ORIGINAL ARTICLE

Caspase-9 and TNF-α Expression in Reversible Pulpitis: In Vivo Study

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ABSTRACT

Introduction: Pulpitis is an inflammation of the pulp that can turn back become normal condition or necrosis. A biomarker of TNF-α characterizes the role of the immune response in the occurrence of pulpitis. Reversible pulpitis can occur due to trauma or bacterial factors that can cause pulp cell death in the form of necrosis or apoptosis. One of the markers of apoptosis marker is Caspase 9. This study aims to determine the expression of TNF-α and caspase-9 in reversible pulpitis. **Material and Methods:** Eighteenth Sprague Dawley rats were divided into three groups: healthy rats (Sham), group II: reversible pulpitis rat model without added materials, and group III: rats made with pulpitis models with added calcium hydroxide (Ca(OH)2). The pulpitis reversible group's, maxillary incisor crown was cut and then prepared with round bur for 3 mm and K-file for perforation of pulp. Termination of rats on 3rd day. Measurement of Caspase-9 and TNF-α expression using ELISA examination. **Result:** the one-way Anova test obtained p= 0,291 so the value of p>0.05, showed that there are no significant differences in the TNF-α expression between the groups. The Kruskal-Wallis Test for Caspase-9 p=0,006 so the value of p<0.05, showed that there were differences between groups. **Conclusion:** The study found an increase in TNF-α expression while the caspase-9 expression increased significantly in male rats (Sprague Dawley) with Reversible Pulpitis as an animal model.

Keywords: Reversible Pulpitis, TNF-α, Caspase 9, inflammation, in vivo study

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INTRODUCTION

The dental pulp contains odontoblasts, fibroblasts, nerves, blood vessels, ground substances, and other cell components. Pulp inflammation is defined as pulpitis (1) The aetiology of pulpitis includes caries bacteria, trauma or iatrogenic action (2). Pulpitis that hasn't been treated can result in severe pain leading to pulp necrosis Dental pulpitis is classified into reversible and irreversible pulpitis. Reversible pulpitis (RP) is a type of pulp inflammation that is mild to moderate, but it can return to its normal state after pulp capping treatment (3). Irreversible pulpitis is a severe inflammation of pulp so the pulp can't back to a normal condition. Treatment planning for irreversible pulpitis is pulpectomy. In this research is focusing in reversible pulpitis because it can back to a normal condition with pulp capping treatment. The pulp capping material that is often used is calcium hydroxide ($Ca(OH)_2$). Still, some drawbacks of calcium hydroxide ($Ca(OH)_2$) include very soluble in saliva, necrosis of the pulp surface, poor adhesion properties, and the formation of tunnel defects in the dentinal bridge(4). The immune response is the main factor in the occurrence of pulpitis. Several immune cells and inflammatory mediators is leukocyte cells polymorphonuclears (PMNs) and tumour necrosis factors-α (TNF-α) (5). Neutrophil infiltration is a key marker of the severity of pulpitis (6). Tumour necrosis factors (TNF) are inflammatory mediators with biological activities, including stimulating and inhibiting several cell components in the immune system. TNF-α can activate cells and induce the synthesis of pro-inflammatory cytokines. Odontoblast cells are cells that are anatomically located in the peripheral pulp, and the odontoblast process extends through the dentinal tubules so that odontoblast cells are the first cells to respond to bacterial antigens such as lipopolysaccharide (LPS) by expressing pattern recognition receptors (PRRs) which can recognize and bind components bacteria (7). The presence of this binding leads to activation of intracellular signal transduction and secretion of interleukins, pro-inflammatory cytokines, and chemokines (8). The

presence of trauma or bacteria can cause odontoblast cell death in the form of necrosis or apoptosis. Cell death is a process that is important for physiological growth. Cell death can take the form of apoptosis, necrosis, and autophagy, which differ in manner and form through the activation of different specific signals. In recent studies, there is a relationship between forms of cell death in the form of interconnection, and overlapping signal pathways (9). Caspase (cysteine-aspartyl protease) is a protease enzyme that plays a role in the mechanism of apoptosis. Caspases are divided into inflammatory caspases (caspases -1, -4, -5, -12) and apoptotic (Capases -2, -3, -6, -7, -8, -9, -1). Caspase-9 acts as an initiator of caspase in the process of apoptosis. Apoptosis is a death program that can be stimulated by triggers and through pathological pathways. The process of apoptosis is controlled by several levels of cells originating from intrinsic and extrinsic triggers. Apoptosis has an important role in stability through homeostasis and the pathogenesis of diseases(10). Apoptosis is in contrast with necrosis, Necrosis is a condition where cells in the body are injured and will result in the death of cells and body tissues. Necrosis is an unregulated and involuntary cell death caused by nonspecific, or non-physiological stress. Necrosis is always accompanied by the inflammatory response. Pulp exposure in pulpitis reversible can cause odontoblast cell death either in the form of necrosis or apoptosis (9). This form of cell death can affect tissue repair through pulp capping treatment using calcium hydroxide. This study aims to determine the expression of TNF-α and caspase-9 in reversible pulpitis.

MATERIALS AND METHODS

Samples

The type of research carried out is experimental laboratory research. The Ethics Committee, Faculty of Medicine, Universitas Muhammadiyah Surakarta had approved with number 3642/A.1/KEPK-FKUMS/VIII/2021. This research is an in vivo study using a Sprague Dawley rat. The experiment was carried out at the Animal Development Laboratory of the Faculty of Medicine, Sultan Agung University, Semarang. This design research used post-test only with a control group. Subjects or samples of this study used male Sprague Dawley rats with a bodyweight of 250-350 grams, no anatomical abnormalities, and normal activity and behavior. The environment of rats should be housed within a temperature of 26-30 °C and the acceptable humidity range is 30% to 70% which they can adapt with minimal stress and physiologic alteration. Animals have adequate bedding substrates and/or structures for resting and sleeping. Animals have given fed and drunk twice a day. The number of research samples was 18 rats and were divided into three groups. Each group sample consisted of 6 rats.

Reversible Pulpitis (RP) Animal Model

Making an Animal Model of Reversible Pulpitis (RP) is done by performing intramuscular anesthesia in mice with 0, 2 cc/kg of ketamine. Subsequently, the crown of the incisors was cut to the marginal gingival margin using a wheel disk, followed by cavity preparation by low-speed casting using a round bur with a diameter of 0.8 mm on the maxillary incisors with a depth of 3 mm then puncture using a size 10 K-file until the pulp perforation. The sign that the animal has had reversible pulpitis is that there is a red spot on the floor of the pulp chamber. In group I, was healthy rats. In group II, pulpitis reversible treatment was carried out, and temporary closure was immediately carried out. In group III, pulpitis reversible treatment then added 0,2 mg calcium hydroxide (Ca(OH)2) (Hydcall, Cemento de Hydroxide, Brazil) using Microapical Placement and closed with a temporary filling Cavit (3M ESPE, UK and Ireland).

Extirpation of pulp tissue

Termination of rat on day 3. The rats were euthanized and the right maxilla bones were removed. Barbed broach or extirpation file were used for extraction of pulp tissue in incisors. The extracted pulp was put into a microtube.

Enzyme-Linked immunosorbent Assay (ELISA) procedure

Enzyme-linked immunosorbent assay (ELISA) use to examine the expression of TNF-α (BioEnzy, BZ-08184670-EB, China) and Caspase 9 (ELK, ELK1531, China). The dental pulp tissue that has been taken is cut into small pieces so that it can fit into a tube measuring 1.5 – 2 ml, then washed with 0.9% NaCl solution and dried. Mash the hollow tissue destroyed. 0.28 ml of Phosphate buffered saline (PBS, Sigma, USA) was added and continued with centrifuge at 3000 rpm at 4 degrees. Take the supernatant for storage. The pellets were added with PBS and then sonicated with a sonicator for 3-5 seconds. After that, it was centrifuged again at the same speed of 3000 rpm at 4 degrees. The supernatant was taken for ELISA testing. ELISA for TNF-α procedure as follows: Prepare the samples, standards and reagents. Add 50µl standard liquid to the standard well. Add 40 µl sample of pulp to sample wells and add 10 µl anti TNF-α

Statistical analysis

All variables performed descriptive analysis. The data of caspase-9 and TNF-α is first carried out a shapiro-wilk test to determine whether the data is normally distributed or not and a homogeneity test, namely the levene's test. If the test results show normal and homogeneous distributed data, it is qualified for a one-way anova parametric test Statistical using SPSS Version 17 with $p < 0.05$ for all tests for statistical differences.

RESULT

The mean expression of TNF-α is shown in Table I. The

results in (Table I) showed that the TNF-α expression of the pulp samples obtained in Group I were 238.90±20.94 (ng/L), Group II was 265.70±43.24 (ng/L), and Group III was 255.99±12.66 (ng/L). The mean results showed that the highest expression of TNF-α occurred in group II.

Table I: The mean expression of TNF- (ng/L)

Group	Number	Mean ± SD	Minimum	Maximum
I	6	238.90 ± 20.94	207.07	264.61
II	6	265.70 ± 43.24	214.57	344.18
II	6	255.99 ±12.66	236.50	270.07

Group I: healthy rats (Sham); Group II: reversible pulpitis rat model without calcium hydroxide Ca (OH)2; Group III: rats made with pulpitismodels with Ca $(OH)_2$).

The results of the one-way Anova test were obtained p= 0.291 so that the value of p>0.05 which showed that there were no significant differences in the TNF-α expression between the groups.

The mean expression of Caspase 9 is shown in Table II. The research results in (Table II) showed that the Capcase-9 expression of the pulp samples obtained in Group I were 3.055±0.92 (ng/L), Group II was 19.12±7.48 (ng/L), and Group III was 6.565±5.00.

Table II. The mean expression of Caspase-9 (ng/L)

Group	Number	Mean ± SD	Minimum	Maximum
I	6	3.055 ± 0.92	1.80	4.24
II	6	19.12 ± 7.48	6.13	25.49
III	6	6.565 ± 5.00	2.14	14.33

Group I: healthy rats (Sham); Group II: reversible pulpitis rat model without calcium hydroxide Ca (OH)2; Group III: rats made with pulpitis models with Ca $(OH)_2$).

The data results are not homogeneous, and can't perform ANOVA, so the Kruskal-Wallis test is carried out. The results of the Kruskal Wallis test got a significance value of 0.006, so that p< 0.05, indicates that there were differences between groups. Furthermore, the post hoc test with Mann Whitney test was carried out between groups to determine the significant differences as shown in Table III.

Table III: The Mann Whitney test for each group

Group	I(Sham)	II (RP)	III RP+Ca$(OH)_2$)
I (Sham)		.004*	.337
II (RP)	.004*		.016*
III (RP+Ca$(OH)_2$)	.337	.016*	

Group I: Healthy Rats.(Sham), II: Reversible Pulpitis, III: Reversible Pulpitis added calcium-Hydroxide(RP+Ca$(OH)_2$) *There were significant differences with p<0,05

There were significant differences between group I (healthy rats) and group II (pulpitis reversible rats, and between group II (pulpitis reversible rats) and group III (pulpitis reversible+Ca$(OH)_2$). There were no significant differences between group I and Group III.

DISCUSSION

There were no significant difference in TNF-α expression. This may be due to the low level of inflammation, so the amount of TNF-α expression in pulpitis compared to healthy teeth was not much different. The presence of TNF-α can cause cell death. Odontoblast cells play a role in resisting bacterial invasion and dental pulp immunity, both innate and adaptive immune responses (11). Research by Pezelj et al reports that TNF-α is more commonly found in symptomatic irreversible pulpitis (12). Research by Kokkas et al (2007) reports that gene expression TNF- in pulp tissue correlates with severity according to signs clinically, this is based on the finding of a significant increase in TNF-α gene expression in irreversible pulpitis compared to the control group, whereas in the pulpitis reversible group there was no difference with the normal group (13). TNF-α is related to the severity of inflammation. Several signal transductions that play a role in the inflammatory response and odontoblast differentiation include the p38 MAPK pathway, TGF-β/Smad, PI-3 AKT/mTOR, NF-kβ and Wnt/β-catenin. The inflammatory response of the pulp leads to the activation of intercellular signalling pathways such as the NF-B and MAPK pathways. The NF-B pathway is activated by receptor cytokines such as TNF- and IL-8. Iκβ phosphorylation can trigger ubiquitination and proteasome degradation resulting in the release of NF-κB. The NF-B complex is activated by posttranslational modifications (phosphorylation, acetylation, and glycosylation), is translocated in the nucleus and induces gene expression (14).

Stronger stimuli can cause primary odontoblast cell death. Under stable conditions, mesenchymal cells act as progenitors or stem cells that differentiate into odontoblast-like cells to form reparative dentin (15). Stem cells are derived from pulp mesenchymal cells, which will form odontoblast like cells. Stem cell chemotactic signals are induced by several factors so that they can move to the lesion area. The ability of the dentin component can be increased if there is an irritant that affects the pulp by inducing the formation of mineralized tissue through the migration of pulp cells (16)

The results of the study show a significant increase in capcase-9. The increase in capcase-9 indicates the initiation of apoptosis. Apoptosis plays an active role in odontogenesis, and cells undergoing apoptosis can trigger tissue remodelling through regulation of cell division, cell death, cell fate, cell migration and remodelling of surrounding tissues(17) The caspase-mediated apoptotic pathway is initiated by releasing cytochrome C from the mitochondria. This can induce caspase activation and cell death (18). Caspase-9 has an important role in apoptosis in pulp cells. caspase-9 can activate caspase-3 more than caspase-8. The role of apoptosis in pulp cells initiated by caspase-9 through intrinsic and extrinsic pathways can affect the repair mechanism for pulp (19). Caspase-9 can prevent the accessibility of cytochrome c to complex III in mitochondria, leading to increased reactive oxygen species (ROS) production, but in the presence of effector caspase activity, ROS production is

stopped (20) .

Reversible pulpitis that added calcium hydroxide made TNF alpha expression smaller, but insignificant. Calcium hydroxide has strong antibacterial properties. Calcium hydroxide can inhibit DNA replication by dividing and killing bacteria that cause infection by hydrolyzing bacterial lipopolysaccharide (LPS). High pH in calcium hydroxide is reported to be able to help release proteins and growth factors (TGF- β1) which are mediators in the formation of odontoblast-like cells (21). On the other hand, strong bases in calcium hydroxide when applied over tissues can cause mitochondrial dysfunction. This condition will increase superoxide radicals that diffuse through the cytosol and then enter the mitochondria so that there is a decrease in function. If the mitochondria die, the cells will lack oxygen and eventually die (22). Strong bases formed from the ionization of Ca(OH)2 in the form of Ca++ and OH- can increase pulp abnormalities and apical periodontitis. The use of Ca(OH)2 material on the average number of neutrophil cells is less effective. The release of hydroxyl ions will form a necrotic tissue about 1-2 mm in the area around the pulp. The pH value of 12.5 which is owned by calcium hydroxide will cause tissue necrosis in the superficial layer of the pulp (23) Pulp capping material using calcium hydroxide showed a lower level of apoptosis in parameter caspase due to calcium hydroxide don't have an antioxidant ability that can reduce ROS. Calcium hydroxide is unable to produce Toll-like Receptor 2 and proinflammatory cytokines below high alkaline conditions. Pulp capping using calcium hydroxide can cause early apoptosis via the external apoptosis pathway (24).

CONCLUSION

The study found an increase in TNF-α expression while the caspase-9 expression increased significantly in male rats (Sprague Dawley) with reversible pulpitis as an animal model.

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