SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of the role of transient receptor potential (TRP) ion channels in oral biology

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Debrecen, 2026

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 1:30 PM on 8th of May, 2026.

Introduction

The dental pulp (pulpa dentis) is a living, highly differentiated connective tissue that plays a fundamental role in the physiological functioning and regeneration of teeth and in the coordination of sensory and immunological responses. During inflammatory processes, such as pulpitis, pulp cells are exposed to oxidative and inflammatory mediators, which can result in tissue damage and pain. Therefore, elucidating the cellular mechanisms of pulpitis is of paramount importance in the development of diagnostic and therapeutic strategies.

An important member of the Transient Receptor Potential (TRP) ion channel family, transient receptor potential ankyrin 1 (TRPA1) is a non-selective cation channel that primarily mediates Ca^{2+} influx. Its activation can be triggered by various physical and chemical stimuli, including reactive oxygen species (ROS) formed during oxidative stress. Although TRPA1 is known to play a role in pain transmission in sensory nerves, there is growing evidence that it also has functional significance in non-nerve cells of the dental pulp, such as odontoblasts and fibroblasts.

Oxidative stress is a key factor in the pathophysiology of pulpitis: inflammatory mediators and bacterial effects increase ROS production, which can lead to cell damage, mitochondrial dysfunction and apoptosis. The direct redox sensitivity of TRPA1 suggests that the channel is an important modulator of oxidative processes and that its activation may further increase the oxidative load on cells and perpetuate inflammation through a positive feedback mechanism. Based on these findings, investigating the role of TRPA1 in pulp cells may be of fundamental importance for a better understanding of the pathomechanism of pulpitis.

The aim of our research is therefore to investigate the expression and function of TRPA1 and its relationship with oxidative stress in an in vitro pulpitis model, and to examine whether inhibition of the channel can mitigate cell damage and mitochondrial dysfunction. For the experiments, we used primary cell cultures derived from human pulp, modelled the inflammatory environment by inducing polyonic acid, and analysed TRPA1 expression at both the genetic and protein levels. Functional activity was measured using Ca^{2+} imaging, ROS production was measured using fluorescent assays, and cell viability was measured using MTT-based assays.

For accurate spatial analysis of gene expression, especially in complex tissues containing many different cell types, such as dental pulp, conventional RT-qPCR is limited because RNA from homogenised samples does not carry information about tissue localisation. In contrast t , in situ hybridisation (ISH) is able to preserve cell-level structure and localised

gene expression. However, ISH is particularly difficult to perform in calcified tissues such as teeth, as acidic or lengthy decalcification procedures can cause RNA degradation. The RNAscope technique is an improved, highly sensitive version of traditional ISH, which is also suitable for detecting partially degraded mRNA through multiple probes and strong signal amplification. Although the method can be successfully applied to decalcified bone samples, little data is available on its optimal application to teeth. The mineralised tissues surrounding the dental pulp – dentine and enamel – significantly complicate sample preparation and the preservation of adequate RNA quality. The literature suggests that conventional decalcification procedures may result in significant mRNA loss, therefore careful optimisation is required when applying this technique.

In order to be able to use the RNAscope technique in our future in vivo studies, in the second part of our study we evaluated five different decalcification methods on mouse incisor samples, examining the preservation of histological structure and mRNA integrity. The aim was to determine a sample preparation protocol that best supports the use of RNAscope in dental tissues.

Objective

1. To investigate the role of TRPA1 in a pulpitis model

- To determine the expression of the TRPA1 ion channel in human dental pulp cells under normal and inflammatory conditions.
- To investigate whether inflammatory stimuli cause changes in TRPA1 expression and functional activity.
- Characterisation of TRPA1-mediated Ca^{2+} signalling in an inflammatory environment.

2. Analysis of the interaction between oxidative stress and TRPA1

- Examination of the development of oxidative stress in an in vitro pulpitis model.
- Determination of how TRPA1 activation contributes to the production of reactive oxygen species.
- Effect of pharmacological and genetic inhibition of TRPA1 on the extent of oxidative damage.

3. Methodological optimisation for RNAscope ISH studies

- Comparison of different decalcification methods on rodent tooth samples in terms of preservation of histological structure.
- Examination of the effect of individual decalcification methods on mRNA integrity using RNAscope in situ hybridisation.
- Identification of sample preparation protocols that simultaneously ensure adequate morphology and molecular sensitivity.

4. Translational relevance

- Establishment of an experimental basis that may contribute to a better understanding of the cellular pathomechanisms of pulpitis.
- Supporting the role of TRPA1 as a potential therapeutic target in inflammatory dental diseases.

Materials and methods

Establishment of primary human dental pulp cell (hDPC) cultures

The primary hDPCs were isolated from intact third molars, which were otherwise removed from healthy patients under local anaesthesia for orthodontic reasons at the Faculty of Dentistry of the University of Debrecen. The patients gave their written consent, and the procedure was approved by the Regional and Institutional Ethics Committee of the University of Debrecen with the following identification number: ETT TUKEB 49849-3/2016/EKU. After extraction, the teeth were disinfected with a 3% sodium hypochlorite solution for 2 minutes, then rinsed with phosphate-buffered saline (PBS; 115 mM NaCl, 20 mM Na_2PO_4, pH 7.4; all from Sigma-Aldrich/Merck, St. Louis, MO, USA) and dried with cotton gauze. An incision was made in the corono-apical direction using a sterilised dental diamond fissure bur (Hager & Meisinger GmbH, Neuss, Germany) using a high-speed handpiece (W&H Dentalwerk Bürmoos GmbH, Bürmoos, Austria) with continuous water cooling until the dentine wall surrounding the pulp chamber remained thin but continuous. The sectioned teeth were placed in transport medium (DMEM-F12 culture medium supplemented with 10% foetal bovine serum (FBS) (both Thermo Fisher Scientific, Waltham, MA, USA), penicillin (500 U/ml), streptomycin (500 µg/ml) and amphotericin (1.25 µg/ml) (all from Sigma-Aldrich). They were placed on ice and transported to the cell culture laboratory for further processing. Under a sterile laminar flow hood, the teeth were broken open with a sterile Bein root elevator (MEDICOR Kéziműszer Zrt., Debrecen, Hungary), and then the coronal and radicular pulp tissue was removed with a sterile endodontic Kerr file (#30; Dentsply Sirona, Charlotte, USA) and dental forceps (MEDICOR Kéziműszer Zrt., Debrecen, Hungary).

The pulp tissue was placed in a new Petri dish containing medium of the same composition and chopped into 1–2 mm pieces using a #20 surgical scalpel (Feather, WPI, Sarasota, FL, USA). The pulp tissue was then digested in a solution of collagenase I (3 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) and dispase (4 mg/ml; Gibco, Waltham, MA, USA) for 1 hour at 37°C. The resulting cell suspension was seeded into 6-well culture plates (Thermo Fisher Scientific, Waltham, MA, USA) and cultured in medium of the same composition at 37°C in a humidified incubator in the presence of 5% CO_2. The cultures were checked daily with an inverted microscope (Olympus Corp., Centre Valley, PA, USA) for possible contamination and cell growth. When the cultures reached 70–80% confluence, they were either passaged for further studies or cryopreserved in liquid nitrogen for later use. During passage,

the cells were thoroughly washed twice with PBS, then trypsinised with 0.05% Trypsin-EDTA (Sigma-Aldrich) for 10 minutes, neutralised with trypsin medium, centrifuged (125 × g, 8 minutes, at room temperature), and resuspended in culture medium (DMEM-F12 + 10% FBS). Only hDPCs that had been passaged no more than 5 times were used for the experiments.

Gene expression studies

The expression of mRNA transcripts was determined by quantitative real-time PCR (RT-qPCR) following reverse transcription. Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions, and the quality of the extracted RNA was assessed using a Nanodrop-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and characterised the nucleic acid content at 260 nm and protein contamination at 280 nm using absorbance measurements. Subsequently, 2 μg of total RNA was transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. PCR amplification was performed using the TaqMan universal PCR master mix protocol (Thermo Fisher Scientific) and TaqMan gene expression assays (assay IDs: Hs00175798_m1 for TRPA1, Hs00202960_m1 for TRPC5, Hs01066091_m1 for TRPM2, Hs00257553_m1 for TRPM3, Hs01066596_m1 for TRPM8, Hs00218912_m1 for TRPV1, Hs00275032_m1 for TRPV2, Hs00376854_m1 for TRPV3, Hs00222101_m1 for TRPV4, Hs00985639_m1 for IL-6, Hs00174103_m1 to IL-8/CXCL8, Hs01551078-m1 to TLR3, Hs00533490_m1 to SOD1 [superoxide dismutase 1], Hs00167309_m1 to SOD2, Hs00156308_m1 to CAT [catalase], and Hs00829989_gH to GPX1 [glutathione peroxidase 1]). As internal controls, we determined the transcripts of peptidyl-prolyl isomerase A (PPIA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin (ACTB) (assay IDs: Hs99999904_m1, Hs99999905_m1, Hs99999903_m1). To ensure the reliability of gene expression (CT values), three technical replicates were used. Transcript quantities were normalised to the expression of housekeeping genes using the ΔCT method, with the geometric mean CT value of housekeeping genes serving as a reference.

Measurement of inflammatory cytokine secretion

The supernatants from hDPC cultures exposed to various treatments for 24 hours (100,000 cells per culture) were collected and tested for the presence of human IL-6 and IL-8

using a commercially available ELISA kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocols. Microtitre plates were coated with capture antibody diluted in coating buffer (0.1 M Na_2CO_3, pH 9.5, adjusted with 10 N NaOH) and incubated overnight at 4°C. The plates were then incubated with assay diluent (10% foetal bovine serum in PBS) at room temperature for 1 hour, while the standard and sample dilutions were prepared, also in assay diluent. The concentration standards and samples were dispensed into the appropriate wells and incubated at room temperature for 2 hours. After 2 hours, the detector working solution (detector antibody + SAv-HRP reagent) was added to each well and incubated at room temperature for 1 hour. Between steps, the plates were washed with wash buffer (0.05% Tween-20 in PBS). After washing, substrate solution (tetramethylbenzidine and hydrogen peroxide in citrate buffer, pH 5.0) was added to each well for 30 minutes in the dark, followed by stop solution (2 N H_2SO_4). Absorbance was measured at 405 nm within 30 minutes after stopping the reaction. The amount of cytokines (pg/ml) was calculated based on a calibration curve prepared by serial dilution of IL-6 and IL-8 standards.

Fluorescent measurements of intracellular Ca^{2+} concentration

Changes in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{IC}$) were measured using the fluorescent, Ca^{2+}-sensitive dye Fura-2. Measurements were performed according to our previously optimised protocol (53). hDPCs were seeded into 96-well plates with black walls and transparent bottoms (Greiner Bio-One, Kremsmünster, Austria) at a density of 20,000 cells/well in culture medium and cultured for 1 day. The next day, the cells were treated with poly(I:C) or vehicle for an additional 24 hours. After treatment, the cells were loaded with 2 µM Fura-2 AM (Thermo Fisher Scientific) dissolved in culture medium at 37°C for 30 minutes. The cells were washed three times and then incubated in Ca^{2+} buffer (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2 \cdot 2H_2O$, 10 mM glucose·xH_2O, 10 mM HEPES, pH 7.4; all from Sigma-Aldrich) (100 µl/well). The plates were then placed in a FlexStation 3 multimodal microplate reader (Molecular Devices, Sunnyvale, CA, USA), and $[Ca^{2+}]_{(IC)}$ was monitored by continuously measuring fluorescence while the cells were treated with compounds at different concentrations. During the measurement, only one concentration of a substance was applied in each well. The measured fluorescence data were given in F340/F380 form, where F340 is the fluorescence measured at 340 nm excitation and 510 nm emission, while F380 is the fluorescence measured at 380 nm excitation and 510 nm emission. The

experiments were repeated in several wells, and the cells in each well were spread, cultured and treated independently of each other. In the data analysis, N denotes these experiments performed in independent wells. According to the experimental design, N = 6, but some samples were excluded from the analysis due to obvious pipetting errors and/or lack of response to ionomycin used as a positive control at the end of the experiments.

To examine the distribution of responding cells, $[Ca^{2+}]_{(IC)}$ was measured in individual cells using a fluorescence microscope. For these measurements, cells were seeded in glass-bottomed Petri dishes (Ibidi, Gräfelfing, Germany) suitable for fluorescence microscopy and treated with poly(I:C) or vehicle control for 24 hours. The cells were then loaded with 2 μM Fluo-4 AM (Thermo Fisher Scientific) and placed on the stage of a Zeiss LSM 5 Live confocal fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) for fluorescence measurement. The compounds dissolved in Ca^{2+} buffer were delivered to the vicinity of the cells under investigation using a gravity-fed local perfusion system, while continuous removal of the buffer was ensured by a perfusion pump-operated suction system, maintaining a continuous flow during the measurements. Fluorescence was monitored at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Changes in $[Ca^{2+}]_{IC}$were characterised in F1/F0 form, where F1 represents the current fluorescence and F0 represents the average basal fluorescence at the start of the measurement, before the application of any compound.

Measurement of mitochondrial superoxide production

Mitochondrial superoxide production was examined using the MitoSOX™ Red assay (Thermo Fisher Scientific), which is based on a superoxide-sensitive fluorescent dye specifically targeted to mitochondria. The dye is oxidized by superoxide radicals generated in the mitochondria and does not react with other reactive oxygen or nitrogen radicals. The oxidized form binds to mitochondrial nucleic acids to produce a fluorescent signal whose intensity is proportional to the rate of mitochondrial superoxide production, thus allowing quantitative comparison of the oxidative effects of different treatments. During the experiments, 2×10^4 cells were seeded in 200 μl DMEM-F12 medium in 96-well, black-walled, transparent-bottom microplates (Greiner Bio-One), and the cells were cultured for 24 hours. The next day, 50 μl of MitoSOX Red fluorogenic dye solution was added to each well and the cells were incubated for 20 minutes at 37 °C. The cells were then treated with polyinosin:polycytidylic acid (poly(I:C); InvivoGen, San Diego, CA, USA) and TRPA1 channel agonists and antagonists

at various concentrations. Antimycin A (10 μg/ml) was used as a positive control. The fluorescent signal was measured with a FlexStation 3 microplate reader (Molecular Devices) at 510 nm excitation and 580 nm emission wavelengths, 60 minutes after treatment. During data analysis, N denoted wells containing independently spread, cultured, treated, and measured cells.

Assessment of cell viability

Cell viability was assessed using two methods that measure mitochondrial and cytoplasmic enzyme activity. Mitochondrial activity was examined through the conversion of a non-toxic tetrazolium compound into a soluble formazan dye, catalysed by mitochondrial dehydrogenases, using the EZ4U (Biomedica, Vienna, Austria) assay, according to the manufacturer's protocol. Cells were seeded into 96-well cell culture plates (2×10^4 cells/well), cultured for 24 hours, and then treated with various compounds for an additional 24 hours. After treatment, 20 μl of working solution containing tetrazolium substrate was added to each well with 180 μl of phenol red-free culture medium, and incubated for an additional hour. After incubation, the absorbance of the formazan product was measured at 450 nm, while the reference measurement was performed at 620 nm using a FlexStation 3 microplate reader (Molecular Devices). The absorbance values were normalised to the average optical density of the control group.

Cytoplasmic enzyme activity was assessed based on the conversion of calcein acetoximethyl ester (calcein-AM), which passes through the membrane, into fluorescent calcein, which does not pass through the membrane and accumulates in the cytoplasm. The cells were lysed and treated in the same way as for the EZ4U assay, then loaded with 2 μM calcein-AM for 30 minutes. After incubation and washing, calcein fluorescence was measured at 490 nm excitation and 520 nm emission using a FlexStation 3 microplate reader. Experiments were performed in multiple wells, and cells in each well were separately lysed, cultured, and treated.

Gene silencing

To silence TRPA1 expression in hDPCs, we used RNA interference techniques, employing small interfering RNAs (siRNAs) targeting TRPA1-encoding mRNA transcripts. Cells were seeded in six-well or 96-well plates and transfected with siRNA probes 24 hours later using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. RNAiMAX and siRNA probes were carefully mixed in Opti-MEM

medium (Thermo Fisher Scientific), incubated for 5 minutes, and then gently added to the cultures. A final concentration of 100 pmol/ml siRNA was added to the cells, and Lipofectamine RNAiMAX was used at a ratio of 3:1000. Cells were treated with poly(I:C), TRPA1 antagonists, or vehicle controls 24 hours after transfection and examined after an additional 24 hours (i.e., 48 hours after transfection). The efficacy of the three TRPA1-targeting sequences (Silencer® Select siRNA, Thermo Fisher Scientific, Assay IDs: s17147, s17148, s17149) were tested in preliminary experiments at 48 hours post-transfection, and the most effective (Assay ID: s17148) was used in the experiments. Non-targeting negative control siRNA (Thermo Fisher Scientific) was used to control for non-specific effects of transfection.

Materials

To induce inflammatory conditions, we used polyinosin:polycytidylic acid (poly(I:C), InvivoGen, San Diego, CA, USA) for 24 hours. The TRPA1 agonists, allyl isothiocyanate (AITC) and cinnamaldehyde (CA), were obtained from Sigma-Aldrich and Santa Cruz (Santa Cruz, CA, USA), respectively. The TRPA1 antagonist A967079 was purchased from MedChemExpress, while HC030031 was purchased from Sigma-Aldrich, as were hydrogen peroxide (H_2O_2) and glutathione. In certain measurements, ionomycin (Abcam, Cambridge, UK) or antimycin A (Sigma-Aldrich) served as positive controls.

Animals

The study was conducted on samples from healthy, male C57BL6/J mice aged between 12 and 16 weeks. The animals were housed in standard polycarbonate cages (330 mm × 100 mm × 130 mm, 2–5 mice/cage) at the Institute of Pharmacology and Pharmacotherapy, University of Pécs, and received standard rodent chow (LT/n, Szinbád Kft., Gödöllő, Hungary) and tap water ad libitum at a temperature of 20–24 °C, relative humidity of 50–60%, and a 12–12 hour dark–light cycle. Healthy, untreated mice were euthanised and their organs and tissues were removed. All procedures were approved by the Animal Welfare Committee of the University of Pécs and the National Scientific Animal Experimentation Ethics Committee (BA/73/00064-4/2025), in accordance with the 1986 Directive of the Council of the European Communities and Hungarian Law XXCIII (1998) on the care and use of animals. We constantly strived to reduce the number of animals used to a minimum and to minimise their suffering as much as possible.

Sample collection

A total of 20 upper and lower incisors and molars were collected from 20 male C57BL/6J mice aged between 12 and 16 weeks. First, deep anaesthesia was induced intraperitoneally with urethane (2.4 g/kg) (Sigma-Aldrich, St. Louis, MO, USA; Item No.: 501838936). The mice were then transcardially perfused with 20 ml of ice-cold, 0.1 mol/l phosphate-buffered saline (PBS, pH 7.4), followed by 150 ml of 4% paraformaldehyde (PFA) solution (Sigma-Aldrich; Item No.: 158127). The upper and lower incisors, together with the surrounding alveolar tissue and tendon, were then post-fixed in PFA solution at 4 °C for three days. The samples were then randomly divided into five groups (n = 4 per group), and each group underwent a different decalcification procedure.

Decalcification procedures

We evaluated the effectiveness of five different decalcification methods. The detailed composition of the solutions used and the incubation conditions are shown in Table 1. All components were obtained from Sigma-Aldrich. Each sample was treated with 50 ml of decalcification solution. In each case, the effectiveness of decalcification was checked manually, and the dehydration process was started when the samples reached their elastic properties.

Decalcifying agent	**Composition of decalcifying solution**	**Decalcification conditions**
Plank-Rychlo solution	8.5 mL hydrochloric acid (Cat. No.: 320331), 5 mL formic acid, 7 g aluminium chloride (Cat. No.: 237051), 100 mL distilled water	6 hours at 4 °C
Morse solution	10% sodium citrate (Cat. No.: S4641), 20% formic acid in distilled water	12 hours at 4 °C
5% formic acid (Cat. No.: F0507	5% formic acid in distilled water	1 week at 4 °C
EDTA (Cat. No.: E9884)	150 g EDTA heated in 1000 mL distilled water until clear, then add 15 g sodium hydroxide (Cat. No.: 221465) (pH 7–7.4)	1 week at 56 °C
ACD bone decalcifying buffer (Cat. No.: 321918)	Ready-to-use formulation. The company treats the composition of the solution as a trade secret	2 One week at 4 °C

Decalcifying solutions and protocols

Micro-CT

The decalcification protocols were performed on mouse tooth samples, and their efficacy was quantitatively evaluated based on the measurement of mineral density in molar teeth using micro-CT, on samples from the same animals from which RNA integrity was also determined in incisors. The images were acquired using a Skyscan 1176 micro-CT scanner (Bruker, Billerica, MA, USA). The voltage was 30 kV, the current was 360 μA, and the exposure time for each scan was 3500 ms, using a 0.5 mm aluminium filter and a 0.7-degree step during a 180-degree rotation. One image took approximately 36 minutes to acquire. One pixel corresponded to 8.74355 μm in reality. Raw images were reconstructed using NRecon (v. 1.7.4.2) software. The selected areas were analysed using CTan (v.: 1.20.8.0+) and ImageJ (version 1.52a, NIH, USA) software. Prior to evaluation, the software was calibrated for dental density measurement.

Sample preparation and sectioning

After dehydration, the samples were oriented and embedded in paraffin. Cross-sectional slices 5 μm thick were prepared from the teeth using a microtome (HM 430, Thermo Fisher Scientific, Waltham, MA, USA). The sections were placed on SuperFrost Ultra Plus slides (Thermo Fisher Scientific; Cat. No.: 1014356190). No samples were damaged during embedding or RNAscope processing.

Haematoxylin-eosin (HE) staining was performed on the tooth sections to determine the effectiveness of the decalcification procedures and to evaluate the integrity of the histological microstructure. During staining, the sections were treated three times for 5 minutes with xylene (Sigma-Aldrich; Cat. No.: X4126) and then twice for 3 minutes with isopropyl alcohol (Sigma-Aldrich; Cat. No.: I9516). The samples were then treated with Mayer's haematoxylin (BioGnost, Zagreb, Croatia; Cat. No.: HEMM-OT-X) for 5 minutes and soaked in tap water for another 5 minutes. The sections were then treated with eosin (BioGnost; Cat. No.: EOY-10-OT-X) for 2 minutes, rinsed with distilled water, and treated with isopropyl alcohol twice for another 3 minutes. The slides were placed in xylene for 2 minutes and then covered with DPX cover slip (Fluka/Honeywell, Seelze, Germany).

RNAscope in situ hybridisation

We used RNAscope in situ hybridisation (ISH) to examine the RNA integrity of paraffin-embedded mouse tooth samples. Pretreatment was performed according to the RNAscope Multiplex Fluorescent Reagent Kit v2 user manual (ACD, Hayward, CA, USA; Cat. No. 323100). The sections were incubated at 60 °C for 1 hour, then deparaffinised in xylene for 2 × 5 minutes, followed by treatment with absolute ethanol for 2 × 2 minutes. The sections were then dried at 60 °C for 5 minutes, treated with hydrogen peroxide for 10 minutes to inhibit endogenous peroxidase, and washed with Milli-Q (MQ) water.

We then tested three different permeabilisation methods for optimisation: Permeabilisation method 1. The sections were incubated in a 20 mg/mL concentration of pre-warmed proteinase K (Sigma-Aldrich; Cat. No.: P2308) solution at 37 °C for 20 minutes. Permeabilisation method 2. The sections were treated in a microwave oven at 700 W for 10 minutes in 100 mL of pH 6 citrate buffer (Sigma-Aldrich; Cat. No.: C9999). After washing with MQ water, the sections were treated with absolute ethanol for 3 minutes and then dried at 60 °C for 5 minutes. After tracing the sections with a hydrophobic pen (Vector Immedge Pen, Vector Laboratories, Newark, CA, USA), the sections were treated with Protease Plus (ACD; Cat. No.: 322331) solution for 30 minutes at 40 °C in a humidified steam chamber. Permeabilisation method 3. The sections were boiled at high pressure for 10 minutes in Target Retrieval solution (ACD; Cat. No.: 322000). After washing with MQ water, they were treated with absolute ethanol for 3 minutes, followed by drying at 60 °C for 5 minutes. After tracing the sections with a hydrophobic pen, Protease Plus (ACD; Cat. No.: 322331) treatment was applied for 30 minutes at 40 °C in a humidified steam chamber.

After completion of the experiments, the subsequent steps were identical for all samples and followed the instructions in the manufacturer's (ACD) user manual. After permeabilisation, the sections were washed with MQ water and then hybridised for two hours with mouse 3-plex positive control probes (ACD, Cat. No.: 320881). This reagent targets three different mouse housekeeping gene-specific sequences expressed at different levels and labelled with different fluorochromes. The low-expression RNA polymerase II A subunit (Polr2a) mRNA was labelled with fluorescein, the medium-expression peptidyl-prolyl cis-trans isomerase B (Ppib) mRNA was labelled with cyanine 3 (Cy3), and the high-expression ubiquitin C (Ubc) mRNA was labelled with cyanine 5 (Cy5). A 3-plex negative control probe targeting bacterial L-2,3-dihydrodipicolinate reductase mRNA (dabP) was used as a negative control. Following hybridisation, signal amplification and channel development steps were performed according

to the manufacturer's (ACD) instructions. Finally, the samples were washed in PBS for 2 × 15 , then stained with 4′,6-diamidino-2-phenylindole (DAPI) (ACD; Cat. No.: 320858) to label the cell nuclei. After washing, the sections were covered with ProLong Gold Antifade (Thermo Fisher Scientific; Cat. No.: P36930) mounting medium and stored at -20 °C until imaging.

Microscopy, morphometry

HE sections were examined using a Nikon Microphot FXA microscope and recorded with a Spot RT colour digital camera (Nikon, Tokyo, Japan). RNAscope ISH stained samples were digitised in analogue mode using sequential scanning with an Olympus FluoView 1000 confocal microscope (Olympus, Hamburg, Germany) in analogue mode using sequential scanning. Fluorescence imaging was performed using the following settings: 80 µm confocal aperture, 3.5 µm optical thickness, 1024 × 1024 pixel resolution, using 40× and 60× objectives. The excitation and emission spectra of the fluorescent dyes were selected using FluoView software (FV10-ASW; Version 0102, Olympus) and illuminated the DAPI, fluorescein, Cy3 and Cy5 dyes with 405, 488, 550 and 647 nm laser beams. The sections were scanned in four channels and then digitally merged. In the digital images, the colours blue (DAPI), green (fluorescein), red (Cy3) and white (Cy5) represent the four different channels. Densitometry was performed using ImageJ software (version 1.52a, NIH, USA) on unedited images to quantify the RNAscope signals for each housekeeping gene across the different decalcification methods. Fluorescence intensity was measured in four manually selected surface areas in the pulp using unedited images. Signal density was corrected for background signal. The mean specific signal density (SSD) for each area was determined based on four sections per animal. The mean of these four values gave the SSD value for a mouse. SSD was expressed in arbitrary units.

Data and statistical analysis

In experiments with cell cultures, treated and untreated samples were compared with samples from the same primary culture; therefore, randomisation was not used. The person performing the statistical analysis was blind to the experimental conditions. The data and statistical analysis comply with recommendations for pharmacological experimental design and data evaluation. Origin 2018 software (OriginLab Corporation, Northampton, MA, USA) was used for data analysis and data presentation. Unless otherwise indicated, individual data are presented as scatter plots with mean ± SD. Logistic concentration-response curves were fitted using the equation y = A2 + (A1 – A2)/(1 + (x/x0)^p), where the calculated parameters are as

follows: A1: initial value (ymin), A2: final value (ymax), x0: centre point (EC50), p: calculated power. Statistical analysis was performed using IBM SPSS Statistics 22 software (IBM, Armonk, NY, USA). In gene expression studies, expression values were subjected to log10 transformation, followed by statistical analysis. Before applying parametric statistical tests, we examined the suitability of the data. We checked the normality of the data using the Shapiro-Wilk test and examined the homogeneity of variances using the Levene test. We used an independent samples Student's t-test to compare two groups. We performed a paired t-test to compare the gene expression of two samples from the same donors. We used one-way ANOVA to compare multiple groups. If the F value was significant, we performed pairwise comparisons using the Tukey HSD post hoc test. When comparing multiple groups to a single control group, we used the Dunnett post hoc test for pairwise comparisons. To examine the interaction between two treatments, we used two-way ANOVA. In all cases, we considered *P < 0.01 to be significant.

Statistical analysis of data obtained from histological samples was performed using Statistica 13.5.0 software. Data are presented as mean ± SEM. All data sets were tested for normal distribution (Kolmogorov–Smirnov) and homogeneity of variance (Levene). The main effects were examined using one-way analysis of variance (ANOVA, variable: decalcification procedure), followed by Tukey's post hoc test. A p-value below 0.05 was considered statistically significant. There was minor variability between animals within the group, but no clear outliers.

Results

Results of I.TRPA1 in vitro studies

Temperature-sensitive TRP channels are expressed in hDPCs

To examine the expression of temperature-sensitive TRP ion channels in primary hDPCs, total RNA was isolated from several donor hDPC cultures and subjected to reverse transcription followed by Q-PCR. hDPCs expressed high levels of heat-sensitive TRPV2 and TRPV4 channels. The expression of TRPV1 transcripts was approximately one order of magnitude lower, while TRPV3, TRPM2, and TRPM3 were detected at low levels and only in a few donors. When examining cold-sensitive TRPs, we detected high TRPA1 expression in most donors, and TRPC5 transcripts showed slightly lower expression. It is important to note that TRPM8, the main cold-sensing receptor in somatosensory neurons, was not detected in any of the samples.

Poly(I:C) induces inflammatory responses and enhances TRPA1 channel expression

To investigate whether the expression of the above TRP channels changes under inflammatory conditions, we also examined their expression in a poly(I:C)-induced inflammatory model. To this end, we treated hDPCs in our primary cultures with poly(I:C) for 24 hours to induce inflammatory conditions. Poly(I:C) markedly and concentration-dependently increased the expression of proinflammatory cytokine transcripts, IL-6 and IL-8/CXCL8, and also increased the release of these cytokines. Poly(I:C) also significantly increased the expression of TLR3, a well-known marker of receptor activation. We also analysed the expression of the above TRP channels in this inflammatory model, in which hDPCs were treated with 20 μg/ml poly(I:C). We found that this had little effect on the expression of heat-sensitive TRP channels. With regard to cold-sensitive channels, TRPM8 expression was not induced under inflammatory conditions and, as in the control cases, was not detectable in poly(I:C)-treated samples. Interestingly, TRPC5 expression was significantly reduced in all donors tested (to ≈14% of the control value). In contrast, TRPA1 expression was significantly increased, on average approximately 37-fold in seven donor samples. In one additional donor, the increase exceeded 10,000- -fold, although the baseline expression of this donor in the control sample was very low.

We further investigated the functional consequences of increased TRPA1 gene expression and monitored changes in $[Ca^{2+}]_{IC}$during the application of TRPA1 channel agonists. We found that AITC and CA elicited a rapid, concentration-dependent increase in $[Ca^{2+}]$IC in control hDPCs. These Ca^{2+} signals were significantly enhanced in cells pretreated with poly(I:C), which was consistent with the increased expression of TRPA1 channels. Importantly, both AITC- and CA-induced responses were almost completely blocked by the TRPA1 antagonists HC030031 and A 967079, supporting the TRPA1-specificity of the agonists used. Measurements at the single-cell level showed that poly(I:C) pretreatment increased the average amplitude of AITC-induced Ca^{2+} responses in individual cells but did not affect the proportion of responding cells, which was similarly high under both control and inflammatory conditions. Examination of the pharmacological properties of CA revealed that both the efficacy (maximum response, Δ(F340/F380): 1.94 ± 0.05 vs. 1.06 ± 0.07) and potency (EC50, μM: 19.3 ± 1.6 vs. 62.6 ± 13.9) were higher in the inflammatory group compared to control values.

hDPCs become more sensitive to exogenous H_2O_2 under inflammatory conditions due to increased TRPA1 expression.

The TRPA1 channel is generally considered to be a cellular sensor for harmful stimuli and is activated by reactive oxygen species (ROS), among other things. Therefore, we hypothesised that upregulation of TRPA1 under inflammatory conditions could result in increased sensitivity to reactive oxygen species. To test this, we treated hDPCs with H_2O_2 and measured its acute effect on $[Ca^{2+}]_{IC}$. Control cells showed only a moderate Ca^{2+} response to H_2O_2 concentrations above 1 mM. In contrast, hDPCs pretreated with poly(I:C) were activated by lower concentrations of H_2O_2 and produced significantly larger responses. Importantly, H_2O_2-induced Ca^{2+} signals were abolished in the presence of HC030031, clearly indicating that the H_2O_2-induced increase in $[Ca^{2+}]_{IC}$- is mediated by TRPA1 channel activation.

Poly(I:C) induces oxidative stress and increases mitochondrial superoxide production

Mitochondria are the primary sites of intracellular ROS production, which is neutralised by the antioxidant defence system. An imbalance between ROS production and antioxidant defence leads to oxidative stress and an increase in intracellular ROS concentration. Since ROS are important signalling molecules in inflammation and can exacerbate inflammation-induced tissue damage, we investigated whether poly(I:C) induces oxidative stress and ROS production

in our inflammation model. We found that the expression of mitochondrial superoxide dismutase 2 (SOD2), an enzyme responsible for converting mitochondrial superoxide radicals to H_2O_2, was significantly increased, while the expression of cytoplasmic SOD1 and H_2O_2-neutralising catalase and glutathione peroxidase 1 (GPX1) remained unchanged. These results suggested severe disturbances in cellular redox homeostasis and indicated mitochondrial oxidative stress. Indeed, we found that poly(I:C) rapidly (within 60 minutes) induced mitochondrial superoxide production in a concentration-dependent manner. Unlike poly(I:C), TRPA1 agonists did not stimulate superoxide production, suggesting that selective activation of TRPA1 does not induce oxidative stress. Furthermore, TRPA1 antagonists had only a partial effect on poly(I:C)-induced superoxide production. Although HC-030031 and A967079 partially reduced superoxide production induced by 10 μg/ml poly(I:C), it remained significantly elevated even in their presence, and the effect of 20 μg/ml poly(I:C) was not affected by TRPA1 inhibition. These results suggested that TRPA1 plays only a partial role in the regulation of mitochondrial ROS production under poly(I:C)-induced inflammatory conditions. TRPA1 antagonists did not reverse poly(I:C)-induced inflammation and did not inhibit the production of inflammatory IL-6 and IL-8/CXCL8.

Poly(I:C)-induced inflammation reduces mitochondrial activity, which is mediated by ROS production and, in part, by TRPA1.

Although TRPA1 only slightly affected mitochondrial ROS production under inflammatory conditions, we hypothesised that the upregulated ion channel could be a target for endogenously produced reactive oxygen species and contribute to the consequences of oxidative stress under inflammatory conditions. Therefore, we examined the effect of poly(I:C)-induced inflammation on cell viability and the possible role of TRPA1 in this effect. To assess cell viability, we measured mitochondrial enzyme activity and cytoplasmic esterase activity of . We found that poly(I:C) caused a significant decrease in mitochondrial function but had little effect on cytoplasmic enzyme activity. Mitochondrial dysfunction was mitigated by the ROS scavenger glutathione in a concentration-dependent manner, suggesting that oxidative stress plays a significant role in the negative effects of poly(I:C) on mitochondrial function. However, cytoplasmic enzyme activity was not restored by glutathione, suggesting that non-oxidative mechanisms may also play a role in some of the effects of poly(I:C).

Since TRPA1 expression was also significantly increased under these conditions, we hypothesized that this ROS-sensitive ion channel may play a role in inflammation-induced mitochondrial dysfunction. We found that poly(I:C)-induced mitochondrial damage was partially prevented by the application of the TRPA1 antagonists HC-030031 and A 967079, but these inhibitors had little effect on cytoplasmic enzyme activity. Furthermore, the effect of poly(I:C) was significantly reduced following TRPA1 RNAi-based silencing. In addition, we found a significant interaction between poly(I:C) treatment and A 967079 or siRNA transfection (two-way ANOVA). These results support the conclusion that elevated TRPA1 expression plays a role in inflammation-related pulp tissue damage, although TRPA1-independent mechanisms are also involved.

II. Development of an optimal decalcification method and tissue preparation protocol

Based on our results to date, our further research plans include *in situ* examination of TRPA1 expression in the pulp. As there are no selective, reliable antibodies available for TRPA1, we plan to perform these studies using in situ RNA hybridisation. The studies are complicated by the hard tissue surrounding the pulp, which has a high mineral content and whose decalcification can significantly affect RNA quality and degradation. Therefore, our additional goal was to optimise a decalcification and sample preparation procedure for histological examination of teeth, which allows for proper sectioning of teeth without damaging mRNA, and is thus suitable for RNAscope ISH. To this end, we tested five different decalcification methods, followed by micro-CT examination to verify decalcification and HE staining to evaluate the microstructure of the samples. RNA integrity was assessed using mouse 3-plex positive control assays, which are capable of detecting high, medium and low expression genes simultaneously.

The effect of decalcification on tooth density

We applied five different decalcification protocols to mouse tooth samples and quantitatively evaluated their effectiveness using micro-CT examination. Mineral density measurements showed a significant decrease in all decalcified samples compared to the untreated, intact control group. One-way ANOVA revealed a highly significant difference between groups ($p = 3.06 \times 10^{-11}$), and post hoc comparisons showed that all decalcification treatments resulted in significantly lower density values than the control ($p < 0.0001$). These results show that all five decalcification protocols effectively removed calcium from the dental tissues.

Effect of decalcification on the histological structure of teeth

In general, all five methods resulted in satisfactory decalcification, and the samples could be sectioned without obvious difficulties. However, moderate differences were observed in the quality of the histological structure, and the duration of the methods also varied significantly. The Plank–Rychlo decalcifying solution contains strong inorganic acid (hydrochloric acid) and

organic formic acid. After decalcification in this solution, the tooth samples were easy to cut, and HE staining showed preserved pulp structure.

The advantage of this method is the relatively short incubation time of only 6 hours at 4 °C. When only organic formic acid was used at a concentration of 5%, the decalcification time required to achieve the desired sectioning condition was much longer, as one week at 4 °C was necessary to achieve the appropriate consistency. The histological structure of the teeth was well preserved, as indicated by HE staining. However, we observed some pulp tissue shrinkage. The use of EDTA solution is a generally recommended method for decalcification when the use of destructive acids should be avoided. According to our results, incubation in a 15% EDTA solution at 56 °C for one week resulted in soft, flexible and easily sectionable samples. Proper decalcification was also indicated by the good preservation of the dentinal tubules, as shown by HE staining. Although the hard tissue was adequately processed, we observed a reduction in pulp volume and separation from the dentine, as was also observed with formic acid decalcification. The use of Morse solution proved to be optimal for rapid dental decalcification. The application of a mixture of 10% citrate and 20% formic acid for 12 hours resulted in rapid and adequate decalcification and excellent sectionable tissue. The histological structure of the teeth was well preserved in both the hard tissue (dentine) and the pulp. Finally, we also used a decalcifying solution recommended by ACD for decalcifying bone samples prior to RNAscope. Our tooth samples were incubated at 4 °C for two weeks to achieve a soft, easily sectionable consistency. The histological structure was intact after decalcification.

Optimisation of permeabilisation and the effect of different decalcification methods on RNA integrity

After confirming that all decalcification procedures were successful in terms of sectionability and microstructure, we performed RNAscope ISH to examine RNA integrity. First, we tested three different permeabilisation methods for RNA recovery. Digestion with proteinase K (permeabilisation method 1) proved ineffective, as the ISH probes did not penetrate the samples adequately and resulted in weak, uncertain staining. Incubation in citrate solution in a microwave oven (permeabilisation method 2) weakened adhesion, causing several samples to detach from the slide after treatment. The most optimal digestion method was treatment with Target Retrieval solution (, ACD) for 10 minutes in a steamer, followed by treatment with Protease Plus (ACD) for 30 minutes at 40 °C (permeabilisation method 3).

During ISH, we used three mouse 3-plex positive control probes specific for mouse housekeeping genes, which provided adequate information on RNA integrity. Samples prepared using the five different decalcification methods described above were tested. Decalcification in Plank–Rychlo solution resulted in severe RNA degradation. All three housekeeping genes examined were barely detectable, although the expected difference in signal intensity between the three housekeeping genes remained recognisable.

A one-week treatment with 5% formic acid resulted in slightly better RNA integrity. All housekeeping genes were clearly detectable by RNAscope ISH, although their levels were lower than expected, suggesting partial RNA degradation in the samples. EDTA solution, the most commonly recommended decalcification method for preserving sensitive, degradation-prone protein antigens, caused severe mRNA damage and resulted in the worst RNAscope signal among the methods tested. As a result, low-expression Polr2a transcripts became undetectable. Medium-expression Ppib transcripts were also barely detectable. Even probes targeting high-copy Ubc transcripts yielded only very weak ISH signals. In contrast to the previous methods, decalcification in Morse's solution and the use of ACD bone decalcifying buffer resulted in high-quality ISH signals. Even low-copy Polr2a transcripts were clearly detectable with high reliability, while probes targeting Ppib and Ubc showed strong, punctate and contiguous signals consistent with their expected expression levels. The above results showed that decalcification with ACD Bone Decalcification Buffer or Morse solution preserves RNA integrity and is well suited for subsequent RNAscope ISH procedures. RNA integrity was assessed after the five different decalcification protocols using RNAscope ISH by densitometric quantification of three different expression levels of a housekeeping gene. The purpose of this analysis was to determine which decalcification method best preserves RNA quality. One-way ANOVA showed a statistically significant difference between the groups ($p = 6.25 \times 10^{-13}$). Post hoc comparisons showed that samples decalcified with Morse's solution and ACD solution retained the highest RNA integrity, and there was no significant difference between these two treatments ( ). In contrast, all other decalcification protocols significantly reduced RNA signal intensity compared to both the Morse and ACD groups ($p < 0.0001$). These results indicate that Morse and ACD solutions best preserve RNA integrity during the decalcification of dental specimens.

Discussion

The sensitivity of dental pulp is primarily related to the trigeminal fibres that innervate it, but non-neural pulp tissue cells can also contribute significantly to sensory transduction. Cell sensitivity is determined by various receptors, among which TRP channels are prominent.

To date, several members of the TRP ion channel family have been detected in dental pulp and the trigeminal fibres innervating it, but their exact role in pulp inflammation is not yet fully understood. In our study, we focused on the non-neural elements of human pulp and systematically analysed the expression of heat-sensitive members of the TRP family in primary human pulp cells (hDPC) under normal and inflammatory conditions, the latter induced by the application of poly(I:C), a potent activator of the TLR3 pattern recognition receptor. Under control conditions, we found significant expression of the heat-sensitive TRPV1, TRPV2 and TRPV4 channels, the cold-sensitive TRPC5 channel, and the TRPA1 channel, which may be sensitive to both noxious cold and heat stimuli. The TRPM2 and TRPM3 channels were only detectable in a few donor samples, and even then at relatively low levels, while the TRPV3 channel was present in only a few samples and in very small quantities. Interestingly, the TRPM8 channel, which is the main cold sensor in somatosensory fibres, was not detected at all in any of the hDPC samples.

Previous studies analysed only a few TRP channels at a time, which is why the results of different models sometimes differed. However, in line with our results, several studies have confirmed the expression of TRPV1, TRPV2, TRPV4, TRPC5 and TRPA1 channels in non-neuronal pulp cells in both rodents and humans. Similar to our findings, the TRPM8 channel was not detectable in rodent odontoblasts and human odontoblasts, although one study demonstrated functional expression in human odontoblasts and pulp fibroblasts. According to immunohistochemical data, the TRPM8 channel is present in the odontoblast layer, but almost absent in the internal pulp of human teeth. It is conceivable that this discrepancy is due to the fact that TRPM8 expression may be limited exclusively to differentiated odontoblasts, thus no longer detectable in proliferating cell cultures. In addition, species differences and the often questionable quality of commercially available antibodies against TRP channels may further complicate the interpretation. The situation is further complicated by the fact that TRPM8 channel agonists, icilin and menthol, can also activate TRPA1 channels at higher concentrations, which makes it difficult to pharmacologically separate the two channels ( ). In an elegant, recent study, Zimmermann's group demonstrated that the role of TRPM8 in cold

sensation is limited to the primary afferent fibres innervating the tooth, while the primary cold sensor in odontoblasts is the TRPC5 channel, but the contribution of TRPA1 has also been confirmed.

The TRPA1 channel is known as a multimodal nociceptor that is sensitive to both thermal and chemical stimuli. Several studies have shown that it is widely expressed in the pulp, and we also found significant expression in hDPC cells. Under poly(I:C)-induced inflammatory conditions, the expression of TRPA1 transcripts increased dramatically—approximately 40-fold—in our samples. TRPC5 expression decreased, while the expression of other heat-sensitive TRP channels did not change significantly. TRPA1 channels appear to be generally involved in inflammatory signalling processes in the pulp, as other studies have also reported that TNFα and bacterial lipopolysaccharide enhance their expression in pulp-derived cells. Although the signalling pathway that enhances TRPA1 channel expression is not known in detail, previous results suggest that NF-κB activation, which is a known downstream signalling pathway of TLR3, may contribute to channel upregulation in our inflammatory model. Activation of NF-κB in response to various stimuli increased TRPA1 channel expression in lung cancer cells, HaCaT keratinocytes, allergic contact dermatitis, and sensory neurons. In fibroblast-like synoviocytes, inflammatory cytokines led to the activation of the hypoxia-inducible factor-1α (HIF1α) transcription factor via the NF-κB-related signalling pathway, which increased TRPA1 expression. The clinical significance of TRPA1 channels in pulpitis is also supported by the fact that increased TRPA1 immunoreactivity has been described in human teeth with caries. Furthermore, H_2O_2-containing tooth whitening gels also enhance the expression of TRPA1 channels in pulp-derived stem cells.

Importantly, our results show that not only expression but also specific functional responses to TRPA1 channel agonists were greatly enhanced in hDPCs cultured under inflammatory conditions. Under inflammatory conditions, the maximum responses elicited by TRPA1 agonists were greater, which correlates well with the increased expression of the channel. In addition, the potency of cinnamaldehyde was also increased, as indicated by a decrease in the EC50 value, suggesting increased sensitivity of TRPA1 channels. ROS produced endogenously during inflammation may contribute to this sensitisation by reacting with intracellular cysteine residues of the channel. These reactive residues may be targets for further inflammatory mediators, such as the electrophilic arachidonic acid derivatives of the e . In addition, PGE2 has been shown to sensitise TRPA1 channels via PKA- and PKCε-mediated

pathways. These findings indicate multiple points of connection between inflammatory signalling pathways and TRPA1 channels, which are likely to be present in hDPCs.

TRPA1 channels are activated by reactive oxygen species (ROS), and our results clearly demonstrated that under inflammatory conditions, hDPCs become more sensitive to oxidative stress, which we modelled using acute H_2O_2application. Co-administration of a TRPA1 antagonist almost completely abolished H_2O_2-induced Ca^{2+}responses, confirming that these oxidative stress-induced Ca^{2+}signals are mediated through TRPA1 channel activity. H_2O_2 is widely used in teeth whitening procedures and is known to damage pulp cells and induce the expression of inflammatory mediators and TRPA1 channels. 2-hydroxyethyl methacrylate, used in dental restorations, is thought to activate TRPA1 channels in hDPC cell lines via ROS. Our results show that inflamed pulp may be even more sensitive to ROS due to increased TRPA1 expression. ROS is released in high concentrations and plays an important role in inflammation. In pulpitis, ROS is produced by infiltrating immune cells to destroy pathogenic bacteria, but this can also damage surrounding tissues. In addition, dental pulp cells are also capable of producing ROS. We found that poly(I:C) stimulation increases mitochondrial superoxide production in hDPCs, accompanied by decreased mitochondrial activity and impaired cell survival within 24 hours. Previous studies have suggested that modulating ROS signalling in the pulp may be a promising therapeutic target for preventing inflammation-induced tissue damage. ROS scavengers inhibited inflammatory signals and prevented pulp cell damage in various models. In our study, glutathione also inhibited inflammation-related mitochondrial dysfunction, suggesting that oxidative stress plays an important role in poly(I:C)-induced damage. Furthermore, inhibition of ROS-sensitive TRPA1 channels or reduction of TRPA1 expression partially prevented inflammation-related cell damage in hDPCs. These results clearly indicate that inhibition of TRPA1 channels may represent a promising therapeutic approach for reducing pulp damage induced by inflammation and oxidative stress.

Previously, the only possible treatment for irreversible pulpitis was root canal treatment with complete removal of the pulp. This was the only option for preserving the tooth and avoiding extraction . However, it had a negative effect on the biomechanical properties of the remaining tooth structure and reduced the long-term life of the tooth. Over the past decade, viable pulp treatments have become increasingly common, and these were initially considered feasible only if a diagnosis of reversible pulpitis could be made prior to pulp inflammation. However, Ricucci's histological studies have confirmed that the clinical diagnosis is not

necessarily consistent with the histological condition of the pulp. In many cases, despite the classic clinical signs and symptoms of irreversible pulpitis, bacterial invasion and inflammatory infiltration are limited to the pulp horns, and in such cases, partial or total pulpotomy may be a more favourable alternative to root canal treatment. However, the success rate of viable pulp therapies varies widely, ranging from 31.8% to 100% under different circumstances. We believe that the use of effective agents that significantly reduce pulp inflammation on the surface of the resected pulp may improve the outcome of these procedures. Our results suggest that TRPA1 channel antagonists and ROS signalling inhibitors may be promising candidates for reducing pulp inflammation. Thus, our findings may contribute to the development of more effective treatments for reducing pulp inflammation, thereby expanding the toolkit for regenerative endodontics.

The second part of our work is essential for examining the expression of ion channels and other molecules involved in inflammation, such as TRPA1, with high spatial resolution and tissue specificity. The comparison of decalcification and sample preparation protocols has allowed us to identify procedures that preserve the morphological integrity of dental tissue while also maintaining mRNA integrity, thus providing a reliable basis for examining the expression pattern and inflammation induction of TRPA1 in animal models. Although there are numerous protocols for decalcification and appropriate histological preparation, particularly for bones, their suitability for RNA ISH has been much less studied, and there is particularly limited information available on the examination of teeth as histological samples.

In our studies, we systematically tested and evaluated five different decalcification methods using RNAscope ISH on mouse incisor samples. The techniques used in the study were originally optimised for bone tissue, so our current goal was to clarify how these different decalcification procedures affect mRNA detectability in mouse dental pulp tissues. RNAscope is a new type of RNA ISH method that opens up new possibilities in qualitative and quantitative in situ gene expression studies. It is suitable for detecting virtually any transcript, even in calcified tissues, with a high degree of specificity and outstanding sensitivity, making it a very competitive alternative to immunohistochemistry in many cases. However, its application in dental tissue poses a significant challenge. Decalcification of dental tissue is an essential step in histological examinations. Without the removal of fluoride and hydroxyapatite crystals and other calcium salts, the microtome blades cannot cut through the tissue, the blades would be damaged and the sample would be fragmented. During decalcification, the tissue softens

sufficiently to allow thin, uniform sections to be prepared for microscopic examination. Proper decalcification removes minerals without damaging soft tissue elements, allowing for clear microscopic visualisation of cells, fibres and extracellular matrix structures, and, particularly important for this examination, preserves RNA integrity. Calcium deposits also interfere with routine histological staining (e.g., HE, trichrome), while decalcification removes these mineral barriers to ensure uniform dye penetration and proper staining. Inadequate or uneven decalcification can cause tears, creases or poor staining in the sections. Conversely, excessive decalcification can damage proteins and mRNAs and distort morphology, so timing and reagent selection are critical.

There are several methods available for decalcification, and the vast majority of these can be classified into three main groups based on the mineral removal agent used. Minerals can be removed with strong inorganic acids, which are generally excellent at dissolving mineral components but are highly corrosive and can easily damage sensitive biomolecules such as protein antigens or RNA. Weaker organic acids, such as formic acid, are a gentler alternative and can preserve sensitive macromolecules more effectively while removing extracellularly deposited mineral salts with similar efficiency. As an alternative, chelating agents such as EDTA are recommended for preserving particularly sensitive antigens during immunohistochemical detection.
In this study, we examined five decalcification methods on mouse incisor samples and compared their effects on histological microstructure and suitability for RNAscope ISH.

Decalcifying agent	Histological structure	mRNA integrity
Plank–Rychlo solution	Well-preserved tissue structure in dentine and pulp	Severe degradation
5% formic acid	Well-preserved dentine structure with slight pulp shrinkage	Partial degradation
EDTA	Well-preserved dentine structure, slight pulp shrinkage	Almost complete degradation
Morse solution	Well-preserved tissue structure in dentine and pulp	Preserved mRNA integrity
ACD bone decalcifying buffer	Intact tissue structure in dentin and pulp	Preserved mRNA integrity

Plank–Rychlo solution contains strong inorganic (hydrochloric acid) and organic (formic acid) acids, as well as aluminium chloride. It is recommended for rapid bone decalcification. In our samples, it resulted in excellent decalcification with a short incubation time; at the same time, it also preserved the morphological structure very well. However, it impaired RNA integrity, as indicated by very weak RNAscope signals. These results are consistent with previous data on bone decalcification. Shibata and colleagues demonstrated in mouse jawbones that Plank–Rychlo solution and other inorganic acid-based decalcifying agents, although excellent at preserving the morphology of the ameloblast layer, significantly reduced both 28S rRNA and osteoblast-specific mRNA signals in conventional ISH.

Formic acid is often used for decalcifying bone tissue and is considered a gentler method than inorganic acids, better preserving antigens for immunohistochemistry. According to our results, formic acid ideally preserved the histomorphology of the tooth, although some pulp tissue shrinkage was observed. The disadvantage compared to the Plank–Rychlo solution was the longer incubation time. It should be noted that RNA integrity was better than in samples treated with inorganic acid; but the relatively weak ISH signal suggested partial mRNA degradation. Similar results have been reported previously in rat and mouse jaws: formic acid decalcification slightly impaired histomorphology due to some vacuolisation. However, the integrity of 28S rRNA was relatively well preserved.

EDTA is considered the gold standard for decalcification when a gentle procedure is required. It is ideal, for example, when the antigens to be detected are sensitive to acid degradation. In our experiment, it did indeed preserve histomorphology ideally. Nevertheless, the use of EDTA led to severe mRNA degradation, as we observed hardly any RNAscope ISH signal. It is important to note that highly variable incubation conditions significantly affect mRNA quality. For example, it is well known that high temperatures or prolonged incubation impair RNA integrity. In our protocol, we incubated the samples in EDTA solution at 56 °C to accelerate decalcification. Nevertheless, it took a week for the mouse sections to become suitable for cutting. In previous studies, raising the temperature of the decalcifying EDTA solution from room temperature to 37 °C reduced the time required for decalcification by half in rat jaws. However, this higher temperature impaired cell morphology despite the shorter incubation time. In contrast, another study found that both rRNAs and mRNAs were preserved in mouse jaws after EDTA decalcification, but the incubation temperature was not reported. In addition to temperature and time, the pH of the solution critically affects the efficiency of EDTA

decalcification. In an acidic medium, EDTA did not cause decalcification, while at neutral pH it provided an ideal effect, and alkaline pH further shortened the incubation time.

According to our results, the bone decalcifying buffer recommended by ACD provided excellent decalcification when used at 4 °C for two weeks without compromising the structural quality of the tissues and also enabled outstanding RNA preservation in the tooth samples. Morse's solution yielded similarly excellent results. It should be noted that all this was achieved after a much shorter incubation time of only 12 hours. This is consistent with previous studies, which found Morse's solution to be an excellent decalcifying agent, comparable to EDTA in both rodent and human dental tissue. Morse's solution was also found to be effective for decalcified cochlear samples, with preserved histological morphology and excellent mRNA quality based on qPCR analyses. Based on our current results and previous observations, both ACD buffer and Morse solution can be recommended for the decalcification of tooth samples intended for RNAscope ISH.

In clinical cases, immunohistochemical examinations are most commonly performed on pulp tissue removed from the tooth. Currently, the use of RNAscope ISH on human pulp is not yet a routine testing method, but its advantages mean that it is likely to become increasingly widespread. To date, only a few studies have reported the use of RNAscope for the detection of long non-coding RNA or viral RNA in human pulp tissue. Importantly, these studies used pulp removed from the tooth ( ) for RNAscope ISH, which significantly limits the quality and reliable assessment of tissue components. Sectioning the entire tooth, including the hard tissues and the soft contents of the pulp chamber, may allow for more accurate localisation of mRNAs within the tooth.

In summary, the aim of the present study was to identify the optimal method that allows consistent comparison between multiple decalcification protocols and is suitable for the RNAscope technique in mouse pulp tissue sections. It should be noted that the differences in temperature and time used in the various protocols may contribute to the variability observed in morphological structure and RNA integrity, and the limitation to mouse sections and a single animal model reduces the generalisability of the research. Future studies should therefore extend to the examination of molar teeth and other animal species.

Novel findings

1. We have demonstrated that poly(I:C)-induced inflammation significantly alters the expression of TRP ion channels in human dental pulp cells.

2. We demonstrated that increased TRPA1 expression during inflammation is accompanied by the appearance of functionally active ion channels.

3. We found that the increased TRPA1-mediated Ca^{2+} response results from an increase in the responsiveness of individual cells, rather than an increase in the proportion of responsive cells.

4. We were the first to demonstrate that under inflammatory conditions, the TRPA1-dependent sensitivity of human dental pulp cells to reactive oxygen species is increased.

5. We demonstrated that poly(I:C) induces mitochondrial oxidative stress and functional decline in human dental pulp cells, a process in which oxidative stress plays a dominant role and TRPA1 plays a partial role.

6. We optimized a tooth decalcification and sample preparation protocol that preserves mRNA integrity and makes tooth samples suitable for RNAscope in situ hybridization assays.

Summary

Inflammatory processes in the dental pulp (pulpitis) are the result of complex cellular and molecular mechanisms in which oxidative stress, calcium-related signalling pathways and redox-sensitive ion channels play a central role. The aim of the present study was to investigate the role of the TRPA1 ion channel in the mechanisms of poly(I:C)-induced oxidative stress and inflammatory cell responses in an in vitro model of human pulp cells. The experiments showed that poly(I:C) treatment significantly increased TRPA1 mRNA and protein levels, which can be attributed to the activation of the TLR3-mediated signalling pathway. The increase in channel expression was accompanied by increased intracellular calcium levels and ROS production associated with TRPA1 activation. TRPA1 inhibition (HC-030031 antagonist or siTRPA1 transfection) had a protective effect: it reduced oxidative stress, improved mitochondrial function and cell viability. These results suggest that TRPA1 may be an important factor in the development of poly(I:C)-induced oxidative damage. Elucidating the role of TRPA1 activation opens up new perspectives for understanding the oxidative mechanisms involved in inflammatory processes in the dental pulp. Modulation of this channel may offer a potential therapeutic strategy for the targeted treatment of oral diseases associated with inflammation and oxidative stress, particularly pulpitis.

In the second part of our work, we investigated which decalcification and sample preparation methods are most suitable for RNAscope in situ hybridisation (ISH) analysis of rodent incisors. We compared five different decalcification methods in terms of morphological integrity, sectionability and mRNA preservation of dental tissues. Based on micro-CT, HE staining and RNAscope positive control tests, we concluded that the methods generally preserve tissue structure well, but some are associated with significant mRNA degradation. We identified two optimal procedures for RNAscope ISH (decalcification with Morse solution or ACD bone decalcifying buffer) that simultaneously preserve the histological structure of the teeth and do not significantly damage the RNA content of the sample, thus providing a reliable basis for gene expression analyses in dental pulp.

Appendix

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Registry number: DEENK/598/2025.PL
Subject: PhD Publication List

Candidate: Árpád Kunka
Doctoral School: Doctoral School of Molecular Medicine
MTMT ID: 10095340

List of publications related to the dissertation

1. Konkoly, J., **Kunka, Á.**, Szentágotai, A., Lisztes, E., Marincsák, R., Racskó, M., Bohács, J., Pintér, E., Gaszner, B., Tóth, I. B., Kormos, V.: Identification of Optimal Decalcification Method and Tissue Preparation Protocol for RNAscope in Situ Hybridization in Rodent Incisor Tooth.
   Dentistry Journal. 13 (11), 1-15, 2025.
   DOI: http://dx.doi.org/10.3390/dj13110538
   IF: 3.1 (2024)
2. **Kunka, Á.**, Lisztes, E., Bohács, J., Racskó, M., Kelemen, B., Kovalecz, G., D. Tóth, E., Hegedűs, C., Bágyi, K., Marincsák, R., Tóth, I. B.: TRPA1 up-regulation mediates oxidative stress in a pulpitis model in vitro.
   Br. J. Pharmacol. 181 (17), 3246-3262, 2024.
   DOI: http://dx.doi.org/10.1111/bph.16386
   IF: 7.7

Total IF of journals (all publications): 10,8
Total IF of journals (publications related to the dissertation): 10,8

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

19 November, 2025

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *