Effect of Combination of Red Pine and $Ca(OH)_2$ as Root Canal Sealer on Fibroblast Cell Apoptosis Through Caspase-3 Expression

Tamara Yuanita[1]*, Sylvia Paulina Panggono[2], Devi Eka Juniarti[1], Setyabudi[1], Dian Agustin Wahjuningrum[1], Iin Indah Aris Wati[2], Adinda Fazzahra Salma[3], Michele Callea[4,5], Niraj Kinariwala[6]

1.Department of Conservative Dentistry and Endodontic, Faculty of Dental Medicine, Airlangga University, Surabaya, Indonesia.
2.Postgraduate Student of Conservative Dentistry, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.
3.Student of Dentistry, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.
4.Meyer Children's Hospital IRCCS, Pediatric Dentistry and Special Dental Care Unit, Florence, Italy.
5.Adjunct Professor of Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.
6.Department of Endodontics, Faculty of Dentistry. Karnavati University, Gujarat, India.

Abstract

Calcium hydroxide is one of the sealers used in root canal treatment, as it qualifies obturation, but hiroxl ions released into free radicals can damage cells. The content of flavonoids, tannins, and phenols in Red pine is a high anti-acidant to ward off free radicals.

This study is expected to show the effect of a combination of red pine and Ca(OH)2 as a root canal sealer on fibroblast cells through caspase-3 expression, so that red pine can be considered as an alternative root canal sealer material. 27 wistar rats were divided into 3 groups. The control group was not treated, the kalsium hidroksidae group and the red pine group Ca(OH)2. Each group in the back area was given material and on the 3rd day an incision was made ± 1cm then a slide was made and stained with HE and IHC.

There were significant differences between each group, caspase-3 expression was lower in the red pine ca(OH)2 group than the kalsium hidroksidae and control groups.

The combination of Red pine and Ca(OH)2 as a root canal sealer may decrease caspase-3 expression in fibroblast cells, low Caspase-3 as an indicator of no cell apoptosis.

Experimental article (J Int Dent Med Res 2023; 16(4): 1483-1488)

Keywords: Red pine, Caspase-3, sealer, apoptosis, fibroblast cells, Immunology.

Received date: 08 November 2023 **Accept date:** 10 December 2023

Introduction

Caries is still a global problem, including in Indonesia. Basic Health Research conducted by the Ministry of Health of the Republic of Indonesia in 2018, there was an increase in the prevalence of active caries in the Indonesian population in 2013, from 53.2% to 57.6%. From that number, if the results of Riskesdas show a prevalence of 57.6% experiencing active caries, namely caries that has not been treated or has not been treated, then in Indonesia there are 98,134,670 people who suffer from active caries.[1] Teeth with necrosis require root canal treatment, which aims to: to clean the pulp chamber from infected pulp tissue, then form a root canal for obturation to form an apical seal.[2] According to Gopikrishna and Chandra[3] 65% of endodontic treatment failures are caused by poor obturation quality, while the success of obturation is influenced by the obturation material and obturation technique used.[4] Calcium hydroxide is one of the sealers commonly used in endodontic treatment, because it has antibacterial properties, and is able to reduce tissue inflammation and can stimulate the formation of hard tissue because it can release calcium ions (Ca^{2+}).[5]

However, in a study conducted by Saziye *et al*[6] , root canal treatment using a calcium hydroxide based sealer failed in 4 teeth (7.7%) due to periapical lesions and pathological resorption. It has been suggested that long-term use of calcium hydroxide may have an adverse effect on physical property of dentin, making it more susceptible to root fracture. In many *in vitro* studies, it has been reported that use of calcium hydroxide dressing for 5 weeks or more,

Corresponding author:
Tamara Yuanita,
Faculty of Dental Medicine Universitas Airlangga
St. Mayjen Prof. Dr. Moestopo No. 47 Surabaya, Indonesia.
E-mail: tamara-y@fkg.unair.ac.id

causes a decrease in root fracture resistance.[7]

The graph in Endodontics[8] reveals some of the shortcomings of Ca(OH)2, namely that it dissolves and leaves an empty space, causing microleakage. -cementogenic activity but there is no further research on this.[8] The hydroxyl ion OH- in the released calcium hydroxide is a high free radical oxidant and shows strong reactivity to cells. The effect of this highly reactive hydroxyl ion is that it combines rapidly with the cell's cytoplasmic membrane. Damage to the cytoplasmic membrane will affect the organic structure contained in the cell membrane, namely proteins that cause protein denaturation. Hydroxyl ions when in contact with DNA will cause replication inhibition resulting in necrosis and the number of lysed cells will increase or this is known as cell death (apoptosis).[9]

Apoptotic events are cell death events, one of which is triggered by DNA damage that fails to repair, infection, cell shrinkage and radiation exposure. Apoptosis has several pathways, extrinsic, intrinsic, and granzyme pathways. The intrinsic pathway generally involves the mitochondria. Apoptosis via the intrinsic pathway is initiated by the release of signals from the mitochondria in cells. Changes in the oxidative phosphorylation of the mitochondrial intermolecular cause the channel to open, the release of cytochrome C activates the initiator Caspase-9 and then Caspase-3. Caspase-3 initiator to enhance apoptosis. Caspase-3 is a marker of intrinsic pathway apoptosis.[10]

Nowadays, the use of traditional plants as alternative medicine is growing rapidly. Red pine extract has a high antioxidant content. Red pine as an alternative medicine reduces the detrimental effects caused by calcium hydroxide because Red pine contains phenol and flavonoid components ranging from 5.7%.[11] Phenols and flavonoids are known to induce the activation of the proapoptotic protein of the Bcl-2 family, the Bax protein. The results of a recent study showed that administration of red pine extract to cells causing DNA damage reduced the expression of BAX protein by eliminating ROS in cells, inhibiting cell damage by increasing the expression of the antiapoptotic Bcl-2. The study also stated that Bcl-2, which is known as an antiapoptotic marker, showed significantly high values in cholesterol-induced rat experiments and red pine.[12] Red pine (Pinus Densiflora) is a type of pine that grows in mountainous areas of Japan, Korea and Japan. China.[13] The content of -pinene, -pinene and camphene is one of the bioactive substances that protect against apoptotic factors and antioxidant action that inhibits apoptosis. The mechanism of -pinene, -pinene, and camphene in red pine as antioxidants is carried out by suppressing p53 protein expression resulting in an increase in Bcl-2 followed by a decrease in Caspase-3 which is an antiapoptotic.[12] Caspase-3 is an important initiator of intrinsic and extrinsic apoptosis pathways. It is commonly found in cells in an inactive state called procaspase and when activated can trigger apoptotic signals followed by cell death.[10] A study on wistar strains for apoptotic index and Caspase-3 expression was carried out on day 3 after application of the active ingredient. On the 3rd day got Inflammatory phase moderate to severe inflammation, PMN cells, leukocytes, hyperemia of blood vessels appear at that time.[14]

Based on the description above, it is necessary to conduct research on apoptosis and Caspase-3 expression in fibroblast cells after administration of a combination of Red pine and $Ca(OH)_2$ as a root canal sealer.

Materials and methods

Research Samples

The number of samples in this study were 27 males Rattus novergicus aged 2-3 months with a body weight of 200-300 grams.

Research Methods

The samples were divided into 3 groups, each group consisted of 9 rats. Red pine extract concentration 0.78%. Group 1 (control group) consisted of 9 rats. The dorsal skin area was treated in an incision of ± 1cm then the subcutaneous area of the rat was sutured with 4/0 silk thread as much as 2 sutures and closed with hipafix. Group 2 (calcium hydroxide group) consisted of 9 rats. The dorsal skin area was treated in an incision of ± 1cm then inserted calcium hydroxide and then sutured with 4/0 silk thread as much as 2 stitches and closed with hipafix. Group 3 (calcium hydroxide and red pine group) consisted of 9 rats. The dorsal skin area was treated in an incision of ± 1 cm then inserted a combination of calcium hydroxide and red pine sealer and then sewn with 4/0 silk thread as much as 2 stitches and closed with hipafix.

The tools used were first disinfected with 95% alcohol. All mice were anesthetized with 100 mg of ketamine (Ketalar, Wamer Lambert, Ireland) (65 mg/kg body weight) and xylazine HCL (Rompun, Bayer, Leverkusen, Germany) dissolved in sterile phosphate buffered saline (PBS). The rats were fixed and then the dorsal skin area was shaved and disinfected with iodine in 5% alcohol. An incision was made in the dorsal skin area with a length of 15 mm using a scalpel. The combination sealer of red pine and calcium hydroxide is mixed according to the 2:1 rule. Insert the sealer combination of red pine and calcium hydroxide into the subcutaneously which has been carefully incised to a depth of 1.5 mm. The wound area was sutured with a single resorbable suture (silk suture 4/0) as many as 3 sutures were disinfected with povidone-iodine and closed with hipafix given antibiotics amoxicillin 0.03 cc and dexamethasone 0.03 cc by injection. Experimental animals were put into disinfected cages given the antibiotic drug amoxicillin 25 mg twice a day, dexamethasone dr 5 mg vitamin b com and eat and drink as usual. Experimental animals were kept for 3 days and then euthanized rats for later histochemical and immunohistochemical testing of tissues that received treatment.[15]

Mice from each treatment group were terminated on the 3rd day after treatment. The treated rats were turned over and injected with anesthesia using ketamine HCl 50 mg/Kg BW and xylazine HCl 10 mg/Kg BW intramuscularly in the caudal extremity of the femoris region. Caspase-3 expression is the number of nerve cells that react positively to anti-Caspase-3 monoclonal antibody which was measured quantitatively by immunohistochemical techniques from specimens under a microscope with 400 times magnification in 10 times the field of view. Apoptosis is the number of fibroblast cells that undergo DNA fragmentation, cell shrinkage, and membrane blisters under microscopic observation, seen and counted under a microscope with 400 times magnification in 4 times the field of view.

To determine the distribution of data used Kolmogorrovsmirnow test. If the data is normally distributed, then Lavene's test is carried out for homogeneity. To find out the differences between groups, the ANNOVA test with a significance level of 0.01 was used, followed by the Tukey HSD test.

Results

From the results of research on the effect of the combination of red pine and Ca(OH)2 as a root canal sealer on apoptosis of fibroblast cells through Caspase-3 expression, the data obtained are as follows:

No	Treatment group	N	*X*	SD
1	Control	9	14	0,3163
2	Calcium hydroxide	9	11	0,2346
3	*Red Pine* and $Ca(OH)_2$	9	6,4	0,1590

Table 1. The mean (X), standard deviation (Sd) and sample size (N) of Caspase-3 expression.

Table 1 above shows that the sample size for each group is 9. The largest average Caspase-3 expression is the control group (-), while the smallest Caspase-3 expression average is the Red Pine and $Ca(OH)_2$ group.

No	Treatment group	Uji Kolmogorov Smirnov test	Levene test
1	Control	P = 0,127	P = 057
2	Calcium hydroxide	P = 0,200	
3	*Red Pine* and $Ca(OH)_2$	P = 0,200	

Table 2. The results of the Kolomogorov Smirnov test and the Levene test for Caspase-3 expression.

Table 2 above can be seen that all treatment groups have a p value > 0.05 in the Kolomogorov Smirnov test. This shows that all treatment groups have normal data distribution. From the results of the Levene test, p value > 0.05. This shows that the treatment group has homogeneous variances.

	Control	Calcium hydroxide	*Red Pine* and $Ca(OH)_2$
Control	-	p = 0,004	p = 0,001
Calcium hydroxide	-	-	p = 0,001
Red Pine and $Ca(OH)_2$	-	-	-

Table 3. Tukey HSD test results for Caspase-3.

Table 3 above can be seen that Caspase-3 expression between all treatment groups has a p value <0.05. This indicates that there is a significant difference in Caspase-3 expression between all treatment groups.

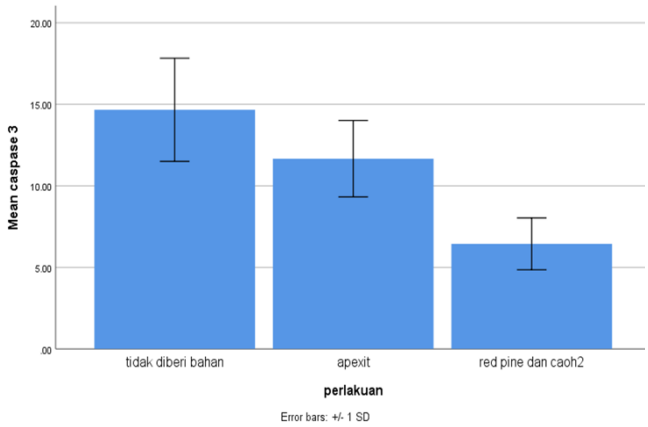

Figure 1. Caspase-3 expression measurement results.

No	Control	N	X	SD
1	Control	9	12	0,2872
2	Calcium hydroxide	9	10	0,1855
3	*Red Pine* and $Ca(OH)_2$	9	5,1	0,2260

Table 4. The mean (X), standard deviation (Sd) and sample size (N) of apoptosis.

From table 4 above, it is known that the sample size for each group is 9. The smallest apoptosis average is the Calcium hydroxide group, while the largest apoptosis average is the control group.

No	Treatment group	Kolmogorov test Smirnov test	Test Levene test
1	Control	P = 0,17	P = 0,245
2	Calcium hydroxide	P = 0,200	
3	*Red Pine* and $Ca(OH)_2$	P = 0,200	

Table 5. The results of the Kolomogorov Smirnov test and the Levene test for apoptosis.

From table 5 above, it can be seen that all treatment groups have a p value > 0.05 in the Kolomogorov Smirnov test. This shows that all treatment groups have normal data distribution. From the results of the Levene test, p value > 0.05. This shows that the treatment group has homogeneous variance.

	No ingredients	Added Calcium hydroxide	Added Red Pine and Ca(OH)2
Control	-	P = 0,002	p = 0,001
Calcium hydroxide	-	-	p = 0,001
Red Pine and $Ca(OH)_2$	-	-	-

Table 6. Tukey HSD apoptotic test results.

*Tukey HSD test is a post hoc test commonly used to assess the significance of differences between pairs of group means.

From table 6 above, it can be seen that apoptosis between all treatment groups has a p value <0.05. This indicates that there is a significant difference in apoptosis between all treatment groups.

Immunohistochemical detection (IHC) can be seen in Figure 2-4. Caspase-3 expression in fibroblast cells in the control group was seen to be high, Caspase-3 expression in fibroblast cells in the Calcium hydroxide group was seen less than the control group and Caspase-3 expression in fibroblast cells in the Red pine and Ca(OH)2 group was seen less than the Calcium hydroxide group and less than the control group.

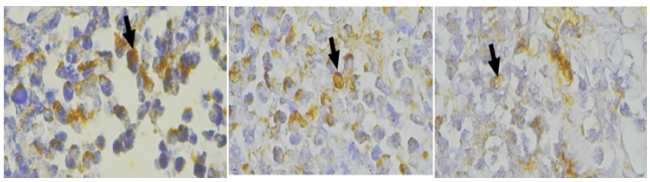

Figure 2. Caspase-3 expression in fibroblast cells in the control group, 3. Caspase-3 expression in fibroblast cells in the Calcium hydroxide group, 4. Caspase-3 expression in fibroblast cells in the Red pine and Ca(OH)2 groups.

Discussion

The cause of the decrease in Caspase-3 expression in this study is through the mechanism of the intrinsic pathway caused by low oxidation and phosphorylation in

mitochondria so that there is no ionic change that causes matrix swelling. The matrix that is maintained on the outside of the cell is not damaged, so that the mitochondrial canal is closed, there is no release of cytochrome c from the mitochondrial intermembrane. Cytochrome c is a heme protein that acts as an electron carrier in mitochondrial oxidative phosphorylation. Apaf-1 as a cytoplasmic protein does not activate the Caspase-9 initiator in the cytoplasm. Caspase-9 will not activate procaspase-3 to Caspase-3 which is an effector caspase carrying out apoptosis.[16]

In the apoptotic mechanism, the extrinsic pathway decreases Caspase-3 expression due to a decrease in TNF-ᵅ expression. The decreased TNF- then stops the extrinsic pathway of apoptosis by inactivating caspase-8 as an initiator caspase through the activation of the Tumor Necrosis Factors receptor (TNF-R). When Caspase-8 is not initiated, Caspase-3 is inactive so that there is no DNA fragmentation and no apoptosis process occurs.[10]

The reason for the decrease in Caspase-3 expression in this study was the combination of Red pine and calcium hydroxide made the chemical reaction between calcium hydroxide and Red pine produce phenolic salts that were beneficial for cells because they could lower pH and irritation. The decrease in Caspase-3 expression in the P3 group, namely the Red pine and Ca(OH)2 groups, is due to the process of oxidation and phosphorylation in cells producing hydroxyl radicals (OH-) which can damage the permeability of fibroblast cells. The content of flavonoids, phenols, camphene and tannins in Red pine has a strong antioxidant effect as a donor for electorn donors. The unpaired electrons are known as reactive oxygen species (ROS). The flavonoid content in red pine can induce the activation of antiapoptotic proteins, namely Bcl-2 and Bcl-xl, these conditions indicate a reduction in BAX protein expression by eliminating ROS in cells, inhibiting cell damage by increasing antiapoptotic Bcl-2. Phenols and flavonoids showed significantly high Bcl-2 values. The ROS in the cell is low so that, cell damage is inhibited by increasing the expression of the antiapoptotic Bcl-2. This is in line with research conducted.[12]

The cause of the decreased expression of caspase-3 is the activity of phenol as an antioxidant associated with the ability to donate hydrogen ions or electrons to DPPH to ward off free radicals. Red pine essential oil contains flavonoids, phenols, -oinene, -pinene, camphene and tannins, which are strong antioxidants that are associated with increased activity of Bcl-2 antiapoptotic group binding to Bax protein to form oligomers on the mitochondrial surface as a channel for the release of cytochrome C into the mitochondria. cytosol. Cytochrome C released through the mitochondrial membrane is an activator of apoptosome formation that plays a role in Caspase-9 activity. The activated caspase-9 forms a holoenzyme that functions to activate the executable caspase, namely Caspase 3 which decreases so that it does not induce endonuclease and then decreases apoptosis. This is in line with the research presented.[14]

The decrease in Caspase-3 expression in this study was due to the balance of Caspase work that was maintained in the presence of Caspase inhibition from the apoptosis inhibitor family (IAPs) which could inhibit the initiator and executor Caspase through several different processes.[17]

The cause of decreased apoptosis of fibroblast cells in this study was the content of -pinene, -pinene and camphene, which are one of the bioactive substances that protect against apoptotic factors and antioxidant action that inhibits apoptosis. The mechanism of -pinene, -pinene, and camphene in red pine as antioxidants is carried out by suppressing p53 protein expression resulting in an increase in Bcl-I2 followed by a decrease in Caspase-3 which is an antiapoptotic. Members of the Apoptosis Stimulating Protein p53 ASPP 1 and ASPP2 do not specifically stimulate the p53 transaction function at the promoter of pro-apoptotic genes such as Bax and p53 inducible gene 3 (PIG 3).[17]

Conclusions

The results of data analysis and discussion in this study can be concluded that there is a decrease in Caspase-3 expression in the combination of calcium hydroxide and Red pin and a decrease in apoptosis of fibroblast cells in the combination of calcium hydroxide and Red pin.

Acknowledgements

The authors are grateful to the Ministry of Research, Technology, and Higher Education, Indonesia.

Declaration of Interest

The authors declare that there are no conflicts of interest.

Ethical policy and institutional review board statement

Ethical clearance had been obtained from the Ethics Commission of the Faculty of Dentistry, Universitas Airlangga, Surabaya (Number :249/HRECC.FODM/V/2020)

References

1. Riset Kesehatan Dasar (Riskesdas). Hasil Riset Kesehatan Dasar (Riskesdas) 2018. Badan Penelitian dan Pengembangan Kesehatan Kementerian RI tahun 2018. 2018: 182. Available at: http://www.depkes.go.id/resources/download/infoterkini/materi_rakorpop_20 18/Hasil%20Riskesdas%202018.pdf . Accessed Agustus 2020
2. Kartinawanti KA, Feduniw S, Ajdacka U, et al. Risk Factors for Anxiety and Depression Among Pregnant Women during the Covid-19 Pandemic: A Web-Based Cross-Sectional Survey. Medicine. 2020;99(30):e21279
3. Candra TT, Sarakoat P, Phutdhawong WS. Major Chemical Composition of Fruit Extracts of Morinda citrifolia L. and their Antibacterial, Antioxidant and Cytotoxicity Properties. Journal of Applied Science. 2020; 19(5): 366-7,371-2.
4. Annisa CN, Prisinda GH, Patel B, Gangaramani S, Desai K. A Novel combination of Herbs as chelating agents: An in vitro SEM evaluation. Indian Journal of Conservative and Endodontics, 2020;1(2):47-51.
5. Ba-Hattab Dennis N, Prasetia, W. The Ability of Root Canal Irrigant With Ethanol Extract of Lerak Fruit (Sapindus Rarak Dc) in Removing Root Canal Smear Layer (A Sem Study). IOSR Journal of Dental and Medical Sciences. 2019;16(01):24-30.
6. Sarı S, Okte Z. Success rate of Sealapex in root canal treatment for primary teeth: 3-year follow-up. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2008; 105(4): 93–6.
7. Yoon Lee. Effect of calcium hydroxide application time on dentine. Restor Dent Endod. 2013; 38(3): 186
8. Grag N, Grag A. Textbook of Endodontics. 3rd edition. Jaypee Brothers: New Delhi; 2021: 239
9. Shaofeng AN, Yan G, Yihua H, et al.Short-term effects of calcium ions on the apoptosis and onset of mineralization of human dental pulp cells in vitro and in vivo. International Journal of Molucular Medicine. 2015; 36(1): 215-221
10. Prasetyo EP, Kuntjoro M, Goenharto S, et al. Calcium Hydroxide Increases Human Umbilical Cord Mesenchymal Stem Cells Expressions of Apoptotic Proteasi-Activating Factor-1, Caspase-3 and Caspase-9. Clin Cosmet Investig Dent. 2021; 13: 59-65
11. Patra K, Jayanta, Kim H, Sung CW, Joon, Baek H, Kwang. Volatile compounds and antioxidant capacity of the bio-oil obtained by pyrolysis of Japanese red pine (pinus densiflora Sirbold and Zucc). Molecules. 2015. 20(3):3986-4006; https://doi.org/10.3390/molecules20033986
12. Seo H, Lee H, Nam, Ryu S. Antioxidant and antiapoptotic effects of pine needle powder ingestion and andurance training in high cholesterol-fed rats. 2014. 18(3):301-9; http://dx.doi.org/10.5717/jenb.2014.18.3.301
13. Min J, Hee KJ, Eun SW, Seong BS, Young. Antidiabetic activites of Korean red pine (Pinus densiflora) Inner brak ekstract. 2019; 47(4):498-508
14. Gilbert KY, Shin D. Volatile components and antibacterial effects of pine needle (Pinus densiflora S. and Z.) extracts. Food Microbiology. 2016; 22(1):37-45.
15. Yuanita T, Wahjuningrum D, Prasita S *et al*. Comparison of ratios combination calcium hydroxide $Ca(OH)_2$ with red pine *(Pinus* densiflore) of viscosity. Indonesian Journal of Dental Medicine Vol 4 Issue 1. 2021: 26-31
16. Wong R. Apoptosis in cancer: From pathogenesis to treatment. J Exp Clin Canc Res. 2011;30(87):1-14
17. Olsson M, Zhivotovsky B. Caspases and cancer. Cell Death Differ. 2011; 18(9):1441-9; doi: 10.1038/cdd.2011.30.