4. Topical Epigallocatechin-3-gallate Hydrogels Regulated Inflammation and Pain

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Topical Epigallocatechin-3-gallate Hydrogels Regulated Inflammation and Pain

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Abstract

The aim of this study is to determine in vivo response of topical Epigallocatechin-3-gallate (EGCG) hydrogels to pain and inflammation through Toll-like receptor 4 (TLR4), Superoxide dismutase (SOD1), Prostaglandin E2 (PG-E2) and Transient receptor potential vanilloid 1 (TRPV1) in pulpal inflammation.

Rats (n=35) were divided into a normal group (N), a positive control group induced with LPS for 6 hours (C1), a group induced with LPS for 24 hours (C2), a treatment group induced with LPS and topical EGCG 120 µg/ml (T1) for 6 hours and a group induced with LPS and topical EGCG 120 µg/ml for 24 hours (T2). Tissues were collected and observed by means of immunohistochemistry (IHC) to detect the expression of TLR4, SOD1, PG-E2 and TRPV1. Data were analyzed using a One-Way ANOVA test for TLR4, PG-E2 and TRPV1, while SOD1 was analyzed using a Brown-Forsythe test and Games Howell test.

Application of topical 120 µg/ml EGCG hydrogels in tooth cavities at 6 hours and 24 hours pulpal inflammation significantly increased the expression of SOD1, while significantly decreasing the expressions of TLR4, PG-E2 and TRPV1 ($P < 0.001$). EGCG can be used as a pain inhibitor, anti-inflammatory agent and antioxidant agent against pulpal inflammation in rat models.

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Introduction

Pulpal inflammation is a pulp disease characterized by pain in the trigeminal nociceptor.[1] One of the causative factors in acute pulpal inflammation is lipopolysaccharide (LPS) which activates macrophages through Toll-like receptor 4 (TLR4). This activation leads to gene transcription of Tumor Necrosis Factor-α (TNF-α). The expressions of TLR4 and TNF-α will increase within 6 hours after LPS induction, reaching a peak after 24 hours.[2]

Macrophage is activated during pulpal inflammation. The metabolism of macrophages can trigger production of reactive oxygen species (ROS). Low to moderate concentrations of ROS are important for physiological cell processes. High concentrations of ROS may lead to adverse modifications to cell components, such as lipids and proteins, which may cause changes in deoxyribonucleid acid (DNA) structure. ROS can induce lipid peroxidation and disrupt the membrane lipid bilayer .[3]

Humans produce endogenous antioxidants to neutralize oxidants. The major enzymatic antioxidants are superoxide dismutase (SOD1), catalase (CAT) and glutathione peroxidase (GSH-Px).[3,4] The increased level of SOD1 will catalyze $2O_2^{\cdot}$ and $2H^+$ into H_2O_2 and O_2. High expressions of CAT will catalyze H_2O_2 into H_2O and O_2.[5] Cell membrane regulation for cell damage repair also produces ROS. Effective antioxidants should immediately be administered to maintain membrane integrity. High levels of ROS accompanied by the presence of cyclooxygenase (COX2) enzyme will lead to catalyzation of arachidonic acid (AA) into prostaglandin-E2 (PG-E2).[6] The expression of

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PG-E2 activates transient receptor potential vanilloid (TRPV1) in the sensory nerve endings of dental pulps located in the outer surface sodium gated channel. The up-regulation of TRPV1 in trigeminal nociceptors can contribute to hyperalgesia under pulpitis conditions.[6] Pulp tissue is located inside the pulp chamber and surrounded by a hard dentine wall. Blood supply and drainage pass through narrow apical foramen. This condition may accelerate inflammation and death of the pulp tissue.[8] In addition, early treatment is needed to inhibit the progress of inflammation in order that endodontic treatment can be rendered unnecessary.

One of the widely used medicaments in the field of dentistry is eugenol that contains an active component, known as phenol. Eugenol has been a popular medicament as topical pain relievers to alleviate toothache and dentin hypersensitivity, for pulp capping material or root canal obturation material. However, eugenol is not entirely a biocompatible material due to previous studies that showed eugenol dressing for 2-3 days had side effect of ulceration and within 48 hours leading to painful ulcer with surrounding erythema. Furthermore, within 2 months will lead to tooth necrosis.[9] It is, therefore, necessary to identify alternative materials for dental medicament.

Epigallocatechin-3-gallate (EGCG) contains an active component, known as polyphenol, that is classified as a catechin. EGCG is an interesting subject of study due to its flavanoid content which can inhibit the opening of the Natrium (Na^+) ion channel and its potential action.[10] In addition, EGCG can act as an antioxidant and anti-inflammatory agent.[11-13] Catechin of EGCG can reduce the level of free radicals and also prevent their chain reactions because EGCG can bind itself to ROS.[12] EGCG capable of passively diffusing into the cell cytoplasm and nucleus through protein pore channels in the cell membrane and EGCG directly binds to membrane component including protein and lipid.[14-15] EGCG flavonoid can bind with 67-kDa laminin receptor (LR) in macrophage cell membranes , inhibit LPS is to mediate anti-inflammatory action and decrease of TLR4, and trigger SOD1 and CAT gene transcription.[16-20] The aim of this study is to determine the extent of the in vivo response of topical EGCG hydrogels at a concentration of 120 µg/ml because, based on previous results, the concentration showed more than 50% cell viability of TLR4, SOD1, PG-E2 and TRPV1 present in pulpal inflammation in Wistar rat models.

Materials and methods

Rats (*Rattus norvegicus)* male (n=35) aged 2.5 months and weighing 22-30 grams were obtained from the Department of Medical Biochemistry, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia. The study reported here was approved and supervised by the Universitas Airlangga Faculty of Dental Medicine Research Ethical Clearance Commision, No 018/HRECC.FODM/ III/2018.

The rats were divided into a normal group (N) without LPS and EGCG application, a positive control group (C1) induced with LPS for 6 hours, a control group (C2) induced with LPS for 24 hours and a treatment group (T1) induced with LPS and topical EGCG 120 µg/ml for 6 hours and T2 induced with LPS and topical EGCG 120 µg/ ml for 24 hours. Each subject in groups C1, C2, T1 and T2 was anesthetized intra-muscularly with 0.2 cc taken from a mixture of 0.5 cc of ketamine (Ketalar®, PT.Pfizer Indonesia) and 0.5 cc of xylazine (Xyla®, PT Tekad Mandiri Citra, Indonesia). A 1.5mm deep cavity with a diameter of 2mm was created in the maxillary left first molar with a new sterile fissurotomy bur (SS White burs Inc., Lakewood, NJ, USA) using a high-speed contraangle (W&H Dentalwerk Burmoos GmbH, Austria). The resulting cavities were subsequently irrigated with sterile saline solution and dried with sterile cotton rolls. Fine paper points were used to carefully inspect any sign of exposure of the pulp horn, such as bleeding from the tooth pulp.[7]

An acute pulpal inflammation model was created using LPS (derived from *Escherichia coli* (*E.coli*), serotype 0111: B4, Sigma Chemical Co, St. Louis, MO, USA; 10 mg/ml; product number L2630). The LPS was then diluted to a concentration of 10 µg/µl, a volume of 0.5 µl and applied using a fine flattened microbrush (Microbrush, USA) before being allowed to dry. This wet and dry procedure was repeated five times.[7)] The N group observed was without LPS induction. The C1 and T1 groups were induced with LPS for 6 hours, with each cavity being sealed with GIC (Fuji 7, GC Corp, Tokyo, Japan). Groups C2 and T2 were induced with LPS for 24 hours and the cavity was then sealed with GIC.

After LPS induction, the GIC restorations were disassembled to open the cavity. EGCG was then applied (Xi'An Rongsheng Biotechnology, China, 2013101512 RS) at a concentration of 120 µg/ml in the form of PEG hydrogels (PEG 400 and 4000) (Sigma-Aldrich, 25322-68-3). Base compounds for PEG 400 and PEG 4000 were measured at a ratio of 5:1. From the calculations, the PEG 400 base was determined at 83.33 grams and the PEG 4000 base at 16.67 grams. Both were poured into a porcelain cup, mixed under heat using a hot plate at a temperature of 100 °C, stirred until homogenous and allowed to cool. Up to1200 µg of EGCG was then poured into 10mL of PEG mixture and stirred until homogenous. In groups C1 and C2, 0.5 ml PEG hydrogels were applied to the tooth cavities. Meanwhile, in groups T1 and T2, 0.5 ml of 120 µg/ml EGCG hydrogels was applied to the respective tooth cavities, prior to their being sealed with GIC restoration. After 24 hours, the administration of PEG was repeated for groups C1 and C2, while EGCG hydrogels was repeatedly administered to groups T1 and T2. The cavity was sealed with GIC restoration and allowed to settle for 24 hours.

The subjects in groups C1 and T1 were sacrified 54 hours after the dentinal application of LPS, while those in the C2 and T2 groups were sacrified after 72 hours. The subjects in group N were sacrificed after 72 hours along with those in groups C2 and T2. The left maxillary first molars were then surgically removed along with the upper jaw to be used as specimens for analysis, the unit of analysis being dental pulp. IHC staining technique was employed to examine the expression of TLR4 (anti-rat TLR4 monoclonal antibody, Santa Cruz Biotechnology Inc., USA), SOD1 (anti-rat SOD1 monoclonal antibody Santa Cruz Biotechnology Inc., USA), PG-E2 (anti-rat PG-E2 polyclonal antibody *Primary Conjugated*, Bioss, USA) and TRPV1 (anti-rat TRPV1 monoclonal antibody, NeuroMab, USA). Two operators were blinded to the samples. They were not informed about the identity of the samples. Operators uniformed as to the technique employed examined the samples. Samples were observed using a light microscope E 100 at 400x magnification from five perspective points (Nikon, Tokyo, Japan).

The surface area of the cells was calculated through observation. In each group, the number of TLR4, PG-E2, macrophage cells and TRPV1 sensory nerve cell expressions was recorded. The images were captured with a digital camera (A7; Sony, Tokyo, Japan) (Figure 1). The homogenity of the distribution of data obtained was then analyzed by means of a One-sample Kolmogorov-Smirnov Test, in combination with a Levene test. A One-Way Analyses of Variance (ANOVA) test and Comparative Analysis of Least Significance Difference (LSD) were performed on the TLR4, PG-E2 and TRPV1 data, while a Brown-Forsythe test and Games Howell test were performed on the SOD1 data.

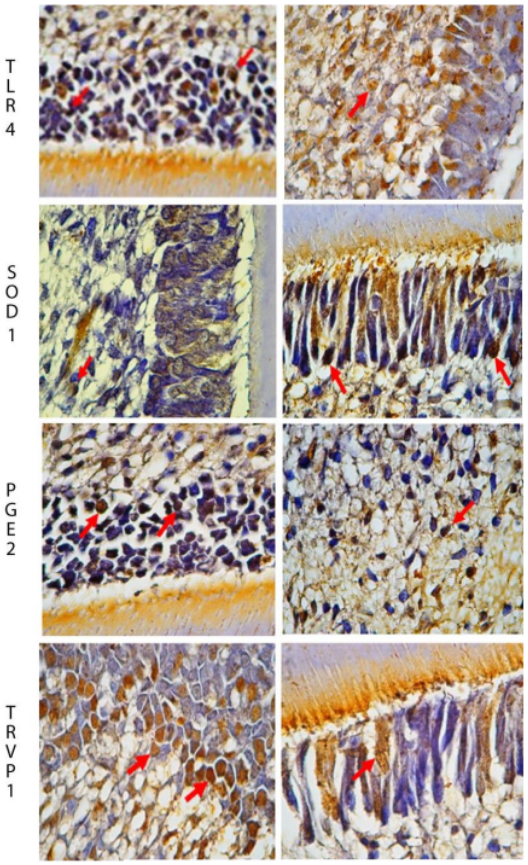

Figure 1. IHC result for TLR4, SOD1, PG-E2 expressions in macrophages cells and TRPV1 in sensory nerve cell expressions. The red arrows indicate positive expressions and examples of cells that were counted.

Results

The test showed that all the data in this research were normally distributed (*P*>0.05). A homogenity test using a Levene test confirmed the data of TLR4, PG-E2 and TRPV1 as homogenous (*P>0.05*). In contrast, the data of SOD1 expressions did not demonstrate homogenous variance (*P<0.05*). A One-Way ANOVA test and Comparative Analysis of LSD (*P*<0.001) were performed on the TLR4, PG-E2 and TRPV1 data. A Brown-Forsythe test and Games Howell comparation test were performed on the SOD1 data the results of which are shown in Table 1. The induction of LPS for 6 hours and 24 hours produced a significant increase in the expression of TLR4, SOD1, PG-E2 and TRPV1 (*P*< 0.001). The topical application of 120 µg/ml EGCG hydrogels on 6-hour and 24-hour pulpal inflammation produced significant inhibition in the expression of TLR4, PG-E2 and TRPV1. There was significantly increased expression of SOD1 (*P*<0.001).

Discussion

Bacterial products such as LPS may activate macrophages cells through TLR4.[2] Activation of macrophages cells may increase the level of ROS which will catalyze AA into PG-E2.[3,6] The activation of PG-E2 will activate TRPV1 in the sensory nerve endings mof the dental pulp which contributes to hyperalgesia under conditions of pulpitis.[7]

In this research, a pulpitis model was created by means of inducing LPS from *E.coli* to freshly cut dentin in the maxillary left first molar. The research method applied during this procedure was non-invasive in order to induce pulpal inflammation, while minimizing any risk of pulpal injury, and to observe only the effects of LPS application without disruption to other unknown inflammation sources.[7] The results of this research showed that TLR4, SOD1, PG-E2 and TRPV1 expressions occurred in the normal group.

In the groups experiencing 6-hour pulpal inflammation, the following approximate increases occurred: 2.75-fold in the expression of TLR4, 1.72-fold in SOD1, 3.40-fold increase in PG-E2 and 2.66-fold in TRPV1. In the groups experiencing 24-hour pulpal inflammation demonstrated the following approximate increases: 0.75-fold in the expression of TLR4, 2.72-fold in SOD1, 8.8-fold in PG-E2, and 7.00-fold in TRPV1 compared with the normal group. This study demonstrated that the dentinal application of LPS up-regulated TLR4, SOD1, PG-E2 in macrophage cells and TRPV1 presetn in the sensory nerve cells present in dental pulp. In the 24-hour pulpal inflammation groups, the following approximate increases occurred: 3.90-fold in TLR4 expression, 1.57-fold SOD1, 2.58-fold in PG-E2 and 2.62-fold in TRPV1, compared to the 6-hour pulpal inflammation model. These figure indicate that in 6-hour acute pulpal inflammation, the degree of inflammation remained low, with the result that the production of TLR4, SOD1, PG-E2, and TRPV1 was also limited. The results obtained show that the elevated level of inflammation could increase the expressions of TLR4, SOD1, PG-E2, and TRPV1. A higher level of inflammation can trigger the production of ROS, cell damage, lead to an increase in the recruitment of endogenous antioxidant enzymes, such as SOD1, and trigger pain.

The antioxidant activity and direct role as a free radical scavenger of EGCG has the potential to indirectly stimulate SOD1 and CAT, thereby increasing their anti-inflammatory , and analgesic.[10,13,19,20] In this research, EGCG was present in the form of hydrogels. The delivery system applied in this research was liquid PEG 400 mixed with solid PEG 4000. The use of a biocompatible polymer such as PEG was expected to maintain cell stability and viability, while also controlling the release of EGCG.[21] Therefore, the absorption of EGCG will become slower, more continuous, more stable and more evenly spread in order to extend the half-life time. In addition, other benefits include: enhanced bioavailability, a minimal absorption rate, protection of the drug against the environment and avoiding the stimulation of, and increasing affinity between, hydrogen and oxygen.[22-24]

The results showed acute pulpal inflammation leading to an increase in SOD1, and decreases in the TLR4 and PG-E2 expressions of macrophage cells and TRPV1 in sensory nerve cells. 24-hour acute pulpal inflammation led to a greater increase in SOD1 and a more pronounced decrease in TLR4 and PG-E2 expression in macrophage cells and reduction in the TRPV1 present in sensory nerve cells than 6-hour acute pulpal inflammation. Topical application of EGCG at a concentration

of 120 µg/ml in 6-hour acute pulpal inflammation led to an approximate 1.27-fold inhibition of TLR4 expressions, while in the 24-hour acute pulpal inflammation it led to an approximate 2.38-fold inhibition in TLR4 expression. The results of this study are in accordance with those of previous research which stated that the galloyl structure of EGCG will inhibit the activity of peptidase and collagenase in LPS, thereby preventing activation of TLR4.[25] Polyphenol derived from EGCG caused the inhibition of protease activity in LPS resulting in a decrease in LPS and consequent reduction in TLR4 expression,[26] EGCG increases the activity of alkaline phosphatase (ALP) and leads to the dephosphorylation of phosphate groups on LPS surfaces. This, in turn, produces the fatty acid lipid A chain of LPS which, initially a hexa acylated lipid A, becomes penta or tetra acylated lipid A. This results in decreasing LPS immunogenity to the point that there is no signal to activate. Chen EGCG can inhibit the synthesis of fatty acids that, themselves, inhibit the production of toxins.[27]

Topical application of EGCG at a concentration of 120 µg/ml in 6-hour acute pulpal inflammation led to an approximate 1.68-fold increase in SOD1 expression, while the 24-hour acute pulpal inflammation precipitated an approximate 2.06-fold increase in SOD1 expression. Increased expression of SOD1 indicates a high potential for dismutase activity, turning superoxide into less reactive hydrogen peroxide.[14] Higher expression of SOD1 in the 24-hour acute pulpal inflammation model compared to the 6-hour acute pulpal inflammation model indicates a high ROS level in 24-hour acute pulpal inflammation. It also suggests greater physiological responses than the stimulus which is probably intended to offer protection and as a SOD1 backup in responding to ROS. The increased SOD1 expression may be caused by SOD1 gene activation.[28] This is due to the fact that SOD1 can cause the dismutase of $2O_2^{\cdot} + 2H^+$ into H_2O_2 and O_2, with the result that $O2^{\cdot}$ will decrease.[5] The antioxidant activity of CAT then finalizes the activities of SOD1 since H_2O_2 is catalyzed by CAT into H_2O and O_2.[4,5] The prevention of new free radical formation plays a role in preventing tissue redox balance disruption, regulating the signaling molecule for a balanced physiological process.[5] The accumulation of ROS will then damage polyunsaturated lipid A (PUFA) through disruption of the integrity of cell membranes by membrane lipid peroxidation and leads to rearrangement of membrane structure.[3] This research proved that the accumulation of ROS can be inhibited by EGCG, as well as by the the increased expression of SOD1.

As a result, the application of EGCG at a concentration of 120 µg/ml in acute 6-hour pulpal inflammation triggered a significant approximate 1.54-fold decrease in the expression of PG-E2, while in 24-hour acute pulpal inflammation, the expression of PG-E2 decreased to an even greater extent, reaching about 1.69-fold. Activation of the phospholipase A2 (PLA) enzyme will catalyze the reaction of AA formation in the cell lipid membrane, while the presence of COX2 and free radicals will catalyze AA to PG-E2.[6] The decreased expression of PG-E2 was due to the inhibition of ROS production caused by the influence of EGCG, thus inhibiting AA catalysis to synthesize PG-E2. This is in accordance with the findings of this study that the application of EGCG in a 6-hour or 24-hour acute pulpitis model will cause increased expression of SOD1. This subsequently plays a role in reducing ROS and acting as scavenger, thereby decreasing ROS and inhibiting AA catalysis to synthesize PG-E2. The presence of saponins and flavanoids in EGCG can inhibit COX2 synthesis, consequently decreasing the synthesis of PG-E2.[10,29] EGCG inhibits the activity of phosphorylation of IKK, resulting in the inhibition of IκB and, consequently, decreasing the activity of NFκB, TNF-α, NO, ROS, and COX2.[30]

The findings of this research show that the topical application of EGCG at a concentration of 120 µg/ml in a 6-hour acute pulpal inflammation model produced an approximate 1.6-fold inhibition of TRPV1 expression, while in the 24-hour acute pulpal inflammation model, it resulted in an approximate 2-fold inhibition of TRPV1 expression. TRPV1 is a transmembrane receptor located on the outer surface of the complex gated sodium channel on the sensory nerve endings of the A- δ fibers in dental pulp. Increased TRPV1 expression plays a role in acute inflammation.[7] Inhibition of TRPV1 expression is caused by MAPKs activation of EGCG resulting in the inhibition of transcription factor p65 NF-κB and the inhibiting of TNF-α, COX2 and PG-E2 expression. Byun EGCG can inhibit the opening of Na + ion voltage-activated channels.[10,31] The phenol group may interact with the vanilloid receptors in the afferent nerve to

block nerve action potential, thereby blocking the opening of ion channels.[32] Stimulation of PG-E2 on TRPV1 causes membrane depolarization. Therefore, the sodium ion channel / channel will open, allowing Na + ions to enter the sensory nerve cells.[33] The influx of Na + ions causes an action potential. Thus, neuropeptides SP and CGRP, neurotransmitters located in the synaptic cleft, are released.[14] The more Na + ions enter the cell, the greater the potential for the membrane to increase and the wider the sodium channel openings, allowing more Na + ions to enter the cells and cause higher TRPV1 expression.[34]

Conclusions

EGCG can be used as an anti-inflammatory agent because EGCG can significantly decrease the expressions of TLR4 and PG-E2. EGCG can also be used as an antioxidant because of its ability to significantly increase expressions of SOD1. In addition, EGCG can be used as a pain inhibitor due to decreased expressions of TRPV1 in rat pulpal inflammation models.

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Declaration of Interest

Conflict of interest declared none.

Group	N	TLR4		SOD1		PG-E2		TRPV1	
		Mean±SD		Mean±SD		Mean±SD		Mean±SD	
N	7	0.80±0.20[a]		2.20±0.52[a]		1.00±0.27[a]		1.20±0.53[a]	
C1	7	2.20±0.55[b]		3.80±0.11[b]		3.40±0.98[c]		3.20±1.08[c]	
C2	7	8.60±1.03[d]		6.00±1.65[c]		8.80±1.25[e]		8.40±2.15[d]	
T1	7	1.73±0.43[c]	< 0.001	6.40±1.78[c]	< 0.001	2.20±1.37[b]	< 0.001	2.00±0.71[b]	< 0.001
T2	7	3.60 ±1.12[b]		12.40±1.33[d]		5.20±0.98[d]		4.20±1.04[c]	

Table 1. The Results of TLR4, SOD1, PG-E2 and TRPV1 Expression.

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1	Sindy Cornelia Nelwan, Soegeng Wahluyo, Firli Cahaya Khairani, Ricardo Adrian Nugraha, Haryono Utomo, Udijanto Tedjosasongko. "The Relationship between Chronological Age, Dental Age, and Salivary Alkaline Phosphatase in Indonesian Children Aged 8-14 Years: A Cross-Sectional Study", Cold Spring Harbor Laboratory, 2021 Publication	1%
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