Effect of Extract and Caffeic Acid Phenethyl Ester on Apoptosis of Odontoblast Cells

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Abstract

Background: Propolis is a resinous hive product collected by bees from tree buds and mixed with secreted bee wax to both avoid bacterial contamination in the hive and seal it. It is known to have a wide spectrum of pharmacological properties, including anti-inflammatory, antioxidant, antibacterial, antiviral, and anti-fungal abilities. Apoptosis tests were performed on odontoblast cells in rats (*Rattus novegicus*) to assess the viability of propolis extract and caffeic acid phenetyl esters (CAPE) as alternative candidates for pulp capping agents in conservative dentistry treatment. **Objective:** To examine the apoptosis activity of propolis extract and CAPE as capping materials on odontoblast cells. **Methods:** This study was designed as a post-test only control group laboratory experiment. The rats were randomly divided into three groups. Pulp exposures were performed on the occlusal surface of the right maxillary first molars. In the first group, the control group, glass ionomer cement (GIC) was directly applied to the pulp exposure. In the second group, the sample group, propolis extract was applied to the pulp exposure, and in the third group, CAPE was applied to the pulp exposure. All cavities were then filled with GIC as a permanent filling. Animals were sacrificed on the first and fourteenth days. The direct counting method of histological examination was based on the apoptotic odontoblasts, using the terminal deoxyribonucleotidyl transferase dUTP nick end labeling (TUNEL) assay technique. **Result:** There was a greater number of apoptotic odontoblasts in the control group, followed by the CAPE group and, lastly, the propolis extract group. **Conclusion:** The apoptosis activity of the propolis extract is lower than that of CAPE.

Keywords*: odontoblast cell, apoptosis, propolis, caffeic acid phenetyl esters, herbal medicine*

Introduction

Homeostasis has an important role to play in the balance of bodily functions. It is, however, influenced by different regulatory mechanisms, such as osmoregulation, thermoregulation, and chemical regulation, all controlled by various cell systems to maintain body stability.[1] One of these mechanisms, moreover, can also occur in dental pulp tissue. If a cell experiences a stimulus or mild stress, it will then react to maintain a balanced state. Some of the cell reactions that have emerged include cell adaptation, reversible injury, irreversible injury, or cell death.[2]

A type of cell death that is commonly found is necrosis. Necrosis involves cellular edema, denaturation, protein coagulation, cellular organelle degradation, cell destruction, loss of the entire plasma membrane, and cytoplasmic disorganization. In general, necrosis involves a number of cells simultaneously.[2]

Apoptosis is defined as internally programmed cell death and has a role in a variety of physiological and pathological conditions. It is also considered as a complex, tightly regulated and active cellular process

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in which individual cells self-destruct without injuring neighboring cells but causing non-necrotic inflammatory reactions. Apoptotic cell death plays an important role in maintaining normal physiological functions, as well as being responsible for the body's disease process. A dysfunctional apoptotic system is involved in the pathogenesis of various diseases, including oral pathology.[4]

Cell necrosis subsequently induces an inflammatory reaction. Necrosis is generally caused by bacterial invasion and heavy tissue damage. Inflammatory mediators involved are histamine, bradykinin, serotonin, interleukins, and arachidonic acid metabolites (PGE2). This inflammatory response causes vasodilation and increased vascular permeability. The tissue becomes swollen due to serum protein infiltration (electrolytes) and fluid from blood vessels, causing necrosis.[5]

The process of apoptosis is of broad biological significance, and is involved in the development, differentiation, proliferation/homoeostasis, regulation, and function of all systems, as well as in removing damaged or dangerous cells.[4] Apoptosis is a very well-programmed, active process, and is characterized by morphological and biochemical changes. In the early stages of apoptosis, chromatin is broken but the cell membrane is still intact (karyorrhexis). In the final stage of apoptosis, the membrane protrudes, the ultrastructural modification of cytoplasmic organelles occurs, and the integrity of the membrane is lost. Phagocytic cells, such as epithelial cells, macrophages, and fibroblasts will usually eat apoptotic cells before the apoptotic body is formed. Apoptotic cells that are not phagocytes, as in a laboratory-produced cell culture, will experience a degradation similar to necrosis, which is called secondary necrosis.[6] Necrosis and apoptosis are different types of cell death. The apoptotic process does not induce an inflammatory reaction, but it is induced by a previous inflammatory reaction.[7]

Propolis is often used in the dentistry and medical fields and is a non-toxic, natural, resinous substance that has antimicrobial, anticancer, anti-fungal, antiviral, and anti-inflammatory properties. The word "propolis" is derived from the Greek words "pro" (meaning outer wall) and "polis" (meaning city), reflecting the protective properties of the substance. Propolis is one of the natural substances created by bees to build and maintain their hives. It kills pathogens and protects the beehives from rain, and because of its adhesive properties, it can prevent external objects passing into the hive.[8]

Propolis is known to contain resins and bioactive materials that include bioflavonoids, artepillin, apigenin, and caffeic acid phenetyl esters (CAPE), which have a role in controlling inflammatory, antioxidant, antibacterial, and antiviral reactions; managing immunomodulators; and stimulating tissue healing. This is consistent with a study conducted by Bankova in 2009, which stated that CAPE can inhibit activation of Nuclear Factor κB (NFκB) so that Tumor Necrosis Factor-α (TNFα) secretion will also be inhibited.[9]

CAPE's anti-inflammatory activity mode inhibits the release of arachidonic acid from cell membranes, then inhibits COX-1 and COX-2 activity and suppresses the activation of genes responsible for COX-2 expression. In inflammation induced by carrageenan, CAPE suppresses the volume of exudate and the relocation of leukocytes.[10] Propolis displays anti-inflammatory activity that can increase the body's immune system. The benefit of propolis in oral health is as an anti-inflammatory agent since it contains CAPE. The CAPE in propolis has various characteristics, one of which is to act as an anti-inflammatory agent. This agent passes easily into cells to inhibit LOX and COX (both involved in metabolic pathways), inhibit the release of inflammatory cytokines, and simultaneously increase the production of anti-inflammatory cytokines, such as IL-10 and IL -4. CAPE can also reduce the infiltration of neutrophil and monocyte inflammatory cells.[11]

CAPE is the main active component of propolis, which is a soft and sticky lipophilic resin collected from beehives, and is collected by honeybees from various plants. Flavonoids and caffeic acid (CA) have antimicrobial, anticancer, anti-fungal, antiviral, and anti-inflammatory properties that have received special attention in medicine and dentistry.[8]

CAPE rapidly developed in dentistry. In vitro and in vivo studies have previously mentioned that propolis has a strong anti-inflammatory effect as a pulp-capping material,[8] and is quite effective in the formation of dentin.[12] Although the use of CAPE as a pulp-capping material is still controversial and requires further

research, it is known that it can reduce inflammation and bacterial infection and stimulate the formation of dentine in pulp degeneration.[12] In another study, it is even stated that the level of CAPE in certain doses can be cytotoxic to cell survival. This cytotoxic effect has been known to cause cell apoptosis.[13]

Moreover, the flavonoids and cinnamic acid derivatives contained in propolis, such as acacetin, quercetin, naringenin, CAPE, and CA, are able to inhibit silica, which induces reactive oxygen species (ROS), and then bind them to induce the release of arachidonic acid, CAPE, and CA. As a result, the release of arachidonic acid, the production of PGE2, and the release of histamine can be induced.[14]

Propolis, as an anti-inflammatory agent, is also able to block lipoxygenase and cyclooxygenase. Lipoxygenase is the main neutrophil enzyme producing leukotriene compounds, while cyclooxygenase produces prostaglandins, which will be mediators in inflammatory reactions. The existence of these obstacles then decreases the production of leukotrienes and affects the activity of neutrophil phagocytosis, so that the inflammatory process is suppressed. Following on from this, the inhibition of lipoxygenase and cyclooxygenase pathways by CAPE will reduce blood vessel vasodilation and blood flow, so that the migration of leukocytes (PMN) to the inflammatory area will also be decreased.[15]

Therefore, this study aims to reveal the effects of propolis extract and CAPE on the apoptosis of Wistar rat odontoblast cells. The apoptosis of odontoblast cells was analyzed after the administration of propolis extract and CAPE.

Materials and Method

The design of this research was experimental and was approved by the Faculty of Dental Medicine, Universitas Airlangga ethics committee (No. 14/KKEPK.FKG/II/2015). This laboratory research study used 42 male Wistar rats weighing 200–250 grams and aged 8–16 weeks as research samples. It was necessary for these rats to have fully grown molar teeth and healthy physiques. Once selected, the samples experienced adaptation for two weeks, and were then divided into six groups. Group (I) was given pulp roof perforation treatment with glass ionomer cement (GIC), and then was observed on day 1 (control I). Group (II) was given the same treatment as Group (I) but observed on day 14 (control II). Group (III) was given pulp roof perforation treatment with propolis extract, and then was observed on day 1. Group (IV) was given the same treatment as Group (III) but observed on day 14. Group (V) was given pulp roof perforation treatment with CAPE, and then was observed on day 1. Group (VI) was given the same treatment as Group (V) but observed on day 14. In groups (III, IV, V, VI) 0.5 mg of each ingredient was applied and then covered using GIC.

The samples were subsequently euthanized ready for tissue sampling followed by decapitation and maxilla separation. The molar teeth were cut, using microtomes, in a parallel series with a long tooth axis of seven microns. The preparations were then stained with hemotoxin and eosin. Finally, the data were analyzed using the Kolmogorov-Smirnov test to determine the distribution of data. As it was known that the data were normally distributed, a homogeneity test was performed using the Levene test. The independent t-test was then performed to find out whether there was an average difference between the two unrelated sample groups.

Result

A histological examination, counting apoptotic cells, was carried out using a 1000x magnification microscope. The following results were observed (Figure 1). The Mean and standard deviation of apoptotic cells on day 1 and 14 can be seen in Table 1.

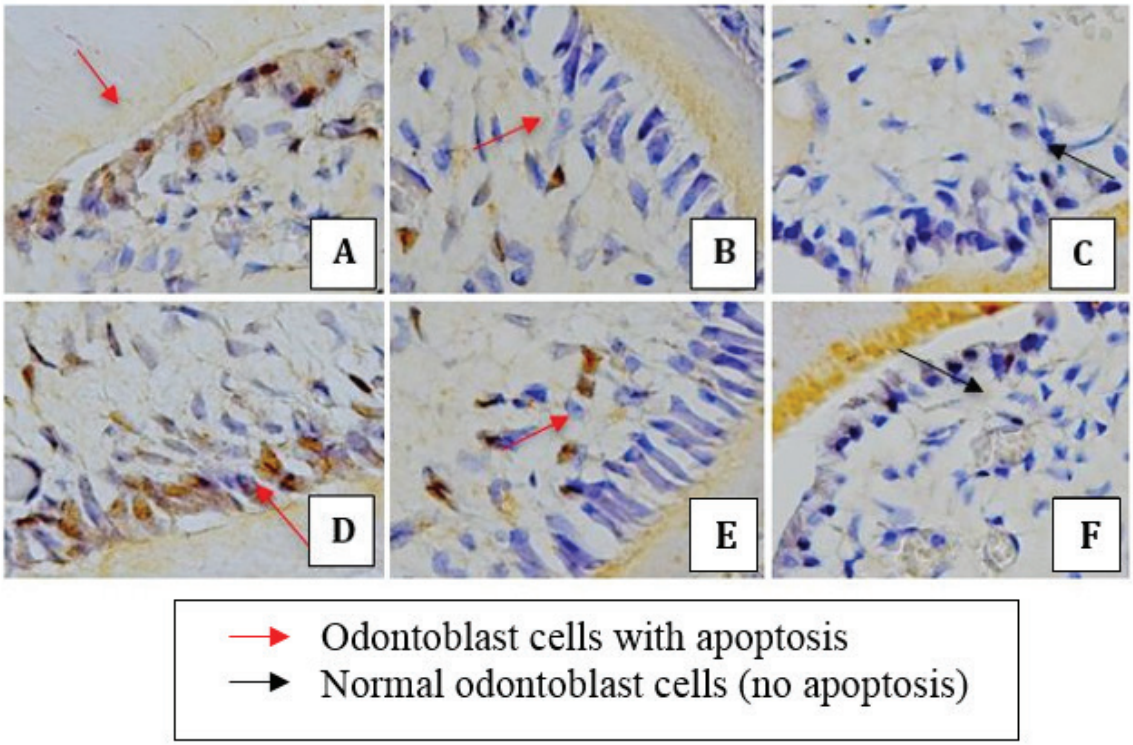

Figure 1. A. Odontoblast cells with GIC day 1, B. Odontoblast cells with propolis extract day 1, C. Odontoblast cells with CAPE day 1, D. Odontoblast cells with GIC day 14, E. Odontoblast cells with propolis extract day 14, F. Odontoblast cells with CAPE day 14.

Data analysis for apoptosis day 1

A normality test was performed for the three research groups to determine whether the data obtained was of a normal distribution. This normality test also determined the appropriate statistical test to be used in the research. A one-sample Kolmogorov–Smirnov test was used for the normality test and a result of $p > 0.05$ means that the data are normally distributed (Table 2).

Table 1. One Sample Kolmogorov - Smirnov Test

Substance	p
Control	0.73
Propolis Extract	0.93
CAPE	0.73

A Levene's homogeneity test was then performed. The Levene's test result of $p > 0.05$ means that the data are homogenous.

Following this, a one-way ANOVA test was performed to determine whether there were significant differences between groups. The test result of $p > 0.05$ means that there are significant differences between the three groups.

The analysis continued with a post hoc test for finding out the details of the differences in each group; the test used was Tukey's honestly significant difference (HSD) test (table 3).

Table 2. Turkey HSD test for apoptosis day 1

	Control (GIC)	Propolis Extract	CAPE
Control (GIC)	-	0.000*	0.000*
Propolis Extract		-	0.000*
CAPE			-

*: There were significant differences with $p < 0.05$

Data analysis for apoptosis day 14

A normality test was performed for the three research groups to determine whether the data obtained was of a normal distribution. This normality test also determined the appropriate statistical test to be used in the research. A one-sample Kolmogorov–Smirnov test was used for the normality test and a result of $p > 0.05$ means that the data are normally distributed (Table 4).

Table 3. One Sample Kolmogrov – Smirnov test

Substance	p
Control	0.71
Propolis Extract	0.74
CAPE	0.98

A Levene's homogeneity test was then performed. The Levene's test result of $p > 0.05$ means that the data are homogenous.

Following this, a one-way ANOVA test was performed to determine whether there were significant differences between groups. The test result of $p > 0.05$ means that there are significant differences between the three groups.

The analysis continued with a post hoc test for finding out the details of the differences in each group; the test used was Tukey's HSD test. The test result of $p < 0.05$ reveals that there are significant differences in the number of apoptotic cells between the groups (Table 5-8).

Table 4. Turkey HSD test for apoptosis day 14

	Control (GIC)	Propolis Extract	CAPE
Control (GIC)	-	0.000*	0.000*
Propolis Extract		-	0.000*
CAPE			-

*: There was significant differences with $p < 0.05$

Data analysis result for apoptosis in control group

Table 5. Significant difference test result between groups

Group	Significant difference test
Control day 1	p= 0.00*
Control day 14	
Propolis extract day 1	p= 0.006*
Propolis extract day 14	
CAPE day 1	p= 0.006*
CAPE day 14	

*p < 0.05: There was significant differences

Discussion

Oral cavities experience a variety of environmental conditions, and materials used in this environment must therefore be able to withstand those conditions. The safety of materials used in the oral cavity is very important, and other properties required for dental materials, such as not irritating the oral cavity and surrounding tissue and being non-allergic, non-carcinogenic, and nontoxic, must also be fulfilled.[16]

Toxicity testing is a part of dental material evaluation, and toxicity information is required for standard screening procedures. Hence, this study aims to determine the potential toxicity of CAPE and propolis extract against pulp cells, based on the number of cell deaths during the application of the pulp-capping material.

Moreover, apoptosis is a very well-regulated active process, which is characterized by morphological and biochemical changes. Apoptosis stimulated by physiological and pathological conditions plays an important role in maintaining normal homeostasis and the pathogenesis of several diseases.[6]

The initial stage of apoptosis is characterized by the expression of Phosphatidylserine (PS) passing through the inner layer into the outer layer of cell membranes. Apoptotic bodies formed at the end of apoptosis then trigger these dead cells to be recognized by macrophages without the release of cellular pro-inflammatory components.[6]

The selection of apoptotic identification method, nevertheless, depends on several factors, including experiment type, cell type, and experience. TUNEL testing is one of the apoptosis detection methods involving DNA fragmentation examination. Apoptosis examination with the TUNEL method can provide an overview of the apoptosis process at the single cell level, so that it is more specific and highly accurate.[17]

The results of apoptosis analysis on the first day using Tukey HSD showed that there were significant differences between the groups. The control group, with GIC, had the highest level of apoptosis, compared with the propolis extract and CAPE groups. Similarly, on the fourteenth day, the control group had the highest level of apoptosis compared with the propolis extract and CAPE groups. The high level of apoptosis in the control group is mostly due to the physical properties of soluble GIC. The level of fluoride contained in GIC can inhibit cell growth and proliferation and disrupt mitochondrial activity and protein synthesis, causing apoptosis in odontoblasts.[18]

Moreover, the results also revealed that the level of apoptosis on the first day in the CAPE group was higher than that in the propolis extract group. Similarly, the level of apoptosis on the fourteenth day in the CAPE group was higher than that in the propolis extract group.

Propolis is known to contain resins and bioactive

materials that include bioflavonoids, artepillin, apigenin, and CAPE, which have a role in controlling inflammatory, antioxidant, antibacterial, and antiviral reactions, managing immunomodulators, and stimulating tissue healing.[9] In this study, odontoblast cell death probably occurred due to the presence of phenol contained in CAPE. CAPE forms hydroxyl ions, which can change the transportation of organic material and nutrients that have toxic effects on cells. Free radicals have high reactivity since they tend to attract electrons, and can convert molecules into radicals, due to the loss or increase of one electron in another molecule.[19] Strong antioxidant activity correlates with high concentrations of CA and phenethyl caffeate.[14]

Cells have natural antioxidants, such as superoxide dismutase (SOD), catalase, reductase, glutathione peroxidase, and other antioxidants, that can protect against the influence of free radicals. However, when the number of free radicals is higher than the level of natural antioxidants the defense capability of cells can be disrupted, so that the normal reduction chain will be broken, and oxidative tissue damage will occur.[20]

The toxic effects of propolis extract can cause changes in the permeability of odontoblast cell membranes that will activate proapoptotic proteins in the cytosol (intracellular fluid). The activation of proapoptotic proteins will then induce the opening of mitochondrial permeability transition pores (MPTPs). The release of cytochrome c is related to the opening of MPTP, which will cause mitochondrial swelling, damage to the outer membrane, and trigger the release of inter-membrane proteins, including cytochrome c in some cells.[21]

Mitochondria contain proapoptotic factors, such as cytochrome c and apoptotic inducing factor. Both are dangerous substrates but can be stored safely in the mitochondria. When both are released into the cytoplasm, this protein can activate the caspase activation pathway. Cytochrome c acts as a water-soluble electron carrier in mitochondrial oxidative phosphorylation. Therefore, when an electron coil occurs via cytochrome c oxidase or complex IV, the change in ionic strength will trigger a wave matrix. Once the inner membrane of the mitochondria has achieved a wider surface permeability than the outer membrane, the wave matrix will cause the permeability pores inside the nonspecific membrane to open, so that cytochrome c passes through the cytoplasm. The cytochrome c that emerges from the cytoplasm then binds to Apaf1 to form a caspase recruitment domain (CARD). Several CARDs are bound into one to form an apoptosome complex, and then bind with pro-caspase-9 to become caspase-9 (caspase initiator). After that, the caspase-9 will activate procaspase-3 into caspase-3, an effector caspase stimulating apoptosis.[22]

Apoptosis of odontoblast cells after propolis extract application is of a lower level than following CAPE application. However, further research needs to be conducted to determine the physical properties of other propolis extracts to be used for pulp-capping treatment.

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