

Involvement of TRPA1 in the cinnamaldehyde-induced pulpal blood flow change in the feline dental pulp

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Objectives: The purpose of this study was to investigate the involvement of TRPA1 in the cinnamaldehyde-induced pulpal blood flow (PBF) change in the feline dental pulp. **Materials and Methods:** Mandibles of eight cats were immobilized and PBF was monitored with a laser Doppler flowmetry at the mandibular canine tooth. To evaluate the effect of cinnamaldehyde on PBF, cinnamaldehyde was injected into the pulp through the lingual artery at a constant rate for 60 seconds. As a control, a mixture of 70% ethanol and 30% dimethyl sulfoxide (DMSO, vehicle) was used. To evaluate the involvement of transient receptor potential ankyrin 1 (TRPA1) in PBF change, AP18, a specific TRPA1 antagonist, was applied into the pulp through the Class V dentinal cavity followed by cinnamaldehyde-administration 3 minutes later. The paired variables of experimental data were statistically analyzed using paired *t*-test. A *p* value of less than 0.05 was considered as statistically significant. **Results:** Administration of cinnamaldehyde (0.5 mg/kg, intra-arterial [i.a.]) induced significant increases in PBF ($p < 0.05$). While administration of a TRPA1 antagonist, AP18 (2.5 - 3.0 mM, into the dentinal cavity [i.c.]) caused insignificant change of PBF ($p > 0.05$), administration of cinnamaldehyde (0.5 mg/kg, i.a.) following the application of AP18 (2.5 - 3.0 mM, i.c.) resulted in an attenuation of PBF increase from the control level ($p < 0.05$). As a result, a TRPA1 antagonist, AP18 effectively inhibited the vasodilative effect of cinnamaldehyde ($p < 0.05$). **Conclusions:** The result of the present study provided a functional evidence that TRPA1 is involved in the mechanism of cinnamaldehyde-induced vasodilation in the feline dental pulp. (*Restor Dent Endod* 2016;41(3):202-209)

Key words: Blood flow; Cinnamaldehyde; Dental pulp; TRPA1

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Introduction

A primary sensory nerve fiber is activated by a variety of stimuli and subsequently releases neuropeptides, which induce vasodilatation, extravasation of protein, and recruitment/regulation of immune cells which are neutrophils, macrophages, lymphocytes and mast cells. This phenomenon is termed neurogenic inflammation.¹ Vasodilatation is the first vascular reactions during neurogenic inflammation in the dental pulp.²

The transient receptor potential ankyrin 1 (TRPA1) is a non-selective calcium permeable cationic channel belonging to the TRP channel superfamily.³ TRPA1 is highly expressed in sensory neurons which have emerged as an important molecular target for several types of pain in dorsal root ganglia and trigeminal ganglia, where it co-

localizes with another TRP channel, TRP Vanilloid receptor 1 (TRPV1).^{4,5} In human teeth, TRPA1 is highly expressed in dental pulp fibroblasts where it might be involved in cold responses and pain associated with mechanotransduction⁶ and the majority of pulpal afferents also express TRPA1.⁷ Radresa *et al.* reported that treatment of TRPA1 antagonist inhibits signs of hypersensitivity in many rodent model study about inflammatory pain.⁸ Therefore, TRPA1 might be considered an important target for the management of dental sensitivity.

TRPA1 acts as a cellular sensor for a variety of irritants. Initially reported to sense noxious cold,⁹ TRPA1 has subsequently been reported to be stimulated by some divalent ions (Ca^{2+} , Zn^{2+}), and by a number of exogenous electrophilic compounds, such as the pungent ingredients in mustard oil (allyl isothiocyanate), ginger and cinnamon oil (cinnamaldehyde),^{10,11} all of which can induce nocifensive behaviors, burning pain sensation, and sensory neuron sensitization in animals and man.⁸ Cinnamaldehyde is one of the main constituents of cinnamon and an aromatic aldehyde which has been reported to have multiple potential therapeutic activities.¹² It has been used as an ingredient of tooth pastes and to control toothache, oral microbiota and to treat halitosis.^{13,14}

The previous studies have shown that both cinnamaldehyde and TRPA1 may be involved in the control of blood flow in skin¹⁵ and heart of rat.¹² Despite oral use of cinnamon, functional relation between cinnamaldehyde and dental pulp circulation remains to be clarified. The purpose of this study was to investigate the involvement of TRPA1 in the cinnamaldehyde-induced pulpal blood flow (PBF) change in the feline dental pulp using laser Doppler flowmetry in order to elucidate neurogenic inflammation in the dental pulp.

Materials and Methods

Animal preparation

All procedures that involved the use of animals were approved by the Institutional Care and Use Committee of the School of Dentistry, Kyungpook National University. The experiments were conducted with eight cats weighing 2.2 - 3.2 kg. Cats were anesthetized with a mixture of ketamine (75 mg/kg) and acepromazine (2.5 mg/kg) by intramuscular injection. Supplemental anesthetics, which were a mixture of alpha-chloralose (40 mg/kg) and urethane (500 mg/kg), were injected through the femoral vein as needed to maintain the appropriate level of anesthesia, assessed by the absence of changes in systemic blood pressure (SBP) or nociceptive reflexes to noxious stimuli. Periapical radiographs of canine teeth were taken to ensure the maturity of apices and to evaluate the size of the pulp. To maintain the airway, tracheostomy was carried out with an endotracheal tube. Femoral vein was cannulated to inject supplemental anesthetics and femoral artery was cannulated to monitor SBP with a pressure transducer (Figure 1).

Both jaws were immobilized by intermaxillary splinting with dental plaster and a steel rod that was anchored to the base of experimental table with a locking device. Body temperature was kept at $37.0 \pm 0.5^\circ\text{C}$ with a heating pad and monitored with a rectal thermometer.

Lingual artery was cannulated to administrate cinnamaldehyde into the dental pulp. For the administration of drugs to the dental pulp, a class V dentinal cavity (1.5 mm in width, 2 mm in length and 0.5 mm in depth) was prepared on the middle third of the

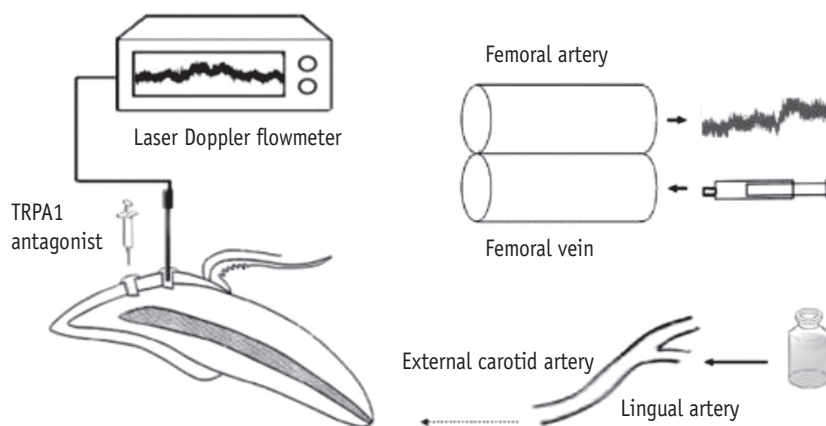


Figure 1. Schematic diagram of experiment design. Pulpal blood flow was measured at tooth surface by a laser Doppler flowmeter. Femoral artery was cannulated for monitoring of systemic blood pressure and femoral vein was cannulated for injection of supplemental anesthetics. TRPA1 antagonist was administered through the dentinal cavity and cinnamaldehyde was injected into the pulp through the lingual artery. TRPA1, transient receptor potential ankyrin 1.

labial surface of the crown with a high speed #701 tapered fissure bur. The exposed dentin surface was etched with 32% phosphoric acid for 10 seconds so that smear layer was removed and the dentinal tubules were open.^{16,17}

Preparation and administration of drugs

Cinnamaldehyde (Sigma Chemical, St. Louis, MO, USA) was dissolved in a mixture of 70% ethanol and 30% dimethyl sulfoxide (DMSO) and diluted to make stock solutions of 10 mg/mL concentration. A solution of cinnamaldehyde at the desired concentration was prepared daily by dilution of the stock solution with the mixture of 70% ethanol and 30% DMSO. In each experiment, cinnamaldehyde was titrated to exert maximum effect on PBF without influencing SBP to minimize or eliminate systemic effect. All injections and measurements were started 60 minutes after the preparation of animal for a stable hemodynamic condition. Earlier studies have reported that a minimum of 30 to 50 minutes is required after cavity preparation for PBF to return to the control level.¹⁷ In order to evaluate the effect of cinnamaldehyde on PBF, 2 mL of 0.5 mg/kg cinnamaldehyde was injected through the lingual artery at a constant rate for 60 seconds, followed by a flush of 0.3 mL isotonic saline. As a control, 2 mL of cinnamaldehyde vehicle (a mixture of 70% ethanol and 30% DMSO) was injected before the administration of cinnamaldehyde.

A specific TRPA1 antagonist, AP18 (Sigma Chemical) was dissolved in 100% DMSO and diluted to make stock solutions of 1 mg/mL concentration. A solution of AP18 at the desired concentration was prepared by dilution of the stock solution with DMSO. AP18 was titrated to exert maximum inhibitory effect on cinnamaldehyde-induced PBF change without influencing PBF. 1 μ L of AP18 (2.5 - 3.0 mM) was placed into the dentinal cavity (i.c.) followed by intra-arterial (i.a.) administration of cinnamaldehyde three minutes later. The cavity was flushed with isotonic saline for the following experiment.

Measurement of pulpal blood flow

Change in PBF was measured by using a laser Doppler flowmeter (PeriFlux 4001, Perimed, Stockholm, Sweden). A shallow cavity was prepared at the cervical third of the labial surface of canine under copious running water to expose the dentin using a high-speed inverted cone bur. It was placed approximately 3 mm from the marginal gingiva to eliminate the influence of the gingival blood flow. A laser Doppler flowmeter probe (PF416, Perimed) was positioned at right angles and 0.1 - 0.2 mm away from the axial wall of the prepared cavity using a micromanipulator (MMN-3, Narishige, Tokyo, Japan). To avoid drying of the dentin, isotonic saline was flooded between the dentin surface and the probe tip. PBF was monitored continuously

and recorded with a computer software (LabScribe2, iWorx Systems Inc., Dover, NH, USA). Control level of PBF was established by recording it for three minutes prior to each drug. To determine the effect of drug, the maximum change of PBF was evaluated.

In order to evaluate the effect of a TRPA1 antagonist, AP18, on cinnamaldehyde-induced PBF change, cinnamaldehyde was administered three minutes after application of AP18 and then PBF was recorded and compared with the control level.

Statistics

All numerical data in the text were expressed as percent change from control and mean \pm standard error of the mean (SEM). The paired variables of control and experimental data were statistically analyzed using paired *t*-test. A *p* value of less than 0.05 was considered as statistically significant.

Results

Effect of cinnamaldehyde on pulpal blood flow

Change in PBF in response to cinnamaldehyde is presented in Figure 2 and Table 1. Application of cinnamaldehyde (0.5 mg/kg, i.a.) showed significant increase in PBF (*p* < 0.05). After cinnamaldehyde administration, the mean peak values of PBF were increased to 88.02 \pm 11.56% (*n* = 19) from the control level. Administration of cinnamaldehyde vehicle, a mixture of 70% ethanol and 30% DMSO, itself did not cause any significant change of PBF.

Effect of TRPA1 antagonist on cinnamaldehyde-induced vasodilation

Typical strip-chart recordings of SBP and PBF in response to cinnamaldehyde with vehicle and cinnamaldehyde with TRPA1 antagonist are presented in Figure 2. SBP remained

Table 1. Effect of cinnamaldehyde on PBF

	No.	% Change of PBF (mean \pm SEM)
Ethanol (70%) + DMSO (30%) (cinnamaldehyde vehicle, i.a.)	19	7.63 \pm 1.17 ^a
Cinnamaldehyde (0.5 mg/kg, i.a.)	19	88.02 \pm 11.56 ^b

Different lowercase letters indicate statistically significant difference (*p* < 0.05).

DMSO, dimethyl sulfoxide; i.a., intra-arterial; PBF, pulpal blood flow; SEM, standard error of mean.

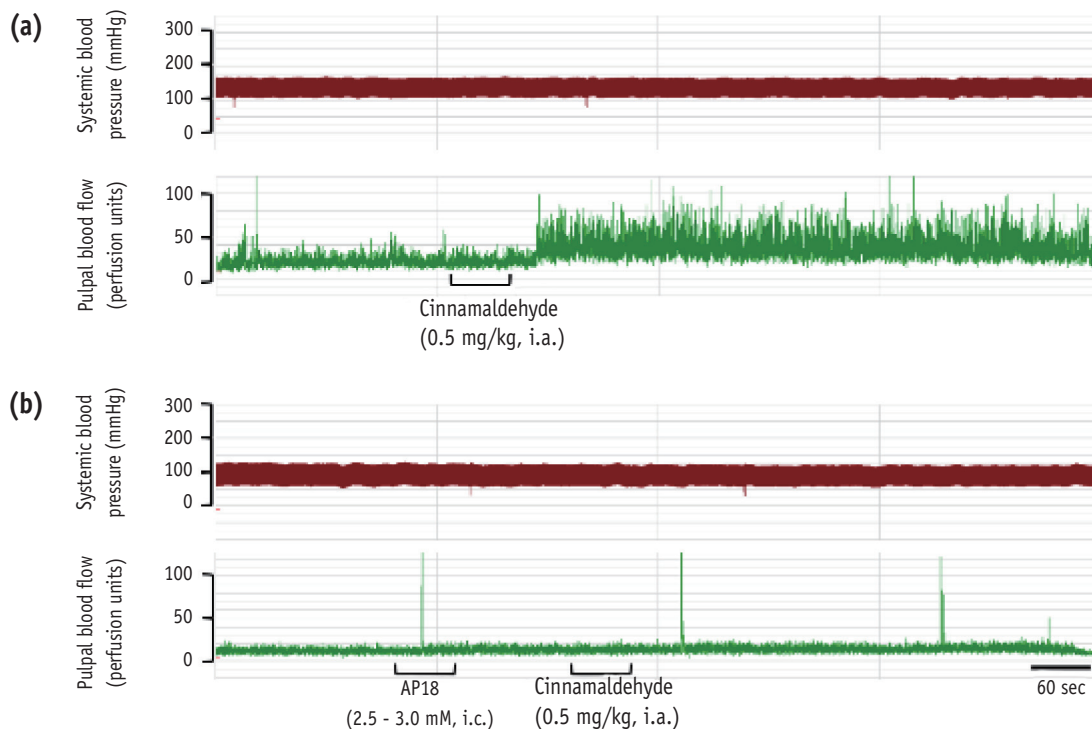


Figure 2. Typical strip-chart recordings of systemic blood pressure and pulpal blood flow (PBF). (a) Cinnamaldehyde (0.5 mg/kg, i.a.) resulted in a significant increase of PBF; (b) TRPA1 antagonist, AP18 (2.5 - 3.0 mM, i.c.), effectively attenuated an increase in PBF induced by cinnamaldehyde (0.5 mg/kg, i.a.) ($p < 0.05$). i.a., intra-arterial; i.c., into the dentinal cavity.

Table 2. Effect of AP18 followed by cinnamaldehyde administration on PBF

	No.	% Change of PBF (mean \pm SEM)
Cinnamaldehyde (0.5 mg/kg, i.a.)	19	88.02 \pm 11.56 ^c
AP18 (2.5 - 3.0 mM, i.c.)	19	7.72 \pm 2.26 ^a
Cinnamaldehyde (0.5 mg/kg, i.a.) after AP18 (2.5 - 3.0 mM, i.c.)	19	21.25 \pm 3.80 ^b

Different lowercase letters indicate statistically significant difference ($p < 0.05$).

i.a., intra-arterial; i.c., into the dentinal cavity; PBF, pulpal blood flow; SEM, standard error of mean.

unchanged both during and after the periods of drug administration.

Administration of cinnamaldehyde (0.5 mg/kg, i.a.) following the application of a TRPA1 antagonist, AP18 (2.5 - 3.0 mM, i.c.) resulted in an increase of PBF by 21.25 \pm 3.80% ($n = 19$) from the control ($p < 0.05$), whereas the antagonist itself did not cause any significant change of PBF (Table 2).

Discussion

In the present study, PBF was increased significantly when cinnamaldehyde was injected into the dental pulp through the lingual artery. TRPA1 antagonist AP18 was shown to effectively inhibit cinnamaldehyde-induced vasodilation. These findings strongly show the evidence that TRPA1 stimulated by cinnamaldehyde is involved in the mediation

of vasodilation in dental pulp.

TRPA1 channels localized in primary afferent neurons may play a crucial role in the vascular reaction of neurogenic inflammation. It has been proposed that TRPA1 promotes inflammation through its mediation in not only neural (direct) but also immune cell (indirect) activation.¹⁸ TRPA1 activation create increased neuronal activity that induces the release of various neuropeptides and neurotransmitters, such as neurokinin A, substance P, and calcitonin gene-related peptide (CGRP). These materials lead to vasodilation and gather immune cells to the site.¹⁹ These immune cells will finally secrete a variety of signaling molecules, including the TRPA1 agonists hypochlorite from neutrophils, hydrogen peroxide from granulocytes, and prostaglandins from mast cells, macrophages and dendritic cells that will reactivate the neuron.²⁰ The tissue damage often accompanies TRPA1 stimulation and produces reactive oxygen species followed by induction of liposome peroxidation and the generation of additional TRPA1 agonists including 4-oxo-2-nonenal and 4-hydroxynonenal.^{21,22} Therefore, TRPA1 plays a role as a key mediator of the feed forward loop that allows local inflammation to maintain.

The vasodilatory effect of cinnamaldehyde has been investigated in previous studies. Pozsgai *et al.* investigated TRPA1-induced responses in the vasculature in response to TRPA1 agonists (allyl isothiocyanate and cinnamaldehyde) using wild-type (WT) and TRPA1 knockout (KO) mice.²³ Cinnamaldehyde triggered a significant increase in the hind paw blood flow in WT, but not in TRPA1 KO mice.²³ Cinnamaldehyde also induced dose-dependent relaxation in WT and TRPA1 KO mesenteric arterial rings *in vitro* and this relaxation was significantly less potent in TRPA1 KO compared with WT arteries, indicating a TRPA1-relaxant component.²³ A reduced but significant response was observed in the absence of endothelium.²³ Xue *et al.* studied the vasodilatory effect of cinnamaldehyde and its mechanism of action using isolated rings of rat aorta.¹² They suggested that cinnamaldehyde dilated both endothelium-intact and endothelium-denuded rings in a dose-dependent manner, which means that cinnamaldehyde-induced vasodilation is related to an endothelium-independent manner.¹² Yanaga *et al.* studied the vasorelaxant effect of cinnamaldehyde using isolated rat aorta and demonstrated that cinnamaldehyde at final concentrations of 1 μ M to 1 mM showed dose-dependent relaxation of the rat aorta.²⁴ In the present study, PBF was increased significantly after cinnamaldehyde administration into the feline dental pulp through lingual artery and this response was effectively attenuated by TRPA1 antagonist AP18. This result corresponds to the results of the previous studies and based on these results, cinnamaldehyde-induced vasodilation in dental pulp might be functionally involved with TRPA1 channel activation in dose-dependent

manner.

The activation of TRPA1 was proposed to cause arterial dilation through two distinctive pathways.²⁵ First, TRPA1 channels expressed in perivascular nerves are stimulated by chemical agonists and CGRP is released from nerve ending, followed by arterial dilation. Second, TRPA1 channels placed in the myoendothelial junction sites cause endothelium-dependent smooth muscle cell hyperpolarization and vasodilation mediated by Ca^{2+} -activated K^{+} channels.²⁵ Kunkler *et al.* studied the role of TRPA1 channels in meningeal vasodilation.²⁶ They suggested that cinnamaldehyde stimulated the release of CGRP from cultured rat trigeminal neurons and this response was blocked by TRPA1 selective antagonist, HC-030031 and CGRP selective antagonist, CGRP₈₋₃₇.²⁶ This study strongly supported that activation of TRPA1 channels present in primary afferent neurons with cinnamaldehyde led to Ca^{2+} influx, followed by release of CGRP at nerve endings.²⁷ CGRP binds to G protein-coupled receptors expressed in smooth muscle cells to produce membrane hyperpolarization, myocyte relaxation and vasodilation.²⁸

Cinnamaldehyde is the main component of cinnamon and there are many reports on its pharmacological effects. Mainly, the sedative effect of decreasing spontaneous motor activity,²⁹ anti-inflammatory effects related to cyclooxygenase 2 (COX-2)³⁰ and antibacterial activity against *Escherichia coli* and *Pseudomonas aeruginosa*³¹ have been reported. In addition, in oriental medicine, cinnamaldehyde is often used to improve blood circulation.^{32,33} In relation to dentistry, cinnamaldehyde has been traditionally used as a component of tooth pastes and to manage toothaches, oral microbiota and bad breath.^{13,14}

It is interesting to note that cinnamaldehyde has a contradictory character in relation to pain and inflammatory reaction. There have been previous studies about an analgesic effect of cinnamaldehyde. Huang *et al.* investigated whether cinnamaldehyde prolonged cutaneous analgesia when co-administrated with local anesthetics in rats.³⁴ In this study, cinnamaldehyde alone provided a dose-dependent block to pinpricks and complete block to pinpricks accomplished in 2% cinnamaldehyde with 0.5% lidocaine and with 0.0625% bupivacaine, which were significantly prolonged compared to lidocaine or bupivacaine alone.³⁴ Boonen *et al.* studied whether cinnamaldehyde inhibited voltage-gated sodium channels expressed in sensory neurons and they found that cinnamaldehyde inhibited tetrodotoxin-sensitive voltage-dependent sodium currents in a concentration dependent manner in mouse trigeminal neurons.³⁵ In a previous study, anti-inflammatory effects of cinnamaldehyde was demonstrated related to COX-2.³⁰ Guo *et al.* found that cinnamaldehyde reduced interleukin-1 β -induced COX-2 activity and consequently inhibited production of prostaglandin E2 in cultured rat cerebral microvascular

endothelial cells.³⁰

Markowitz *et al.* suggested that the biological effects of eugenol, which has similar molecular structure with cinnamaldehyde, varied greatly, depending on its concentration.³⁶ They found that eugenol may have beneficial effects which were prostaglandin synthesis, nerve activity, and white blood cells chemotaxis inhibition with concentrations ranging from 100 nM to 100 μ M, but may have cytotoxic effects which were cell death, cell growth, and respiration inhibition with concentration lower than 1 mM.³⁶ In another study, Klein *et al.* investigated activation of rat trigeminal ganglion cells by sequential application of menthol and/or cinnamaldehyde.³⁷ They found that trigeminal ganglion cells exhibited significant self-desensitization to cinnamaldehyde at 400 μ M but not 200 μ M and cinnamaldehyde at a concentration of 400 μ M but not 200 μ M also cross-desensitized menthol-evoked responses.³⁷ The difference of effect of cinnamaldehyde on pain and inflammation might be caused by its concentration and it is needed to clarify the mechanism of action of cinnamaldehyde in pain and inflammatory response.

As mentioned above, cinnamaldehyde has been reported to influence the cardiovascular system. In previous study that evaluated the toxicological effects of cinnamaldehyde, 5 - 10 mg/kg of cinnamaldehyde was applied through vein of Mongrel dogs and decrease of blood pressure and increase of respiratory rate and femoral blood flow were observed.³⁸ In another study, A fall in blood pressure was also observed in male guinea pigs after application of cinnamaldehyde at a dose of 1 mg/kg through intravenous administration.³⁸ Heart rate was reduced by 15% compared to baseline after application of cinnamaldehyde at a dose of 5 mg/kg through vein, while femoral blood flow was observed to be raised.³⁸ In the present study, cinnamaldehyde at a dose of 0.5 mg/kg was administered into the pulp through lingual artery and it was ascertained that systemic blood pressure was not influenced by this concentration which was much lower than those of previous studies.

Many TRPA1-activating materials including cinnamaldehyde are electrophils which can react with cysteines. The nucleophilic mercapto group of cysteines may attack the α,β -unsaturated bond of cinnamaldehyde and then TRPA1 is activated through covalent binding of cinnamaldehyde to cysteines.³⁹ In the present study, in order to evaluate the role of TRPA1, AP18 was used as a TRPA1 antagonist. While the mechanism of action for AP18 which is an oxime derivative is not exactly confirmed, AP18 may be capable of covalent modification of TRPA1 reversibly, but unable to initiate a conformational change that would lead to channel activation.⁴⁰ In previous studies, AP18 showed high selectivity and no agonist activity for TRPA1 whereas it was relatively insensitive to

other TRP channel family members.⁴¹ Petrus *et al.* showed dose-response relationships for block of the calcium influx by AP18 into CHO cells expressing mouse and human TRPA1 elicited by cinnamaldehyde.⁴² Moreover, AP18 reversibly blocked cinnamaldehyde-induced TRPA1 currents in excised patches from *Xenopus oocytes*.⁴² Importantly, AP18 significantly blocked cinnamaldehyde-induced but not capsaicin-induced nociceptive events which are related to TRPV1, demonstrating efficacy and specificity.⁴² In the previous study in our lab, AP18 was applied to dental pulp through the dentinal cavity, not through the lingual artery and it resulted in significant inhibition of TRPA1 activation. Based on these studies, AP18 was determined to be administered through dentinal tubule in the present study.

Conclusions

The results of the present study provided a functional evidence that TRPA1 is involved in the mechanism of cinnamaldehyde-induced vasodilation in the feline dental pulp. Further study is needed to clarify the mediation of CGRP in the cinnamaldehyde-induced vasodilation in the dental pulp.

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Conflict of Interest: No potential conflict of interest relevant to this article was reported.

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