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INVOLVEMENT OF THE NLRP3 IN ACTIVATING THE IMMUNE RESPONSE AFTER RESIN HEMA EXPOSURE IN DENTINE-PULP COMPLEX

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ABSTRACT : This study aimed to investigate the involvement of the NLRP3 in activating the immune response after resin monomer 2-hydroxyethyl methacrylate (HEMA) exposure in dentine-pulp complex. The research samples were 28 male Sprague Daley rats in randomized post-test only control groups. The rats were randomly divided into 4 groups, with 7 rats per group. Group 1, the control group was treated with glass ionomer cement restoration (Fuji IX LC) without HEMA application after the lower molar was drilled using a low speed round bur. Group 2-4, the experimental groups were treated with HEMA liquid (Sigma Aldrich) and sealed with glass ionomer cement restoration after the lower molar was drilled using a low speed round bur, and the teeth in each group were extracted after 24, 48 and 72 hours accordingly. The teeth were soaked into EDTA for 8 weeks, then cut 5milimicron by microtome then made the paraffin block. Immunohistochemistry staining was smeared using anti NLRP3 antibody monoclonal. The samples were then statistically analyzed using ANOVA and Tukey HSD. The NLRP3 expression of odontoblast reached the highest number at 48 hours of experimental time. NLRP3 has a role in activating the immune response after resin HEMA exposure in dentine-pulp complex.

Key words : NLRP3, resin HEMA, odontoblast, medicine, dentine-pulp complex.

INTRODUCTION

Composite resin is an aesthetical restorative material commonly used in dentistry for filling dental cavities. The adhesive materials have become one of the most important things for the practice of the conservative aesthetical dentistry. The adhesive materials enable procedures such as bonding of direct and indirect resin restorations, posts, brackets and aesthetical correction (Matos *et al*, 2017). The dentine bonding agents are resin-based materials used to create a composite bond between dentine and enamel (Gupta *et al*, 2014).

Most of the adhesive materials consist of 22-hydroxyethyl methacrylate (HEMA). HEMA is a stable bonding material, which functions as a base and/or as mixed material. HEMA has a good chemical bond strength and hardly degradable; therefore, it makes a proper and durable restoration (Anusavice *et al*, 2013). HEMA diffusion into the dentin tubules can cause irritation on the pulp. The chemical materials can be observed from the ability to stimulate cell death. Residual monomers from HEMA can induce an inflammatory reaction from the pulp. HEMA contains free radicals hydroxyl that can stimulate oxidative stress. ROS is an important feature that can be obtained from the resin monomer exposure (Paeanjpe *et al*, 2005; Widjiastuti *et al*, 2019).

The body has a defense system to protect itself against injury called the immune system. The immune system is composed of an innate and adaptive immune system. The innate immune system is the front liner in eliminating infections. Firstly, to recognize antigens that enter the body, pattern recognized receptors (PRR) are needed. Cellular receptors recognize the microbes and foreign objects as pathogen-associated molecular patterns (PAMPs) and damaged associated molecular patterns (DAMPs). PRR is in the plasma membranes or endosomal membranes and cytosols of various cell types. If the cells bind to PAMP and DAMP molecules, the transduction signal will be activated to protect the host (Abbas *et al*, 2012). DAMP molecules can be produced as a result of the cell damage caused by infections and sterile lesions such as toxins from chemicals, trauma, burns, compression force, or decreased blood supply (Nugraha *et al*, 2020). The innate immune system enhances the ability of cells to recognize pathogens through PRR that can detect infection or cell damage in the cytoplasm.

NOD-like receptors (NLRs) is a leucine-rich repeat domain protein that helps host identifying damage and pathogens in the cytoplasm and NLRs are involved in innate immunity. Odontoblasts express the NLRP3 that can form a multiprotein complex called inflammasomes. NLRP3 inflammasomes induce the activation of caspase-1 then secretes IL-1â dan IL-18 for inflammatory response. Odontoblast is located on the surface of dentine-pulp, so it can be the first cell that is activated by pathogen invasion. Therefore, odontoblast is the first line of the dental immune system (Bergsbaken *et al*, 2010). This study aims to investigate the involvement of the NLRP3 in activating the immune response after resin monomer 2-hydroxyethyl methacrylate (HEMA) exposure in dentine-pulp complex.

MATERIAL AND METHODS

This study was approved by the Health Research Ethical Clearance Commission of Faculty of Dental Medicine, Universitas Airlangga (15/KKEPK.FKG / IV / 2014). The samples of the study were 28 Sprague Daley rats in randomized post-test only control groups. The rats had to be male, weighted 300-350g and healthy. The rats were divided into 4 groups with 7 rats per group. Each of the rats firstly was given 0.2cc/kg intramuscular combine anesthesia of Ketamine HCl and Diazepam (100mg: 10mg). Cavity preparation was performed on the occlusal side of the left lower molar using 0.84mm low-speed diamond bur. The depth of the preparation should be around 1-1.5mm. Group 1, the control group, was treated with glass ionomer cement restoration (Fuji IX LC) without HEMA application after the lower molar was drilled by a low speed round bur. Group 2-4, the experimental groups, were treated with 97% HEMA liquid (Sigma Aldrich) and sealed with glass ionomer cement restoration and the teeth in each group were extracted after 24, 48 and 72 hours accordingly. HEMA was smeared into the cavity using fine micro brush then sealed with glass ionomer cement.

The teeth were soaked into 10% buffer formalin in 24 hours, then replaced the buffer formalin with EDTA for 60 days (renewed every day for the decalcification). The teeth then washed with Phosphate Buffer Saline (PBS) 3-5times to clean the teeth from the contaminants then fixated to 10% formalin. Dehydration process was performed using alcohol (30%, 50%, 70%, 80%, 96%, and absolute concentration accordingly) 60 minutes each. The clearing was done using xylol in 60 minutes, two times. Infiltration with the soft paraffin was done in 60 minutes at 48p C. The block was made from the hard paraffin and left alone in 24 hours. It was attached to the holder then cut 5milimicron by microtome then mounted on to the object glass with 5% gelatin.

The object glasses were soaked into xylol 2 times of 5 minutes. Dehydration processes were performed using alcohol (Absolute concentration, 96%, 80%, 70%, 50%, and 30% accordingly) 5 minutes each then rinsed with aqua dest for 5 minutes. The slides were washed using PBS pH 7.4 for 5 minutes then stained with hematoxylin for 10 minutes. The slides were soaked into aqua dest for 10 minutes. Dehydration was performed using alcohol (30% and 50%) 5 minutes each, stained with eosin in 3 minutes, washed with 30% alcohol, and then rinsed off with aqua dest for 5 minutes while drying. The slides were washed using PBS pH 7.4 for 1 minute, then were endogenous, blocked with 3% H_2O_2 for 20 minutes. The slides rewashed 3 times using PBS pH 7.4 for 5 minutes. The protein unspecified were blocked using PBS 5% (consist of 0.25% Triton X-100), then washed again 3 times using PBS pH 7.4 for 5 minutes. The slides were incubated using anti NLRP3 antibody monoclonal for 60 minutes, then rewashed 3 times using PBS pH 7.4 for 5 minutes. The slides were incubated using anti HRP conjugated for 40 minutes, then rewashed 3 times using PBS pH 7.4 for 5 minutes. The slides were dropped with DAB (Diaminobenzine) and incubated for 10 minutes, then rewashed 3 times using PBS pH 7.4 for 5 minutes, then washed with aqua dest for 5 minutes. The slides were then counterstained, mounted, and sealed using cover glass and observed under a light microscope.

RESULTS

Table 1 : Mean and standard deviations of NLRP3 expression in the control group and HEMA treatment in the three experimental time groups (24, 48 and 72 hours).

	N	$\bar{x}$	SD
Control 24	7	2.2857	0.9511
HEMA 24	7	9.0000	2.5819
Control 48	7	2.5714	0.7868
HEMA 48	7	12.5714	1.7182
Control 72	7	3.8571	1.3451
HEMA 72	7	7.0000	2.2360

The results described in the form of data analysis of NLRP3 in 3 experimental time groups (24, 48 and 72 hours). The normality test with Kolmogorov Smirnov in all experimental time groups showed a normal distribution of data ($p > 0.05$). Homogeneity test with Levene's test showed NLRP3 expression had homogeneous variance ($p > 0.05$) $p = 0.239$. The mean and standard deviations for the NLRP3 expression for 24, 48, and 72 hours of observation were 9.000 +2.581, 12.571 + 1.718 and 7.000 + 2.236, respectively.

ANOVA test in all experimental groups showed a

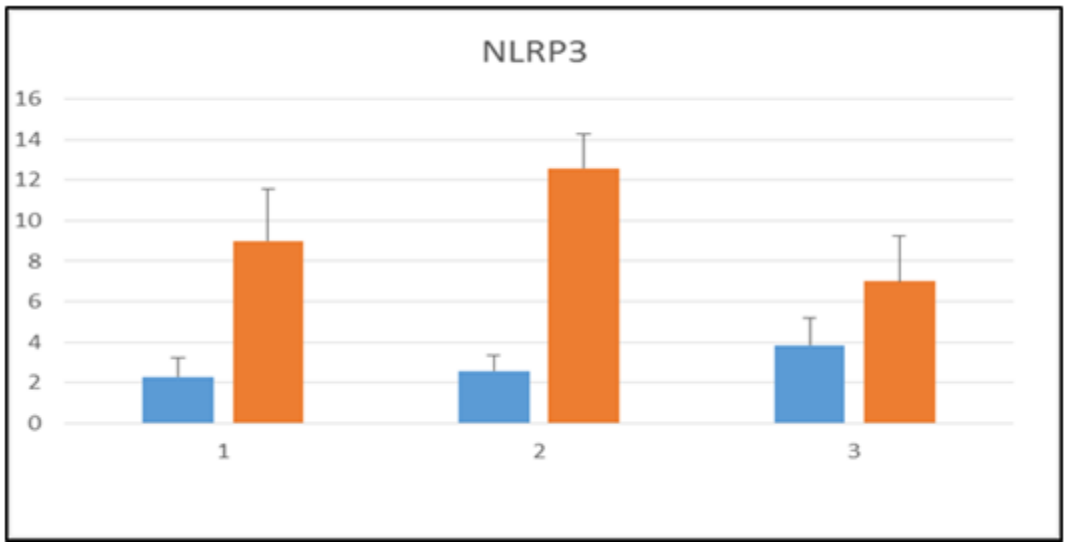

Fig. 1 : Mean and standard deviations of NLRP3 expression in the control group and HEMA treatment in the three experimental time groups (24, 48 and 72 hours).

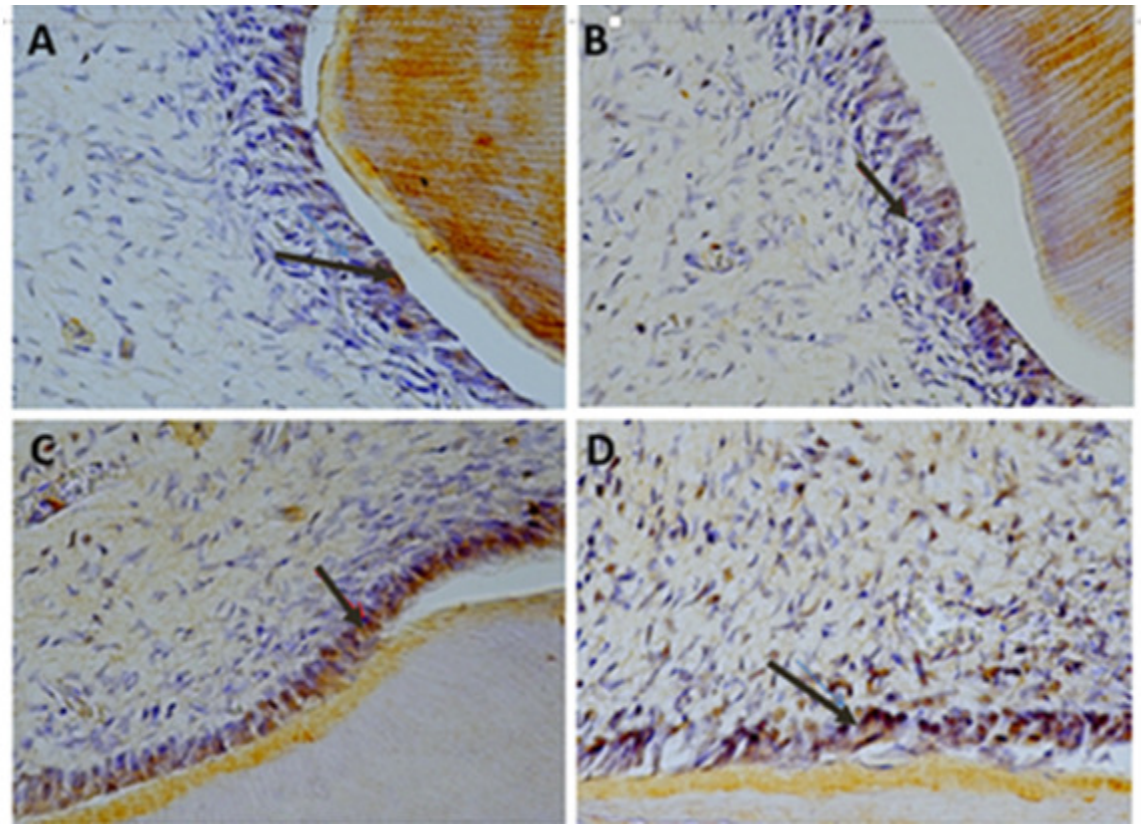

Fig. 2 : NLRP3 expression by immunohistochemical examination at 400x magnification. The Arrows show the cells that positively express NLRP3, brown and round shape. A. Control. B. 24 hours treatment. C. 48 hours treatment. D. 72 hours treatment.

significant difference ($p = 0.000$). Tukey HSD test was performed to find out the differences between treatment groups. The Tukey HSD test results between the experimental time groups 24, 48 and 72 hours showed a difference ($p < 0.05$) $p = 0.000$. In the HEMA group, NLRP3 expression from 24 to 48 hours was significantly increased number ($p < 0.05$) $p = 0.015$. The results from 48 to 72 hours showed significantly decreased number in NLRP3 expression ($p < 0.05$) $p = 0.006$. Based on the results, it can be concluded that the highest NLRP3 expression on odontoblast cells occurred at 48 hours of examination time. This proves that NLRP3 plays a role in activating the immune response due to exposure to HEMA resin.

DISCUSSION

HEMA exposure increased NLRP3 expression

compared to the control group. The significant decrease showed in the 72 hours group. The results showed the highest increase in NLRP3 expression at the 48 hours from the resin HEMA application time. The increase of ROS causing oxidative stress was obtained from exposure to HEMA resin to odontoblast cells, which are cells that were first exposed to pathogenic lesions by both microbes and sterile lesions. It showed the effort of the body to send the signals to eliminate the lesion in the dentin-pulp complex. NLRP3 is responsible for the non-microbial pathogen that makes way to the tissue by identifying and binding the necessary DAMP molecules in the cytoplasm. This danger signal caught, which in this case, comes from resin HEMA material. NLRP3 is an excellent receptor from the NLRs because it can identify the injury, both in microbes or non-microbes (Martinon *et al*, 2009).

ROS plays a role in activating NLRP3. The binding between ROS with the thioredoxin complex and the thioredoxin-interacting protein (TXNIP) is one of the pathways as a factor in activating NLRP3 by ROS due to oxidative stress (Zhou *et al*, 2010). Inflammasome NLRP3 is activated as a response to various signals that are harmful to the host such as tissue damage, metabolic stress, ROS abd infections of micro-organisms (Bostinci *et al*, 2011). NF-κβ transcription factors play a role in activating NLRP3. Induction of ROS in NFKβ signaling mediates an increase in NLRP3 and IL-1β transcription (Bauernfeiend *et al*, 2011). Concurrent activation of NLRP3, ASC and caspase-1 will trigger the release of mature IL-1β and generate an immune response (Huang *et al*, 2013).

Some authors also mentioned that ROS is one of the main factors in activating NLRP3 in the condition of the non-pathogenic sterile injury (Sun *et al*, 2013). Other authors also emphasized that NLRP3 is the most essential component in responding to sterile inflammation (inflammation caused by non-microbial lesions) (Rubartelli *et al*, 2011). The expression of NLRP3 does not only respond to bacterial invasion but also danger signal, which in this case, comes from toxin materials such as HEMA resin (Chen *et al*, 2011). The activation of NLRP3 does not only trigger the innate immune response to eliminate pathogens and cellular damage but can also trigger an adaptive immune response (Sutterwala *et al*, 2006; Wang *et al,* 2013).

CONCLUSION

This study showed a significant increase in NLRP3 expression in the first 48 hours from the resin HEMA and decrease in 72 hours of experimental time. It showed that NLRP3 inflammasome is activated as a response to various signals that are destructive to the host. Therefore, the activation of NLRP3 stimulates the activation of the immune response, not only the innate immune response to the exclusion of pathogens and cellular damage, but also the adaptive immune response to HEMA resin exposure in the dentine pulp complex.

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Conflict of interest

The authors declare no conflicts of interest.

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