

Therapeutic Efficacy of Topical Epigallocatechin-Gallate as a New Therapeutic Strategy for Inhibition of Pain Conduction on Rat Models with Acute Pulpal Inflammation

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ABSTRACT

The pulpal inflammation can lead to elicit pain in trigeminal nociceptor, produces prostaglandin (PG-E2), activating transient receptor potential vanilloid (TRPV1), causing substance P (SP) release. Here, we investigated the potential of epigallocatechin 3-gallate (EGCG) for topical medication to inhibit pain conduction in pulpal inflammation. This research on 28 male Wistar rats (*Rattus norvegicus*) as rat models with pulpal inflammation. Topical 0.01% or 0.1% EGCG was applied to the tooth cavity of pulpal inflammation. The treated pulp tissues were then observed using immunohistochemistry (IHC), and calculation of the amount of PG-E2, TRPV1 and SP. Results show the treatment with topical 0.1% EGCG in 24 hours pulpal inflammation showed a significant inhibit the expression of PG-E2, TRPV1, and SP compared with 0.01% EGCG, and untreated controls ($P < 0.001$). In conclusion, the results suggest a potential to inhibiting pain conduction of 0.1% topical EGCG in pulpal inflammation.

INTRODUCTION

In daily dental practice, pulpal and periapical pains are the cause for patients seek dental care (Estrela et al., 2011). Dental pain originated from pulpal inflammation and stimulation of dental pulp nerve fibers (Jain et al., 2013). Recent studies in pulpal inflammation exhibit evidence for the over expression of tumor nekrosis factor - α (TNF- α) (Zhan et al., 2007), and reached the peak in 24 hours (Ji et al., 2015). The cellular metabolism process in pulpal inflammation triggers excessive reactive oxygen species (ROS) and results in malondialdehyde (MDA) cause surface cell damaged (Burdan et al., 2006), and cause the enzymatic conversion of arachidonic acid (AA) (Jain et al., 2013). High ROS and cyclooxygenase (COX2) would catalyze AA into PG-E2 (Fukata et al., 2006). The expression of PG-E2 which activates TRPV1 on sensory nerve endings on dental pulps (Moriyama et al., 2005), occurs which triggers neuropeptide caused pain impulse conduction along the sensory nerve membrane. The two keys component of pulpal inflammation are microcirculation and the activity of nerve fibers produces augmentation caused by the action of neurokinins, particularly SP released from afferent fibers (Jain et al., 2013). Neuropeptide SP is increased at the initial stage of pulpal inflammation (Reader et al., 2009; Chung and Oh., 2013).

Neuropathic pain originated from injury or disease to the peripheral or central nervous network. Neuropathic pain via activation of production of inflammatory cytokines (Kiguchi et al., 2012), and the stimulation of nuclear factor kappa B (NF- κ B) cascade (Niederberger and Geisslinger., 2008). Therefore, the establishment of new pharmacological agents could be a therapeutic answer for the neuropathic pain.

Epigallocatechin 3-gallate (EGCG) the most important active catechin, is known to have therapeutic properties in many systems,

including the nervous system. Several investigations have a multiple phenolic units in their chemical structure (Shay et al., 2015). These beneficial effects have been attributed cutback of pro-inflammatory cytokines expression (Kuang et al., 2012; Khalatbary and Ahmady and., 2011). EGCG could block TLR4 (Kuang et al., 2012), thus it can reduce the expression of TNF- α (Kuang et al., 2012; Tipoe et al., 2010). The ability of EGCG to inhibit inflammatory process starts from the first 1-2 hours after the exposure (Song et al., 2006). Some experimental works showed antinociceptive effect of EGCG. Lately, it has been revealed in rats that EGCG may inhibit the opening of voltage gated channel of Na⁺ ion, and therefore alleviate the sharp pain (Kim et al., 2009). Intrathecal injection of EGCG reduce mechanical allodynia and thermal hyperalgesia after chronic constriction nerve injury (Kuang et al., 2012)

Nevertheless, there are only few studies disclosing the antinociceptive effect of EGCG in pulpal inflammation. Therefore, this research aimed to reveal the inhibition of pain conduction in pulpal inflammation by using topical 0.01% and 0.1% EGCG was applied to the tooth cavity of pulpal inflammation. The teeth of Wistar rats (*Rattus norvegicus*) suffering from pulpal inflammation within 24 hours were utilized as the models, while the variables to analyze included the expressions of PG-E2 macrophage cells, TRPV1 and SP sensory nerve cells.

MATERIALS AND METHODS

Materials

The starting materials, Lipopolysaccharides (LPS), (obtained from *Escherichia coli* (*E.coli*), serotype 0111: B4, Sigma Chemical Co, St. Louis, MO, USA; product number L2630), EGCG (Xi'An Rongsheng Biotechnology Co., Ltd., made in China, batch number 2013101512 RS), Ketamine (Ketalar®, PT.Pfizer Indonesia), Xylazine base (Xyla®, PT Tekad Mandiri Citra, Indonesia), glass ionomer cement (GIC) (Fuji 7, GC Corp, Tokyo, Japan), round-shaped diamond bur, 1 mm diameter, and a straight fissurotomy bur, 1 mm diameter at the tip (SS White burs Inc., Lakewood, NJ, USA), rabbit anti rat PG-E2 polyclonal antibody, primary antibody (Bioss-USA; catalog number: bs-2639 R), mouse anti rat IgG antibody (H+L), Biotin, conjugated secondary antibody (Bioss-USA; catalog number: bs-0293M-Biotin), mouse anti rat TRPV1 monoclonal antibody (NeuroMab; University of California at Davis/NIH NeuroMab Facility, catalog number: N 221/17), mouse anti rat SP monoclonal antibody (Abcam; ab14184).

The acute pulpal inflammation modelling and procedures of topical EGCG

The study outline accepted by the Health Research Ethics Committee (KKEPK) in Faculty of Dentistry, Universitas Airlangga based on the Ethical Clearance No:181/KKEPK.FKG/XII /2014. This research is an experimental laboratory, using 28 male rats (*Rattus norvegicus*, Wistar strain), and aged 2.5 months. The samples were divided into normal group (N), positive control group (C) which induced with 0.5 microliters LPS for 24 hours, and treatment group (T1) which induced with 0.5 microliters LPS for 24 hours, and then, topical of 0.01% EGCG, and T2 induced with 0.5 microliters of LPS for 24 hours, and then, topical of 0.1% EGCG for 24 hours.

Every rat in the group C and T, was intra-muscularly anesthetized with 0.2 cc of the mixture of 0.5 cc ketamine and 0.5 cc xylazine. The teeth were prepared in the disto-cervical region of the upper right incisors with a new sterile round-shaped diamond bur, and a straight fisturotomy bur. The preparation was made with 1.5 mm deep and a diameter of 2 mm, creating open dentin tubules without exposing the pulp chamber. The cavities then irrigated with sterile saline solution and then dried with sterile cotton pellet. The pulpal inflammation modeling for group C, T1 and T2, was performed using 0.5 microliters of LPS, applied to freshly cut dentin (Chung et al., 2011). To apply LPS by using fine flattened microbrush was utilized as a bearer to convey liquid to the cavity, and then allowed to dry and filled with GIC. After 24 hours, in group C, 0.5 microliters aquadest was applied onto the tooth cavities, while in groups T1, 0.5 microliters of 0.01% EGCG was applied onto the tooth cavities, and in groups T2, 0.5 microliters of 0.1% EGCG was applied onto the tooth cavities and then filled with GIC. After 24 hours, the topical aquadest and EGCG was repeatedly, the cavities were filled with GIC, and settled for 24 hours. Rats were then euthanized on day 4 for molecular studies. The rats were put down in order to conduct surgery to remove the first right maxillary incisors along with the jaw (± 12 mm) to be used as analysis specimens, the analysis unit was the dental pulp. Immunohistochemistry (IHC) staining was then conducted using peroxidase staining of PG-E2, TRPV1 and SP. Two operators who were blinded to the technique examined section by a light microscope at x 400

magnification with 5 perspective points (E 100 Dr; Nikon, Tokyo, Japan). In each group, the number of PG-E2 Macrophage cells, TRPV1 and SP Sensory nerve cells were recorded. The images were captured with a digital camera (A7; Sony, Tokyo, Japan) (Fig.1, 2 and 3). Data were analyzed with *kolmogorov-smirnov test*, *levene's test*, and *one-way anova-test*. The level of significance was set at $P < 0.001$.

RESULTS AND DISCUSSION

Table 1 Effects of topical EGCG 0.01% and 0.1% on the expressions of PG-E2 in Macrophage cells, TRPV1, and SP in Sensory nerve cells

Group	PG-E2	TRPV1	SP
	Mean \pm SD	Mean \pm SD	Mean \pm SD
N	2.30 \pm 0.70 ^a	2.17 \pm 0.75 ^a	2.33 \pm 0.52 ^a
C	19.17 \pm 0.75 ^c	18.33 \pm 2.65 ^c	16.10 \pm 0.98 ^c
T1	17.67 \pm 1.03 ^c	17.00 \pm 1.90 ^c	15.00 \pm 1.41 ^c
T2	8.83 \pm 0.98 ^b	9.83 \pm 2.04 ^b	5.50 \pm 2.35 ^b

The result of the conducted data normality test, the data in this research are in a normal distribution ($p > 0.05$). Table 1 shows the number expressions of PG-E2 in Macrophage cells, TRPV1 and SP in Sensory nerve cells in each group. The application of LPS for 24 hours produced significantly increased in the expression of PG-E2, TRPV1 and SP. Topical 0.1% EGCG in 24 hours pulpal inflammation showed a significant inhibit the expression of PG-E2, TRPV1, and SP compared with 0.01% EGCG, and untreated controls ($P < 0.001$). Topical 0.01% EGCG also showed a inhibit the expression of PG-E2, TRPV1, and SP, but no significant. The analysis of IHC for the expression of PG-E2, TRPV1, and SP in rat teeth is shown in the following pictures.

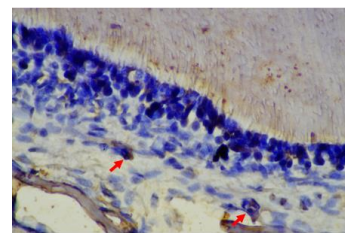


Fig 1 IHC coloring technique with 400 time magnification Positive expression of PG-E2 in Macrophage cells arrows on cytoplasm

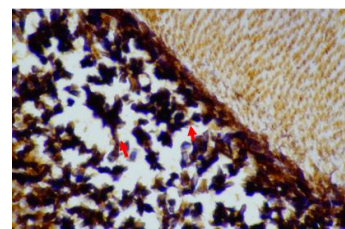


Fig. 2 IHC coloring technique with 400 time magnification Positive expression of TRPV1 in Sensory nerve cells is shown by the red arrows on cytoplasm

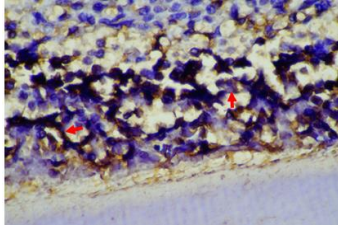


Fig. 3 IHC coloring technique with 400 time magnification Positive expression of SP in Sensory nerve cells is shown by the red arrows on cytoplasm

The pictures above depict groups with a number of cells with expressions of PG-E2 in Macrophage cells (fig. 1), TRPV1 and SP in Sensory nerve cells (fig. 2 and 3). The comparison of the number of cells with expressions of PG-E2, TRPV1 and CGRP between normal group, control group and treatment group can be seen in table 1.

Pulpal inflammation is a complex and multifactorial inflammatory condition. The cellular metabolism process in pulpal inflammation triggers excessive ROS and results in MDA, cause surface cell damaged (Burdan et al., 2005). High ROS and COX2 would catalyze AA into PG-E2 (Fukata et al., 2006). Previous studies have shown that the expression of PG-E2 which activates TRPV1 on Sensory nerve endings on dental pulps (Moriyama et al., 2005), occur increased of SP caused pain impulse conduction along the sensory nerve membrane (Reader et al., 2009; Chung and Oh., 2013).

Former research indicated that induction of LPS causes the up-regulation of TRPV1 in trigeminal nociceptors as a result of bacterial infection could contribute to hyperalgesia under pulpitis conditions (Chung et al., 2011). TRPV1 is a nonselective cation channel expressed in sensory neurons and initiated by various noxious stimuli (Moriyama et al., 2005). The expression of TRPV1 increased on inflamed dental pulp (Ingle et al., 2008). Initiates TRPV1 channels on sensory nerves causing in the opening of a cation channel and increasing calcium entry through this channel and through voltage-gated calcium channels activated by sodium-induced depolarization. These effect increase the release of SP from sensory neuron (Gazzier et al., 2007; Sacardote and Levrin., 2012). Macrophages induced by SP create the inflammatory mediators of PG-E2 and tromboxane as well as the proinflammatory cytokines IL-1, IL-6 and TNF. All of these molecular occurrences finally sustain the synthesis and release of new SP, thereby perform the vicious cycle and further increasing pain sensitivity (Jain et al., 2013).

The result of this research reveals that the expression of PG-E2, TRPV1 and SP can be recognized in inflamed and non-inflamed pulp tissue. The dentinal application of LPS for 24 hours present increase in some inflammatory mediators. An 8.3-fold increase in PG-E2, an 8.4-fold increase in TRPV1, an 6.9-fold increase in SP compared with normal groups.

In this paper we describe the ability of the topical 0.01% and 0.1% EGCG to act as an inhibitory of pain conduction in pulpal inflammation on rat models. The mechanism of inhibitory pain conduction of EGCG could probably be due to blockade of the effect or the release of endogenous substances that excite pain nerve ending. Recently, EGCG have been revealed to employ inhibitory effect on various mediators on inflammation. This comprises repression of LPS- stimulated activation nuclear factor kappa B (NF- κ B)

(Kim, 2009), TLR4 (Kuang et al., 2012), TNF- α (Kuang et al., 2012; Tipoe et al., 2010), COX2 and PG-E2 bio-synthesis by metabolism pathway of AA (Altavilla et al., 2009). EGCG pertains the capability to both directly or indirectly scavenge ROS through chemically reacting with ROS or by modulating pathways that regulate ROS scavenging and various enzymes (Shay et al., 2015). The structure of the benzene ring will capture free radicals so as to prevent the continuation of chain reaction continues and free radicals are no more produced (Barceloux, 2008). Inhibition of ROS and COX2 would inhibition catalyze AA into PG-E2 (Fukata et al., 2006).

This study differs from the earlier reports on the analgesic activity of EGCG, primarily with respect to the expression of PG-E2, TRPV1, SP, and the doses employed. We demonstrate that topical 0.01% EGCG showed no significantly inhibit the expression of PG-E2, TRPV1, and SP. Topical 0.1% EGCG significantly decreasing the expression levels of PG-E2, TRPV1, and SP are higher compared with normal groups. Topical 0.1% EGCG significantly decreasing the expression levels of PG-E2 (2.17 fold), TRPV1 (1.86 fold), and SP (2.92 fold) compared with pulpal inflammation 24 hours. Previous studies have shown that EGCG could block TLR4 (Kuang et al., 2012), thus it can reduces the expression of TNF- α (Kuang et al., 2012; Tipoe et al., 2010).

Inhibition expression of TLR4, can reduce sintesis of COX2 and PG-E2 bio-synthesis (Kim et al., 2009; Zhang et al., 2013). Polifenol can inhibit the expression of TNF- α and production of ROS, cause inhibit surface cell damaged and PG-E2 expression (Altavilla et al., 2009; Zhang et al., 2013; Ishida, 2007). Inhibition of PG-E2 expression can't activates TRPV1 and decreased of SP expression. The earlier reports demonstrate that EGCG may inhibit the opening of voltage gated channel of Na⁺ ion on the culture neuron cell of the rat dorsal root ganglion (DRG) and therefore alleviate the sharp pain (Kim et al., 2009). EGCG may inhibit the opening of voltage gated channel of Na⁺ ion on the primer culture neuron cell of *rat hippocampal CA1* (Deng et al., 2008). Based on that researches, results showed that topical 0,1% EGCG can inhibit pain conduction in acute stage. We related this effect inhibition of the PG-E2, TRPV1 and SP. Our findings give a reasoning for further pre-clinical investigation on therapeutic regimen based on SP inhibitors in acute stage of pulpitis to current therapies.

CONCLUSION:

The results suggests a potential to inhibiting pain conduction of 0.1% topical EGCG in pulpal inflammation through to PG-E2 expression suppressing, with the effect inhibiting TRPV1 and SP neuropeptides release.

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CONFLICT OF INTEREST

Conflict of interest declared none

REFERENCES

- Altavilla, D., Squadrito, F., Bitto, A., Polito, F., Burnett, B.P., Stefano, V.D., Minutoli. 2009. Flavocoxid, a dual inhibitor of Cyclooxygenase and 5-Lipoxygenase, blunts pro-inflammatory phenotype activation in endotoxin-stimulated macrophages. *British J. Pharmacol.* 157, 1410-1418.
- Barceloux, D.G. 2008. *Medical Toxicology of Natural Substances*. Food, Fungi, Medicinal Herbs, Plants and Venomous Animals, Wiley, Hoboken, N.J., USA. pp. 22-33.
- Burdan, F., Chalas, A., Szumilo, J. 2006. Cyclooxygenase and prostanoids-biological implications. *Postepy Hig Med Dosw (online)*. 60, 129-141.
- Chung, G; Oh, S.B. 2013. TRP channels in dental pain. *The open pain J.* 6, 31-36.
- Chung, M.K., Lee, J., Duraes, G., Ro, J.Y. 2011. Lipopolysaccharide-induced Pulpitis Up-regulates TRPV1 in Trigeminal Ganglia. *J. Dent. Res.* 90, 9, 1103-1107.
- Deng, H.M., Yin, S.T., Yan, D., Tang, M.L., Li, C.C., Chen, J.T., Wang, M., Ruan, D.Y. 2008. Effect of EGCG on voltage-gated sodium channels in primary cultures of rat hippocampal CA1 neurons. *Toxicology*. 252, 1-8.
- Estrela, C., Guedes, O.A., Silva, J.A., Leles, C.R., Estrela, C.R., Pecora, J.D. 2011. Diagnostic and clinical factors associated with pulpal and periapical pain. *Braz. Dent. J.* 306-311.
- Fukata, M., Chen, A., Klepper, A., Krishnareddy, S., Vamadevan, A.S., Thomas, L.S., Xu, R., Inoue, H., Arditi, M., Dannenberg, A.J., Abreu, M.T. 2006. Cox-2 is regulated by toll-like receptor-4 (TLR4) signaling: Role in proliferation and apoptosis in the intestine. *Gastroenterology*. 131, 3, 862-877.
- Gazzier, D., Trevisani, M., Springer, J., Harrison, S., Cottrell, G.S., Andre E., Nicoletti, P., Massi, D., Zecchi, S., Nosi, D., Santucci, M., Gerard, N.P., Lucattelli, M., Lungarella, G., Fischer, A., Grady, EF., Bunnett, N.W., Geppetti, P. 2007. Substance P released by TRPV1-expressing neurons produces reactive oxygen species that mediate Ethanol-induced gastric injury. *Free. Radic. Biol. Med.* 43, 4, 581-589.
- Ingle JI, Bakland LK, Baumgartner JC., 2008. *Ingle's Endodontics* 6. BC. Decker Inc, Hamilton. 382-387.
- Ishida, I., Kohda, C., Yanagawa, Y., Miyaoka, H., Shimamora, T. 2007. Epigallo catechins gallate suppresses expression of receptor activator of NF- κ B ligand (RANKL) in *Staphylococcus aureus* infection in osteoblast-like NRG cells. *J. of. Medical. Microbiology*. 56, 1042-1046.
- Jain, N., Gupta, A., Meena, N. 2013. An Insight Into Neurophysiology of Pulpal Pain: Facts and Hypotheses. *Korean. J. Pain.* 26, 4, 347-355.
- Ji, G.L., Jia, J.L; Ni, X.L. 2015. Melatonin attenuates inflammation of acute pulpitis subjected to dental pulp injury. *Am. J. Transl. Res.* 7,1, 66-78.
- Khalatbary, A.R., Ahmadyand, H. 2011. Anti-inflammatory effect of the epigallocatechin gallate following spinal cord trauma in rat. *Iranian. Biomedical. J.* 15, 31-37.
- Kiguchi, N., Kobayashi, Y., Kishioka, S. 2012. Chemokines and cytokines in neuroinflammation leading to neuropathic pain. *Curr. Opin. Pharmacol.* 12, 55-61.
- Kim, T.H., Lim, J.M., Kim, S.S., Kim, J., Park, M., Song, J.H. 2009. Effect of (-)-epigallocatechin-3-gallate on Na (+) currents in rat dorsal root ganglion neurons. *Eur. J. Pharmacol.* 604, 1-3, 20-26.
- Kuang, X., Huang, Y., Gu, H.F., Zu, X.Y., Zou, W.Y., Song, Z.B., Guo, Q.L. 2012. Effect of intrathecal epigallocatechin gallate, an inhibitor of Toll-like receptor 4, on chronic neuropathic pain in rats. *Eur. J. Pharmacol.* 676, 1-3, 51-56.
- Moriyama, T., Higashi, T., Togashi, K., Lida, T., Segi, E, Sugimoto, Y, Tominaga, T., Narumiya, S., Tominaga, M. 2005. Sensitization of TRPV1 by EP1 and IP reveals peripheral nociceptive mechanism of prostaglandins. *Mol Pain.* 17,1-3.
- Niederberger, E., Geisslinger, G. 2008. The IKK-NF- κ B pathway: a source for novel molecular drug targets in the pain therapy ? *The FASEB J.* 22, 3432-3442.
- Reader, A.V.R.E., Mary, A.V., Kumar, R.A, Srinivasan, M.R., Indira, R, Ramachandran, S. 2009. Role of neuropeptides in pulpal pathosis-an overview. *The internet. J. of Dent Sci.* 8, 2, 1-4.
- Sacardote, P., Levrin, L. 2012. Peripheral mechanisms of dental pain: the role of substance P. *Mediators. Inflamm.* 2012, 1-6.
- Shay, J., Elbaz, H.A., Lee, I., Zielcke, S.P., Malek, M.H., Huttemann, M. 2015. Review article. Molecular mechanisms and therapeutic effect of (-)-epicatechin and other polyphenols in cancer, inflammation, diabetes, and neurodegeneration. *Oxid. Med. Cell Longev.* 2015, 1-13.
- Song, X.Z., Bi, Z.G., Xu, A.E. 2006. Green tea polyphenol epigallocatechin 3-gallate inhibits the expression of nitric oxide synthase and generation of nitric oxide induced by ultraviolet B in HaCaT cells. *Chinese. Medical. J.* 119,4, 282-287.
- Tipoe, G.L., Leung, T.M., Liong, E.C., Lau, T.Y.H., Fung, M.L., Nanji, A.A. 2010. Epigallocatechin-3 gallate (EGCG) reduces liver inflammation, oxidative stress and fibrosis in carbon tetrachloride (CCl₄) induced liver injury in mice. *Toxicology.* 273, 1-3, 45-52.
- Zhan., Jin, B., Chen., Reecy., Li, Y.P. 2007. TACE (TNF- α Converting Enzyme). Release of TNF- α Mediates Mechanotransduction-induced activation of P38 MAPK and Myogenesis. *J Cell. Sci.* 120, 237-239.
- Zhang, Y., Jia, Y.Y., Guo, J.L., Liu, P.Q., Jiang, J.M. 2013. Effect of (-) Gallocatechin-3-Gallate on Tetrodotoxin-Resistant Voltage-gated Sodium Channels in Rat Dorsal Root Ganglion neurons. *Int J. Mol. Sci.* 14, 9779-9789.