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Cytotoxicity of Sodium Hypochlorite, Chlorhexidine and Propolis On Human Periodontal Ligament Fibroblast Cell

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ABSTRACT

Irrigation is one of the most important steps in endodontic therapy. Irrigation solution is at risk of extrusion of to periapical tissue which can delay the healing of periodontal ligament. Therefore, irrigation solution must have minimal cytotoxicity properties. Propolis contains flavonoid and phenolic acid that can be considered as an alternative to irrigation solution. To assess the cytotoxicity of NaOCl, CHX and Propolis on HPDLFc. HPDLFc was obtained from the apex of the first premolar. This cell was divided into several groups and exposed to several concentration of Propolis. The count of fibroblast will be measured by the spectrophotometer. The percentage of cytotoxicity will be calculated to obtain lethal concentration(LC)50 value. (LC)50 of NaOCl is 0,25µl/ml or greater. (LC)50 of CHX is 0,016 µl/ml or greater. (LC)50 of Propolis is 92,70 µg/ml or greater. NaOCl, CHX and propolis have cytotoxicity effect on HPDLFc at a certain concentration.

INTRODUCTION

Endodontic treatment is consist of 3 important procedures including preparation, sterilization and obturation. Mechanical preparation using preparation instrument must be followed by chemical preparation by irrigation. This is called biomechanical preparation (Grossman LI.2010). Root canal irrigant can be derived from synthetic chemical as well as natural material. The common chemical irrigation materials used in endodontic treatments are sodium hypochlorite (NaOCl) and chlorhexidine (CHX).

. NaOCl 0,5-5,25% is used in root canal treatment because it has a characteristic as a broad spectrum antibacterial as well as having the ability to dissolve organic materials and necrotic tissue (Navarro-Escobar *et al.*,.2010). Chlorhexidine (CHX) is considered as "gold standard" as an oral antiseptic. Chlorhexidine has bactericidal properties and is effective against gram-positive and gram-negative, anaerobic and facultative anaerobic bacteria, as well as fungi and some viruses (Gomes *et al.*,.2013).

Some natural ingredients are known to have antibacterial power so that natural irrigation materials can be used as an alternative to avoid the cytotoxic effects of chemical irrigation materials. One of the ingredients that can be used as an alternative to natural irrigation materials is propolis. Propolis is derived from honeycomb and has a high flavonoid content. Flavonoids have many functions such as antioxidant, antibacterial, antifungal, antiviral, and anti-inflammatory (Al-Shaher *et al.*,.2004). Research shows that propolis has antibacterial power against Enterococcus faecalis, which is a bacterium that is often found in cases of root canal treatment failure (Mattigatti *et al.*,.2012). Other studies have suggested that propolis at a concentration of 1.5 mg / ml is not toxic to BHK-21 fibroblasts (Kartika *et al.*,.2010).

The ideal irrigation material should have antibacterial properties and be able to dissolve organic and inorganic tissues and have minimal toxicity to periapical tissue. This is because the use of root canal irrigant for debridement and disinfection of the root canal has the risk of irrigation extrude to periapical through the periapical foramen. This can inhibit the healing and regeneration of periodontal tissues (Navarro-Escobar *et al.*,.2010).

The cytotoxicity of an irrigant to the cell can be seen from the median lethal concentration (LC50), which indicates the material's ability to cause 50% cell culture death (Zhang, Ming *et al.*,.2007). A substance is said to be toxic if the percentage of living cells after exposure of the substance is less than 50% (Marion *et al.*,.2012). The cells required in the regeneration of periodontal ligaments are fibroblast cells. The fibroblast cell is the first cell to come into contact when the irrigant extrudes to the root canal. This cell is also the main cell that reacts to endodontic substances in the periapical tissues (Hidalgo *et al.*,.2002).

MATERIAL AND METHODS

This research is an experimental laboratory research using post test only control group design. The number of samples required in this study is 2 in each group according to the calculation of lemeshow.

The materials used for this study were propolis solution (4 mg/ml, 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125mg / ml), Sodium Hypochlorite (1%; 0, 5%, 0.25%, 0.125%, 0.0625%, 0.03125%), chlorhexidine solution (1%, 0.5%, 0.25%, 0.125%, 0.0625%, 0.03125 %) and Human Periodontal Ligament Fibroblasts, phosphate-buffered solution, trypsin/EDTA, Dulbecco's modified Eagle's medium (DMEM) 10%, MTT, PBS, Stop Solution

Culture Preparation of HPDLFc

Premolar tooth extracted for orthodontic interest is rinsed with saline solution. The tooth is placed in a 15 ml tube containing Dulbecco's modified Eagle's medium (DMEM) which has been added with fungizone and penstrep. Tooth is scraped with tweezers on 1/3 apical then placed on a small petri dish and covered with sterile glass deck, then a 10% complete DMEM medium is added, then put on a

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large petri dish and incubated in a 5% CO2 incubator. Cells were observed to 90% confluent, then peridontal tissue was discarded and medium washed 3 times with PBS. Trypsin EDTA is added to the medium. Centrifuge for 5 minutes 1200rpm, then the supernatant formed discarded. A complete DMEM medium of 1 ml was added to the pellet and incubated in a 5% CO2 incubator. If the cell looks 80% confluent, then the cell is ready for treatment

Treatment Stage
On the 96 well plates, add each of the 100 ul cell susupencies with a density of 2X104/20,000 cells/well. Then let it stand for 1-2 hours. After that add 100 ul extract with various concentration dose. Incubation in CO2 incubator for 24 hours (5% CO2, 37oC temperature, 98% moisture).
After 24 hours see under a microscope. After that remove the existing medium. Add 100 ul MTT (5 mg MTT + 1 ml PBS + 9 ml complete medium RPMI) at each well. Incubate for 4-6 hours. Add Stop Solution 100ul at each well. Incubation over night. Calculate the optical density value of ELISA Reader at 550 nm wavelength. The living fibroblast cells will be stained with formazan to blue colour, while the dead cell does not form blue colour.

RESULT AND DISCUSSION

The result of reading by spectrophotometer will be known as optical density from each sample. Optical density results will be used to find the percentage of deaths caused by each material by using the formula:

$$\% \text{ Mortality} = \frac{\text{OD control} - \text{OD sample}}{\text{OD Control}} \times 100\%$$

Based on the formula will be known concentration that causes % mortality closest to LC50. The result will then be analyzed with Probit Analysis to get LC50 value.

Table 1 Result of NaOCL

Concentration µl/ml	OPTICAL DENSITY			% DEATH CELL
	REPLICATION1	REPLICATION 2	REPLICATION 3	
1	0,119	0,150	0,174	87,1
0,5	0,499	0,506	0,444	57,92
0,25	0,584	0,537	0,564	52,17
0,125	0,619	0,627	0,633	45,47
0,0625	0,702	0,748	0,759	35,88
0,03125	0,718	0,590	0,825	38,06
Control	1,256	1,009	1,180	

The concentration of NaOCl which gives the percentage of death closest to LC50 is 0.25µl / ml. This result is incorporated into Probit Analysis so that the NaOCl concentration which causes LC 50 is 0.254 µl / ml

Table 2 Result of Chlorhexidin

Concentration µl/ml	OPTICAL DENSITY			% DEATH CELL
	REPLICATION1	REPLICATION 2	REPLICATION 3	
1	0,1	0,101	0,104	93,00
0,5	0,082	0,088	0,091	93,92
0,25	0,075	0,071	0,071	94,89
0,125	0,11	0,013	0,125	91,54
0,0625	0,354	0,418	0,417	72,3
0,03125	0,724	0,767	0,776	47,23
Control	1,467	1,443	1,383	1,431

The concentration of CHX which raises the percent of deaths closest to LC50 is 0.03125 µl / ml. This result is incorporated into Probit Analysis so that the CHX concentration which causes LC 50 is 0.016 µl / ml.

Table 3 Result of Propolis

Concentration µg/ml	OPTICAL DENSITY			% DEATH CELL
	REPLICATION1	REPLICATION 2	REPLICATION 3	
4000	0,027	0,016	0,008	88,74
2000	0,036	0,037	0,032	88,30
1000	0,061	0,053	0,065	86,97
500	0,093	0,085	0,083	80,79
250	0,116	0,105	0,123	74,83
125	0,213	0,226	0,415	50,77
Control	0,478	0,466	0,415	0,453

The concentration of CHX which raises the percent of deaths closest to LC50 is 125 µg / ml. This result is incorporated into Probit Analysis so that the CHX concentration which causes LC 50 is 92,70 µg / ml.

This study was conducted to determine the cytotoxicity of each irrigation material that is NaOCl, CHX and propolis. NaOCl is the most commonly used irrigation solution in the endodontic due to its antimicrobial ability and its ability to dissolve organic material (Marion *et al.*,.2012). The results of the cytotoxicity test of NaOCl showed that NaOCl can cause 50% of cell death at concentrations of 0.254 µl / ml. That concentration shows that NaOCl is very toxic at low concentrations

Sodium hypochlorite solution in its function as an irrigation solution will release chlorine ions and hydroxyl ions. Chlorine ions will cause an increase in free radical formation that will increase ROS (Reactive Oxygen Species). The release of hydroxyl ions can cause cell death through 2 mechanisms, by increasing ROS directly or by decreasing ATP (Hidalgo *et al.*,.2002).

A decrease in ATP may cause disruption of cellular metabolism. When the oxygen supply to the cell is reduced, it will cause anaerobic glycolysis to increase. Anaerobic glycolysis will results in a decrease in pH that can decrease cellular enzyme activity. A decrease in ATP also causes the failure of the Ca2 + pump causing damage to cellular components (Chang *et al.*, 2001; Kumar *et al.*, 2015; Estrela *et al*., 2002).

Reactive Oxygen Species (ROS) is a type of oxygen derived from free radicals that play an important role in cellular damage. ROS is produced normally in mitochondrial respiration but will be degraded by the body's immune system. Excess free radicals are commonly referred to as oxidative stress involved in cell jejas. The presence of free radicals causes lipid peroxidation reactions on the plasma membrane and organelle. The bonding between Fatty acid and unstable free radicals can cause damage to more severe membranes. Free radicals can also cause the oxidation of amino acid chains, the formation of covalent bonds of proteins, and the oxidation of proteins. This will lead to the destruction of protein structures, increasing proteasomal protein degradation. In addition, free radicals can cause DNA damage and cross-linking DNA chains. It is the mechanism that causes cell death that shows high levels of NaOCl cytotoxicity (Chang *et al*,. 2001; Kumar *et al*., 2015; Estrela *et al*., 2002).

Chlorhexidine (CHX) is considered a "gold standard" as an oral antiseptic. Chlorhexidine has bactericidal properties and is effective against gram-positive and gram-negative, anaerobic and facultative anaerobic bacteria, as well as fungi and some virus (Gomes *et al*., 2013). The results of the CHX cytotoxicity study showed that CHX could cause 50% cell death at concentrations of 0.016 µl / ml. The concentration shows that CHX is very toxic at low concentrations. This is less appropriate with the American Association Of Endodontic study which states that CHX has a low toxicity (American Association of Endodontists.2011).

Chlorhexidin solution can cause an increase in intracellular calcium. Increased calcium can cause leakage of lysosomial enzymes. It increases the enzyme that has potential for cell death: phospholipase (membrane damage), proteases (membrane damage and cytoskeletal proteins), endonuclease (breakdown of DNA and chromatin) and ATPase (accelerates the reduction of oxygen). Continuous increase of calcium will cause calcium buildup. The buildup of calcium in mitochondria can lead to the opening of mitochondrial permeability transition pore which will cause stimulation of tricarboxylic acid

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reactions and electron flowing. This may lead to an increase in ROS and result in failure of oxidative phosphorylation and reduced ATP. The process can lead to an increase in cell death, which indicates high chlorhexidine cytotoxicity (Giannelli *et al.*,2008).

Propolis is a product produced by insects (honeybee) Apis mellifera. High polyphenol content in propolis has important function as an antibacterial, anti-viral, anti-fungal, antioxidant, anti-inflammatory, and boost the immune system (Kaihena *et al.*,2013).

The effect of propolis on dental pulp regeneration occurs because of its ability to inhibit inflammatory reactions, infections, and pulp necrosis. Propolis also stimulates the formation of dentine tubules through stimulation of the stem cells. Stimulation of the dental pulp is due to its flavonoids content. Because of its ability to reduce inflammation in periapical tissues and protective effects on periodontal tissues, propolis can be used for root canal disinfection (Kaihena *et al.*,2013). Use of propolis as an antibacterial can be considered for its antimicrobial ability equivalent to NaOCl (Al-Qathami *et al.*,2003).

The results of cytotoxicity of propolis showed that propolis can cause 50% cell death at concentration 92,70 µg / ml. The concentration indicates that propolis is a safe enough material for periodontal tissue. Kumar (2013) states that Propolis is considered safe in low doses. Propolis has a lower cytotoxicity in gingival fibroblasts than chlorhexidine.

High doses of propolis may also cause cytotoxicity in the periodontal tissues. Propolis contains high phenolic acid and flavonoids. Properties Phenolic components such as coumaric acid, cinnamic acid, Pinocembrin and other derivatives cause the opening of mitochondrial permeability pore. The opening of mitochondrial permeability pore causes depolarization resulting in reduced ATP in the cell. Reduced ATP in this cell is the cause of cell death The mitochondrial membrane damage can also cause DNA damage that can also end up into cell death (Campos *et al.*,2015).

CONCLUSION

(LC)50 of NaOCl is 0,25µl/ml or greater. (LC)50 of CHX is 0,016 µl/ml or greater. (LC)50 of Propolis is 92,70 µg/ml or greater. NaOCl, CHX and propolis have cytotoxicity effect on HPDLFc at a certain concentration.

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