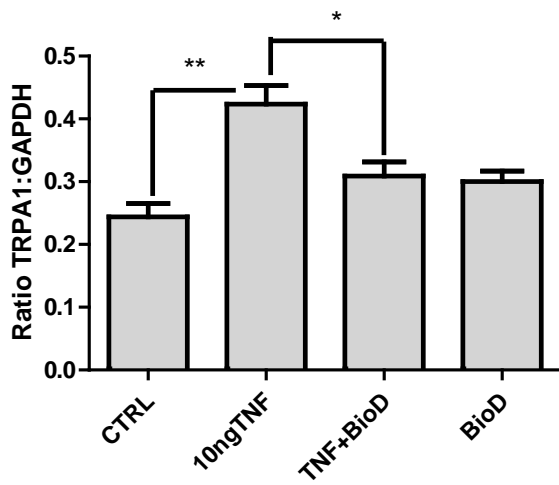
Figure 3



(B)

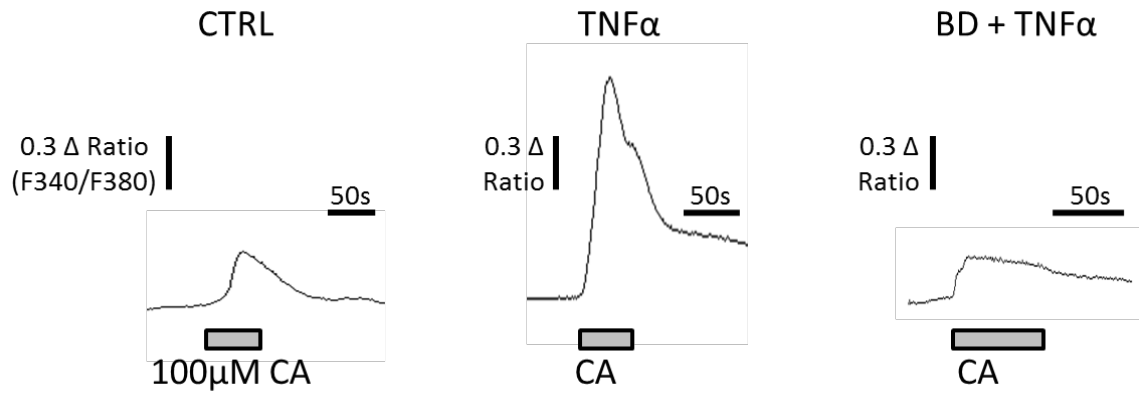


(C)

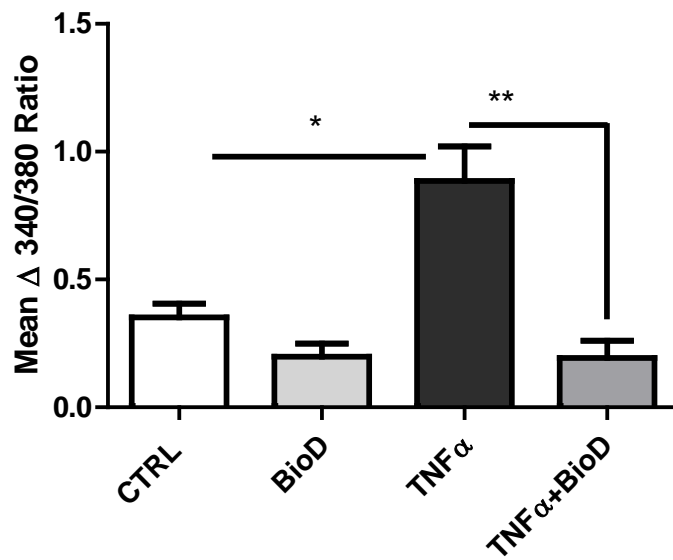


**Figure 4**

(A)

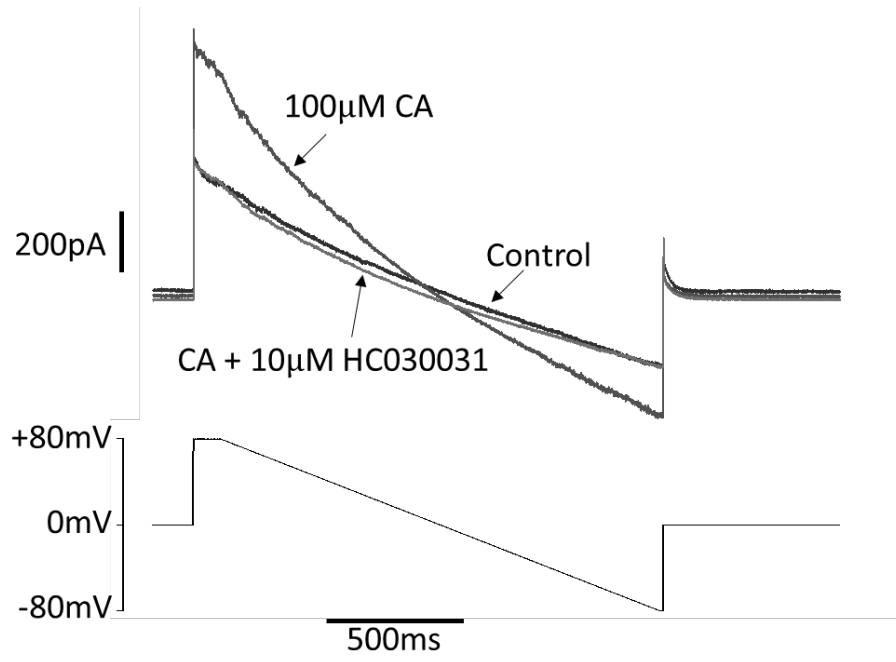


(B)





(C)



(D)

