Effect of pulp out® on caspase 3, and interleukin-1β expression in pulp teeth: A paste contained jatropha, sidaguri, and melittin

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Abstract

Objective: To examine the expression of caspase-3 and IL-1β following application of Pulp Out® on rabbit’s pulp teeth as well as histopathology.

Material and Methods: The study was conducted on the maxillary incisors rabbits. The teeth were prepared and Pulp Out® was inserted at the base of prepared cavity, then restored with RM-GIC. After 24 hours, the animals were then euthanized and processed for histopathology and immunohistochemistry evaluation.

Results: Our results indicated that Pulp Out®, when administered to the pulp teeth, increased the number and diameter of blood vessels. The expression of caspase-3 and IL-1β showed similarities to the expression of commercial devitalizing agent.

Conclusion: Our results revealed that Pulp Out® is a valuable devitalization agent that should be further explored for its safety before clinical application.

Keyword: Caspase-3, Dental pulp, Interleukin-1β, Pulp Out®


Introduction

Cell death is a fundamental process for maintaining homeostasis, removing unwanted cells or damaged cells, and ensuring the repetition of the cellular cycle in promoting growth and differentiation. Two different ways of cell death are commonly studied, apoptosis and necrosis that involves caspase as the proteolytic enzyme in controlling cell death and inflammation.1

Caspase is a cysteine-aspartic protease and has 15 types of caspses that been identified. Caspase-3 is the main executor in cell death and is one of the caspses that are able to activate other caspsases such as caspase 6 and caspase 7 that cause amplification of cell damage.2,3 Activated caspase-3 will trigger cell apoptosis. Cell apoptosis is programmed and controlled cell death, characterized by the activation of the caspase protein, which breaks down proteins to initiate and support apoptotic signals.4 Cells death may induce inflammation which the cells body release some proinflammatory cytokines that are intracellularly stored and released upon cellular disintegration.5,6

Interleukin-1 (IL-1) is a main proinflammatory cytokine that regulates inflammation by controlling a variety of innate immune processes. IL-1 is expressed in a wide range of tissues and a variety of cells in response to infection, toxins and other inflammatory stimuli. It also has a wide range of biological functions, including acting as a leukocytic pyrogen, an inducer of several components of the acute-phase response. IL-1 has been linked to inflammation and pain. IL-1β stimulated cyclooxygenase-2 (COX-2), a rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins (PGE) synthesis, a lipid mediator that contributes to inflammatory pain.7,8

Pulp devitalization agents have been practised for root canal treatment to devitalize the pulp. Paraformaldehyde is a devitalization agent commonly used that causes vasodilation and bleeding, resulting in loss of pulp vitality or cell death. Paraformaldehyde also causes very sharp pain when applied to the cavity.4 Studies have been conducted to find alternative materials for devitalization, including materials derived from natural plants widely available in Indonesia.

Previous studies have reported a combination sap of jatropha (Jatropha curcas) and root of sidaguri (Sida rhombifolia) as an alternative devitalizing agent.10 This article evaluate the effect of Pulp Out® (a paste contain sap of jatropha, root of sidaguri, and melittin) on the cavity caused porosities and decreased the microhardness of tooth structure (in publish). It might be assumed that Pulp Out® might cause cell death. However, its mechanisms are unknown. Therefore, this study was conducted...
to evaluate the expression of caspase-3 and IL-1β of rabbit’s pulp teeth following Pulp Out® application through immunohistochemical (IHC) studies as well as histopathological evaluation.

Material and Methods

Sample preparation

The roots of sidaguri were taken from Bone, South Sulawesi, Indonesia, washed, cut into small pieces. About 500 g was then dried using an oven at the temperature of 50±5°C to obtain a dry root, mashed and macerated with 96% ethanol for 3 days and stirred occasionally. The liquid extract was dried with a rotary evaporator (Buchi) to produce dried root extract (16.05 g). The dried extract was stored in a desiccator until used. The sap of jatropha was also collected from Bone, South Sulawesi, Indonesia, freeze-dried by lyophilization (Buchi), kept in a vacuum desiccator till used. While melittin was purchased from Sigma Aldrich (St Louis, MO, USA).

Paste formulation

The sap of jatropha, root of sidaguri and melittin were suspended in a combination of macrogol and propylene glycol in a ratio of 1:1.

Animal

The study was conducted on the maxillary incisors in rabbits of 2 – 4 months old with a weight of 2 - 3 kg. The animals were housed 24 hours before the research was conducted. The animals were injected intramuscularly in the thighs of the rabbits using ketamine by 10 mg/kg body weight (BW) (Troy Laboratories, Australia) for general anaesthesia. Hypersalivation was managed by applying atropine sulfate 0.05 mg/kg BW intramuscular (Ethica, Jakarta). The protocol was approved by the Ethics Commission of the Dental and Oral Hospital, Faculty of Dentistry, Hasanuddin University (No 0114/PL.09/KEPK FKG-RSGM/UNHAS/2018).

Experimental design

The teeth were prepared using the 0.3 mm round diamond bur (BR-49, Mani Inc., Japan) at high speed with water cooling on the labial surface until it reaches the pulp. The teeth were then isolated in the labial section and disinfected with povidone-iodine (Betadine, Mahakam Jakarta). About 5.0 mg Pulp Put® at 2 dosage (25% and 50%) was inserted at the base of the prepared cavity, then restored with RM-GIC. After 24 hours, the animals were euthanized using pentobarbital 100 mg/kg BW (Euthal, Jurox, Rutherford NSW), extracted, and fixed in a formalin solution (Merck, Germany).

Histopathological examination

The pulp teeth specimens from each rabbit were immediately preserved in 10% v/v formalin in normal saline and dehydrated using escalating formalin concentrations (70-100%). The paraffin-embedded organs were sectioned at a thickness of 5 mm. Following that, routine staining with haematoxylin and eosin (HE) was performed, including deparaffinization, hydration, staining, rinsing, and clearing in xylene. Slides were examined using a light microscope, and photomicrographs were obtained using a Leica DM750 Camera Microscope.

Immunohistochemistry

According to the manufacturer’s instructions, a standard IHC protocol using the DAKO Envision+ staining kit (DAKO, Carpinteria, CA) was used for IHC reactions. Following dewaxing and rehydration, pulp teeth were incubated in milk skim for 30 minutes. Caspase-3 and IL-1β primary antibodies (Finetest) were utilized at a 1:100 dilution with bovine serum albumin (DAKO). At room temperature, the primary and secondary antibodies were incubated for 60 minutes each. The intensity value of immune staining for caspase-3, and IL-1β in pulp teeth was evaluated using ImageJ.

Data represent means ± SE of 3 independent experiments. Statistical significance of differences was determined using the one-way ANOVA, Kruskal Wallis, and Mann Whitney test. Differences resulting in probability (p) values less than 0.05 were considered statistically significant.

Results

Histopathological Observation

The histopathological image of rabbit tooth pulp following Pulp Out® application can be seen in figure 1. Cell lysis depends on the dose given. The higher the dose given, the more blood vessels and the diameter of blood vessels.

From the observations, the treatment group showed a numerical increase in the number of blood vessels which was significantly difference compared to the control group. The higher the dose given, the more the number of blood vessels. Similarly, the diameter of blood vessels in each group also showed a significant difference compared to the control group (p<0.05) table 1. To find out whether there were differences in the number of blood vessels and the diameter of blood vessels between the two groups, further tests were used using LSD test and the Mann Whitney test.
In the treatment group, the number of blood vessels was significantly different from the control group. LSD statistical analysis showed significant differences in the number of blood vessels between the 50% Pulp Out® and the 25% Pulp Out® (p<0.05), as well as between the 50% Pulp Out® and the control group (p<0.05). However, no significant difference in the number of blood vessels was observed between the treatment group 25% Pulp Out® and the control group (p>0.05)

Table 2 Differences between the two treatment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Blood Vessels</th>
<th>Diameter of Blood Vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>p value</td>
</tr>
<tr>
<td>50% Pulp Out*</td>
<td>3.00</td>
<td>0.019*</td>
</tr>
<tr>
<td>25% Pulp Out*</td>
<td>1.67</td>
<td>0.128*</td>
</tr>
</tbody>
</table>

Notes: *LSD Test, **Mann Whitney Test

Immunohistochemical Findings
Due to extremely damage on dental pulp, this assay only evaluated at dose 25% Pulp Out®. The expression of caspase-3 and IL-1β was represent as red area. The large area represented an over-expression. Strong immunostaining of caspase-3 occurred diffusely in the dental pulp of the Pulp Out®, similar to commercial devitalizing agent figure 2A and figure 2B and relate to IL-1β figure 2C and figure 2D. However, an increasing intensity in caspase-3 and IL-1β also no significant in statistical analysis figure 3.

Discussion
Cell death is an important component of various biological processes. Cell death can occur in cells that are exposed to mechanical, thermal, chemical and bacteria stimulation. Cell death that occurs passively is marked by inflammation which is...
associated with changes in microvascular function and shape. Changes in microvascular function are associated with the number of blood vessels. In this study, the number of blood vessels in the 50% Pulp Out was higher than the 25% Pulp Out and the control group. An increase in the number of blood vessels could be associated with an inflammatory process that is closely related to angiogenesis. Angiogenesis is the growth of blood vessels in pathological conditions that will undergo regeneration when they are damaged in order to maintain their survival.

The presence of inflammation following application of Pulp Out was confirmed with the expression of IL-1β under immunohistochemistry evaluation. Higher red color of IL-1β expression was identified after application of 50% Pulp Out however its intensity was not significantly different compared to control. IL-1 has long been associated with inflammation and innate immunity however the family member of IL-1 has a wider role than inflammation. Together with IL-18 and IL-33, they play diverse roles in directing innate immunity and inflammation in response to microbial and environmental challenges. The contribution of the IL-1 system in neuroinflammation has been reported showing that IL-1 (and TNF-α) induces neuronal death directly or indirectly by activating glial production of neurotoxic substances. Previous study showed that application of extract of Jatropha sap caused lysis of dental pulp cells. This might relevant to the expression of IL-1β following Pulp Out application.

Changes in blood vessel diameter are associated with vascular changes. In this study, changes in the diameter of blood vessels in each group were also observed. In the 50% Pulp Out, the blood vessels were smaller than the 25% Pulp Out and the control group. This is related to the collapse of blood vessels, causing the diameter of blood vessels to shrink. The higher the dose given more lysis observed. This is in line with research conducted previously that cell death has been shown at a dose 50% Pulp Out following application of the combination of jatropha and sidaguri which is characterized by vascular lysis and the appearance of blood spots (hemorrhage). Blood vessels undergone lysis in a dose-dependent manner, and greater number of blood spots observed histopathologically.

Pulp Out is a combination of natural extracts consisting of jatropha sap, sidaguri and melittin, which is considered to fasten devitalization on pulp cells. Compared to chemical-based devitalization agents such as paraformaldehyde, necrosis occurs after some days of application. This might be explained that paraformaldehyde undergoes slow depolymerization and penetrates the pulp, causes irritation, and finally necrosis occurred. Jatropha contains alkaloid compounds that could disintegrate peptidoglycan in cells that cause cell death. Alkaloids in jatropha also called jatrophine could precipitate peptidoglycan in cells that cause cell death.

The morphological characteristics of cell necrosis are characterized by cell swelling, rupture of the plasma membrane, the disintegration of the cell membrane structure which causes loss of the cell’s ability to survive. A change in the cell nucleus by loss of cell integrity due to damage to the cell membrane is characterized by shrinking, dense, nuclei that have irregular boundaries and become very basophilic (dark in colour). Cells that die in necrosis will enlarge and then disintegrate and lysis in one area that is a response to inflammation. Cell death after the application of the Pulp Out was observed to be the same as the picture of cell death by necrosis marked by the presence of lysis and changes in the cell nucleus.

In this study, cell death can be observed in caspase-3 pathway death. Caspase-3 is an enzyme that acts as the main executor of apoptotic cell death. The expression of caspase-3 after the application of devitalization 50% Pulp Out showed that the colored area of the cell was not significantly different compared to control. It can be assumed that the cell death pathway of Pulp Out has similarities with the cell death pathway of devitalization material. This is coagulation necrosis and protein precipitation.

Caspase was identified by its capacity to activate the IL-1. It can be assumed that the cell death pathway of Pulp Out was mediated by inflammation. Apoptosis has been implicated in regulating IL-1 release via caspase-8-dependent pathways, however the release of IL-1 has implied in necrosis. As a result of cell death, cellular components are released into the external environment and are sensed by the body that are generally termed as damage-associated molecular patterns (DAMPs) that evoke inflammatory responses. Prostaglandin E2 (PGE2) is the most abundant eicosanoid lipid mediator in the inflammatory milieu. PGE2 is the main product of two cyclooxygenase, COX-1 and COX-2, of which COX-1 is constitutively expressed, while COX-2 is induced by various stimuli such as cytokines. Sidaguri root contains large amounts of steroids compared to other chemicals, which are considered capable of suppressing COX-2 which plays a role in the synthesis of PGE2 and could exert analgesic effects on Pulp Out materials. Whether this may cause less pain compared to commercial devitalizing agent should be further studied.
Under the limitation of this study, it can be concluded that expression of caspase-3 and IL-1β following application of Pulp Out® showed similarities as commercial devitalizing agent.

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Conflict of Interest
The authors Report no conflict of interest.

References