Nf-Kb Expressions on Rat Dental Pulp Mechanically Exposured after Pomegranate Fruit Extract Administration

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Abstract

The Objective of this study was to analyze the activity of pomegranate extract on rat dental pulp mechanically exposured on the expression of NF-kB. Eighteen male Wistar rats aged 2.5 months and has a weight of about 190-230 grams were divided into 3 groups. The first group (A) was rat dental pulps mechanically exposured group which cappped with carboxy methyl cellulose (CMC-Na) 3% gel as control, the second group was cappped with calcium hydroxide(B), the third group was cappped with pomegranate fruit extract gel (C). The group consists of 6 rat each. The expression of NF-kB was examined using immunohistochemical techniques after one day of treatment. Data were analyzed using ANOVA and followed by HSD test.

The Results showed that there were significance difference among the groups ($p < 0.05$). Pomegranate fruit extract as a pulp capping material were found to inhibit translocation of NF-kB into the nucleus of cell, it could be proved by lowering NF-kB expression.

In Conclusion, these data indicate that pomegranate fruit extract decreased NF-Kb expresion and thus lowering the production of pro inflammatory cytokine so accelerate inflammation process.

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Introduction

Pulp capping material usually used in Faculty of Dentistry is *calcium hydroxide* [$Ca(OH)_2$]. In the dental pulp, calcium hydroxide has been used as a pulp capping agent because of its ability to stimulate mineralization. Nevertheless, *calcium hydroxide* still has disadvantages, such as irritating the pulp tissue and resulting in inflammation in the pulp tissue. Calcium hydroxide produces inflammation within the underlying pulp, which can last for up to 3 months in human teeth.[1] The pulp inflammation is caused more by irritation due to pulp capping material than by bacterial invasion due to microleakage.[2]

Dental pulp exposure due to mechanical trauma, moreover, requires *direct pulp capping* treatment. *Direct pulp capping* is administration of pulp capping material onto the exposed pulp, and then the cavity was filled with restorative materials.[3] Mechanical trauma to the pulp treatment procedure actually can not always be avoided, especially if the teeth need extensive restoration or are handled by less experienced operators in *pulp capping* treatment.

Actually, there are many different types of plants that have anti-inflammatory, anti oxidant and anti-bacterial activities, widely studied for the benefit of human health. Pomegranate (*Punica granatum Linn*) is one of plants widely studied since it has been used traditionally and believed to have had medical benefits.[4] *Ellagic acid* contained in pomegranate extract, for instance, has an inhibitory activity against translocation of *nuclear factor kappa beta* (NF-kB) into the nucleus of cell.

In other words, inflammation will occur in pulp mechanically exposured. Consequently, NF-kB in the nucleus will be increased, triggering an increase in production of proinflammatory cytokine. Furthermore, NF-κB induces cytokines that regulate the immune response (such as

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TNFα, IL-1, IL-6 and IL-8), as well as adhesion molecules, which lead to the recruitment of leukocytes to sites of inflammation.[5]

If cells are not stimulated, NF-kB will be located in the cytoplasm and bind to IKB, preventing NF-kB to translocate into the nucleus. But, if cells are stimulated, NF-kB would be translocated into the nucleus and bind to a specific target gene that stimulates gene transcription.[6,7] *Nuclear factor kappa beta,* therefore, is a transcription factor which must be controlled because the higher the NF-kB is translocated into the nucleus of the cell, so the higher the production of proinflammatory cytokines, thus prolonging the healing process of pulp mechanically exposed.

The effects of pomegranate fruit extract on healing process of exposed pulp due to mechanical trauma have not been studied. As a result, it is necessary to analyze the effects of pomegranate extract on healing process of exposed pulp due to mechanical trauma through observing NF-kB expressions. Therefore, this research aimed to analyze the effects of pomegranate extract on exposed pulp, especially in reducing NF-kB expressions.

Materials and methods

Animal. Eighteen Wistar strain of Rattus norvegicus aged 2.5 months weighed 190-230 g, moreover, were used as animal models in this research. Those rats were adapted for one week before and were randomly allotted into three groups. Group A as a control group (exposed pulp capped with CMC-Na), group B (exposed pulp capped with $Ca(OH)_2$) and group C (exposed pulp capped with pomegranate fruit extract). The group consists of 6 rat each. Eighteen healthy rats were utilized in this research , were fed a standard chow and tap water ad libitum. The experiment was approved by the Ethical Committee of the Faculty of Dental Medicine, Universitas Airlangga, Indonesia.

Materials. The following materials were used : CMC-Na 3 % as the basic ingredient of gel, *calcium hydroxide* (Darmstadt, Germany), glassionomer (GC Fuji II, Japan) and standardized pomegranate extract powder containing 40% *ellagic acid* (Xi'an Biof Bio-Technology Co., Ltd. of China.). Concentration of pomegranate extract used in this research was 2.5%, concentration of 2.5% was prepared by mixing 2.5 grams of pomegranate fruit extract into 97.5 grams of gel base material, equivalent to the content of 1% *ellagic acid*. Materials used for immunohistochemical staining were anti NF-kBp65 monoclonal antibodies (ab 16502, Abcam).

Experimental procedure. The rats were intramuscularly anesthesized with a combination of *ketamine* and *diazepam HCl* (100 mg: 50 mg at a dose of 0.5 ml / kg body weight). Class I cavity was prepared on the occlusal surface of the right maxillary first molar using a *low-speed round diamond bur* (intensively, Swizerland) with a diameter of 0.84 mm. The pulp was then exposed at the cavity floor using a dental explorer (Martin, Germany) with a tip diameter of 0.35 mm.[8] The exposed pulps were capped with CMC-Na 3 % as a control group (A), capped with $Ca(OH)_2$ (B) because Ca(OH)2 is commonly used and it is generally the most well established material, and capped with pomegranate fruit extract in the treatment group (C), and the cavities were immediately restored with glassionomer. The observation period lasted 1 day. Those rats then were sacrificed and their maxillary jaw together with their three right maxillary molar was taken approximately along 12 mm. The jaws and those teeth were soaked in buffered formalin. After 24 hours, buffered formalin was replaced with 10% ethylene-diamine-tetra-acetic acid. It was then replaced every day for about 30 days at a room temperature in order to make the maxillary bone tissues and the teeth become soft.

Preparation of immunohistochemical specimens. Embedding was carried out using paraffin solution at a temperature of 56^0-59^0C and paraffin blocks cut with a thickness of ± 5µm. Immunohistochemical staining then was performed using anti NF-kB monoclonal antibodies. After that, measurement was conducted by counting the number of cells expressing NF-kB in the observed specimens using immunohistochemical techniques. The calculation then was carried out on immunoreactive cells with brown color in cell membranes or cytoplasm with 400 x magnification with a light microscope. Observation focused on neutrophils and the total number of immunoreactive cells was used as data.

Statistical analysis. The data obtained from this research expressed as mean ± standard deviasions (SD). One-way analysis of

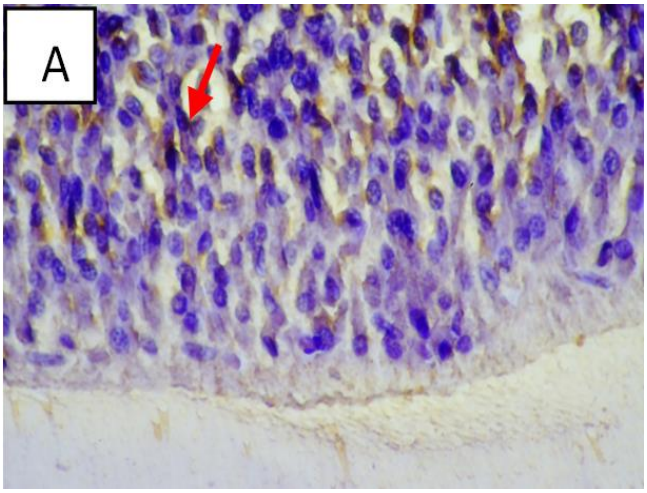
variance (ANOVA) followed by HSD test was applied to assess the statistical significance of the differences between the study group at $p<0.05$.

Results

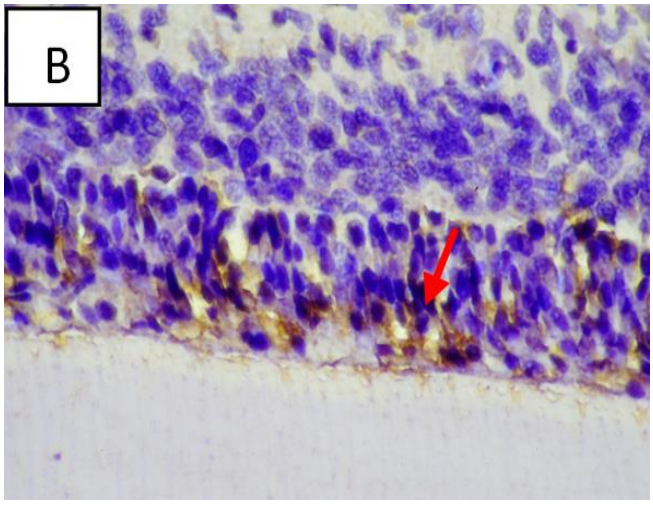
Data were presented in Table 1. Based on the results of observation and calculation on cells expressing NF-kB, the group exposed pulp, capped with pomegranate fruit extract have a lowest expression of NF-kB.

Group	N	Mean of NF-kB	SD
A (control)	6	19.3333[a]	2.33809
B ($Ca(OH)_2$)	6	10.3333[b]	1.75119
C (Extract)	6	3.6667[c]	1.21106

Table 1. Mean and standard deviation of each group.



A



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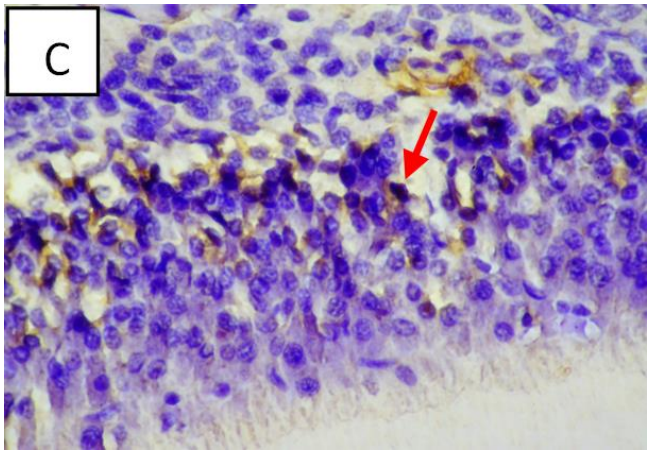

C

Figure 1. Cells expressing NF-kB in the control group A (CMC-Na), the group B ($Ca(OH)_2$), and the group C (extract)
Description: → (Positive)
Immunohistochemical staining with anti NF-kB monoclonal antibodies using 400 x magnification with a light microscope and camera brand Olympus CX 21. Observation focused on neutrophils.

The results of *One way Anova* test showed that there were significant differences in the number of cells expressing NF-kB between the groups ($p = 0.000$). Thus, statistical analysis was continued with HSD test. Cells expressing NF-kB in the group A, B and C were presented in Figure 1.

Discussion

Applications of pulp capping materials are the standard treatment for accidentally injured pulp with no other symptoms, such as mechanically pulp exposured. An ideal treatment outcome of pulpal exposure during restorative procedures is to regain the primary structure of tubular dentin as well as maintain the vitality and healthiness of the dental pulp. *Pulp capping* material still used nowadays is $Ca(OH)_2$ which serves to maintain and sustain the vitality of the pulp. This research showed that administration of $Ca(OH)_2$ to cap an exposed pulp triggered NF-kB expressions higher than that of pomegranate extract. This is because $Ca(OH)_2$ has an alkaline pH, so a few minutes after the contact of pulp tissue with calcium hydroxide, the formation of necrotic areas begins called cauterization zone. Consequently, inflammation occurred in the tissues surrounding. However, since $Ca(OH)_2$ does not have anti-inflammatory activity, it could not inhibit the translocation of NF-kB into the

nucleus, so the degree of pulp inflammation increased. As a result, NF-kB expressions was higher than in the group capped with pomegranate extract gel.

Nuclear factor kappa beta is a transcription factor that regulates the transcription of many genes mainly involved in immune response and inflammation.[5] In other words, *nuclear factor kappa beta* plays an important role in immune and inflammatory responses through the regulation of genes encoding pro-inflammatory cytokines, *adhesion molecules, chemokines, growth factors, inducible enzymes*, such as *cyclo-oxygenase 2* (COX2), and *inducible nitric oxide synthase* (iNOS).[9] *Nuclear factor kappa beta* also can be activated in a few minutes by pro-inflammatory stimuli, including cytokines, *growth factors,* and *stress inducers*. Previous research even showed another function of NF-kB, which is describing 'good and evil' aspects since NF-kB is required for immunological function, but harmful if not regulated.[10] It could be proved in this study, the control group exhibited highest NF-kB expression, it indicated that NF-kB was unregulated.

The administration of pomegranate extract to cap the exposed pulp aimed to regulate NF-kB, moreover, successfully demonstrated the lowest expressions of NF-kB compared to the group capped with CMC-Na and $Ca(OH)_2$ since pomegranate extract contains *ellagic acid,* which has anti-inflammatory activities. The mechanism of anti-inflammatory activities is by inhibiting the activation of *IKB kinase* (IKK). As a result, the degradation of IKB bound to NF-kB is inhibited, and NF-kB translocation into the cell nucleus will decrease. This condition then made the activation of NF-kB responsive genes decreased, so the production of pro-inflammatory interleukin 6 decreased one day after treatment.[11]

Consequently, inflammatory phase will be accelerated and the next phase of wound healing occurs faster so it accelerate the wound healing process of exposed pulp. Interleukin-6 is an interleukin produced in areas of inflammation and have an important role in the inflammatory response of acute phase. Interleukin-6 has to be controlled, because if not controlled, the production of IL-6 to be sustainable so that inflammation leads to a chronic condition.[12]

Based on the results of this research, the highest NF-kB expressions was found in the control group capped with CMC-Na gel. The high expressions of NF-kB demonstrated an increase in translocation of NF-kB into the nucleus, then increasing activation of NF-kB responsive genes. This condition then resulted in the high production of pro-inflammatory cytokines. It indicates that inflammation was uncontrolled so that the wound healing process would take longer time than the groups capped with $Ca(OH)_2$ and pomegranate fruit extract.

Therefore, NF-kB expressions in the group capped with $Ca(OH)_2$ was lower than in the control group capped with CMC-Na gel. This is due to the characteristics of $Ca(OH)_2$ having dissociation into calcium and hydroxyl ions. The action of these ions on tissues explains the biological properties of this substance. The role of calcium ions are as *protective material* in the pulp healing process.[13]

Thus, this material was assumed to help wound healing process in a longer time than the group capped with pomegranate fruit extract. Calcium ions play significant roles in dentin repair by inducing expressions of gene regulating mineralized tissue formation.[14] At the moment, although calcium hydroxide promotes in the dental pulp a superficial necrosis, it encourages mineralization and maintains the pulp health. The cauterization effect of $Ca(OH)_2$ is essential for the repair of exposed pulp. It has been suggested that the pH increases due to the presence of free hydroxyl ions may initiate mineralization.[15]

Conclusions

Administration of pomegranate fruit extract as pulp capping material on pulp mechanically exposured decreased NF-kB expressions.

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Declaration of Interest

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References

1. Accorinte ML, Loguercio AD, Reis A, Carneiro E, Grande RH, Murata SS, et al. Response of human dental pulp capped with MTA and calcium hydroxide powder. Oper Dent. 2008;33:488-95.
2. Vesna D, Elena K and Vesna L. Histological evaluation of odontoblast-like cells response after capping application of calcium hydroxide and hydroxilapatite in dog's pulp. Acta Veterinaria (Beograd). 2007; 57(5-6): 573-84.
3. Kim JH, Hong JB, Lim BS, Cho BH. Histological evaluation of direct pulp cappingwith DSP-derived synthetic peptide in beagle dog. J Kor Acad Dent. 2009; 34(2):120-28.
4. Jurenka, MT. Therapeutic Applications of Pomegranate (Punica granatum L): A Review. Alternative Medicine Review. 2008;13(2): 128-44.
5. Tripathi P and Aggarwal A. NF-kB transcription factor: a key player in the generation of immune response. Current science. 2006; 90(4):519-31.
6. Yao K, Zhang L, Zhang YD, Ye P, Zhu N. The flavonoid, fisetin, inhibits UV radiation-induced oxidative stress and the activation of NF-κB and MAPK signaling in human lens epithelial cells. Molecular Vision. 2008; 14:1865-71.
7. Wang Z, Wesche H, stevevs T, Walker N, Yeh WC. IRAK -4 inhibitors for inflammation. Curr Top Med Chem. 2009;9:724-37.
8. Oliveira MF,Giro EMA, Ramalho LTO, Abbud R. Tissue response to direct pulp capping with calcium hydroxide preceded by corticosteroid or corticosteroid/ antibiotic dressing: a histological study in rats. Rev. odonto ciênc. 2009;24(4):377-82.
9. Baldwin AS. Series introduction: the transcription factor NFkappaB and human disease. J Clin Invest. 2001;107:3-6.
10. Karin, M. and Ben-Neriah, Y. Phosphorylation meets ubiquitination: the control of NF-kB activity. Annu. Rev. Immunol. 2000; 18: 621–63.
11. Nirwana I. The Activities of pomegranate fruit extract (punica granatum linn) as a pulp capping material to IL-6, IL-10, TGFβ-1, MMP -1 and tipe I collagen on dental pulp mechanically exposured. Dissertation Medicine Science Program, Universitas Airlangga. 2012; p 85-7.
12. Jones SA, Richards PJ, Scheller J, Rose-John S. IL-6 transsignaling : the in vivo consequences. J Interferon Cytokine Res. 2005;25:241-53.
13. Estrela c and Holland R. Calcium hydroxide: study based on scientific evidences. J Appl Oral Sci. 2003;11(4): 269-82.
14. Janebodin K, Horst OV, Osathanon T. Dental pulp responses to pulp capping materials and bioactive molecules. CU Dent J. 2010;33:229-48.
15. Cavalcanti BN, Rode SM, Marques MM. Cytotoxicity of substances leached or dissolved from pulp capping materials. Int Endod J. 2005; 38:505-9.