Reduction of Endotoxin from Human Root Canals by Calcium Hydroxide Nanoparticles

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Abstract. This study was aimed to evaluate the reduction of endotoxin using the passive ultrasonic irrigation (PUI) of calcium hydroxide nanoparticles (CHNPs) compared with the PUI of calcium hydroxide (CH) and the CH medication. Forty-seven single-root human premolars were used. The root canals were enlarged and sterilized. Escherichia coli endotoxin was inoculated into the root canals. Thirty-six root canals were assigned into three experimental groups: CHNPs irrigation, CH irrigation, and CH medication. For the irrigation groups, the PUI was activated for three min. For the CH medication, the CH was filled into each canal and incubated at 37°C for seven days. The reduction of endotoxin was evaluated by quantitative chromogenic limulus amoebocyte lysate assay. Data was analysed by Kruskal-Wallis and pairwise comparison tests, with a level of significance set at $P < 0.05$. The CHNPs irrigation was more effective in against endotoxin than the CH irrigation ($P < 0.05$). No significant difference was found between the CHNPs irrigation and the CH medication ($P > 0.05$). The irrigation of CHNPs could eliminate most of the endotoxin from the root canals and approximately from 300 µm of root dentin. CHNPs might be potential as an adjunctive irrigation for endotoxin removal.

1. Introduction

Gram-negative bacteria are predominantly demonstrated through the infected pulp and apical periodontitis. They produce by-products and virulent factors that invade into periapical tissues such as lipopolysaccharides (LPS), or endotoxin, within their outer cell wall. LPS can induce inflammatory reaction and periapical bone resorption[1]. Thus, the main goal of root canal treatment in necrotic pulp with apical periodontitis is based on reduction or elimination of the microorganisms as well as inactivation of the endotoxin from the infected root canal, which lead to the healing of periapical tissues[2]. The chemo-mechanical procedures are effectiveness in reducing bacteria but there are not able to eliminate endotoxin from all root canals due to the irregularities of the root canal system[3]. Therefore, the intracanal medication or adjunctive approach has been recommended as an additional step for the root canal debridement. Passive ultrasonic irrigation (PUI), an adjunctive approach, which ultrasonic energy is transmitted through the root canal irrigant by the free movement of an activated file[4]. Previous studies have indicated that the PUI can reducing endotoxin in necrotic root canals[5, 6].

Calcium hydroxide (CH) is the most commonly used for intracanal medication, its use being for antimicrobial action, detoxifying LPS, and dissolving organic tissue debris. The effectiveness of CH can
be limited by the buffering capacity of dentin, which affects the penetration of hydroxyl ions into dentinal tubules.[7] Nano-sized materials, with dimensions less than 100 nm diameter, have become interesting in dental applications. Higher charge density and surface areas of the nanoparticles enable greatly antibacterial efficacy.[8] Recently, Calcium hydroxide nanoparticles (CHNPs) were studied in endodontic researches such as its antimicrobial properties[8], penetration depth[9], and cytotoxicity.[10] However, the effect on the reduction of endotoxin has never been evaluated. Therefore, the aim of this study was to evaluate the reduction of endotoxin using the PUI of CHNPs compared with the PUI of CH and the CH medication.

2. Materials and Methods
The methods of this study were previously approved by the Human Experimentation Committee, Chiang Mai University, Thailand (protocol no. 014/2561). Forty-seven non-curious, single-rooted, permanent lower premolars which extracted for orthodontic reasons were used. The teeth were transversely cut at the cementoenamel junction with diamond discs. The roots were cut into fragments 10 mm long. The root canals were prepared by using peeso-drills (VDW GmbH, Munich, Germany) no #1 #2 and #3, respectively, and irrigated with 2.5% sodium hypochlorite after the use of each instrument. The roots were submerged and ultrasonicated in 17% EDTA for one min, 2.5% sodium hypochlorite for three min, and pyrogen-free saline for one min, respectively. The specimens were sealed at the apex and coated outer surfaces twice with nail varnish, except at the cervical opening.

All specimens were randomly divided into three experimental groups (n=12) and two control groups (n=9). The specimens were fixed with the chemical-cured acrylic resins in 24-cell culture plates (SPL, Pocheon-si, Gyeonggi-do, Korea). All specimens and equipment were previously sterilized by 60 Co gamma rays (Thailand Institute of Nuclear Technology, Nakornnayok, Thailand) at 20 KGy for six hours in order to eliminate the pre-existing endotoxin. Twenty microliters of *Escherichia coli* 055:B5 endotoxin (Sigma Aldrich, St Louis, MO, USA), at 400 EU/mL concentration, was subjected into each root canal, except in the negative control. The root canals were closed and vortexed for 15 min. All plates were incubated at 37±1°C in a 100% humidified atmosphere for 24 hours, then, the fillings were removed. Two teeth were selected for baseline samples.

2.1. Endotoxin removal procedures

2.1.1. PUI of CH and CHNPs. First, the root canals were irrigated with one mL of freshly prepared CH (Unilab; Ajax Finechem Pty. Ltd., Taren Point, NSW, Australia), 25 g/L dispersed in pyrogen-free water (A.N.B. Laboratories Co. Ltd., Kannayao Bangkok, Thailand), delivered by using disposable syringe with 22-gauge needle which was inserted to two mm short of root length. Second, the solution was activated by ultrasonic file #20/21 (IrriSafe tip; Satelec, Acteon Group, Merignec, France), driven by the ultrasonic device (Newtron®P5, Satelec) at a power setting of 4 for 30 s, with the probe tip placed at two mm short of root length. Then, each canal was refilled with another one mL of CH for 30 s and repeated the irrigation cycle twice to complete the irrigation in three min. The root canal was finally irrigated with five mL of pyrogen-free water for 60 s. To neutralize the activity of CH, the canal was rinse with five mL of 0.5% citric acid for 60 s and followed by five mL of pyrogen-free water for 60 s. Each root canal was dried, and the endotoxin sampling was collected. For the CHNPs group, the protocols were performed as same as the CH group which described above. The premixed form of CHNPs (CaLosiL® E25, IBZ-Salzchemie GmbH & Co.Kg, Halsbrücke, Germany), 25 g/L CH dispersed in ethanol, was used instead of the CH and delivered by using disposable syringe with 27-gauge needle.

2.1.2. CH medication. CH (UltraCal XS; UltraDent Products, Inc., South Jordan, UT, USA), 35% in water, was delivered into each root canal by using a Navi-Tip (UltraDent Products, Inc.). All root canals were sealed with glass ionomer cement (GC Fuji IX GP®; GC America Inc., Alsip, IL, USA) and incubated at 37±1°C in a 100% humidified atmosphere for seven days. When completed, the filling was removed and neutralized with five mL of 0.5% citric acid and followed by five mL of pyrogen-free water for 60 s. The endotoxin sampling was collected.
2.1.3. Positive control (n=6) and negative control (n=3). The specimens were divided into two subgroups according to the solvents of the irrigation used. In the pyrogen-free water group, the root canals were irrigated with 24 mL of pyrogen-free water for seven min, delivered by disposable syringe and 27-gauge needle at the same length. Another subgroup was irrigated with four mL of 75% v/v ethanol for three min followed by 20 mL of pyrogen-free water. In the negative control, the root canals were irrigated by 24 mL of pyrogen-free water for seven min.

Each canal was filled with pyrogen-free water, a pyrogen-free paper point was inserted into the full length of each canal for 60 s. The sampling was kept in the glass tube containing 1 mL of pyrogen-free water. Afterward, the root dentin was drilled by no #4 #5 and #6 peeso-drills (VDW GmbH) to assess the endotoxin from the different depths; 100, 200, and 300 µm. The endotoxin was extracted from the root dentin according to the method has been previously described[11]. All tubes were centrifuged for 20 min at 1000 rpm. The clear solution was collected and kept at temperature -20°C for the endotoxin analysis.

The quantitative chromogenic limulus amoebocyte lysate (LAL) assay was performed by using Pierce LAL chromogenic endotoxin quantitative kit (Thermo scientific Inc., Waltham, MA, USA) according to the manufacturer’s instructions. The endotoxin standard curve was created for calculation of the concentration of endotoxin. The coefficient of determination of the standard curve was 0.99. The test was performed in a 96-well microplate in a heating block at 37 ºC throughout the assay. The plates were measured by using a spectrophotometer at an absorbance of 405 nm (Sunrise®; Tecan, Männedorf, Switzerland). The mean absorbance value of the blank was subtracted from the mean absorbance of the samples to calculate the mean absorbance. The endotoxin concentration (endotoxin unit per milliliters; EU/mL) was determined from the standard curve because the absorbance value was directly proportional to the concentration of endotoxin. All data was analyzed by Kruskal-Wallis and pairwise comparison tests. Significant level was considered at 5% (P < 0.05).

3. Results

Baseline samples were confirmed that the amount of endotoxin was distributed into the root canals. The mean values were 35.92 EU/mL, and the mean values of the root dentin layers at depth of 100, 200, and 300µm were 15.93, 10.51, 12.06 EU/mL, respectively. The reduction of endotoxin was extremely reduced after the PUI or the CH medication. Table 1 provided the values of endotoxin from the experimental groups. In the root canals, The PUI of CHNPs was more effective than the PUI of CH (P < 0.05). No significant difference between the PUI of CHNPs and CH medication (P > 0.05). In the root dentin, there was no statistically significant difference among the experimental groups at every depths of the root dentin (P > 0.05). The endotoxin was found in all levels of the samples in the positive control group, on the other hand, the endotoxin was not detected in the negative control group (data not shown).

Table 1. The median and range values of endotoxin concentration in the root canal samples collected from the experimental groups (EU/mL)

|                     | PUI of CHNPs          | PUI of CH            | CH medication       |
|---------------------|-----------------------|----------------------|---------------------|
| Root canal          | 0.29 (0.02-0.75)       | 0.70 (0.28-0.97)     | 0.29 (0.02-0.75)    |
| Root dentin at 100 µm| 0.05 (0.02-0.53)       | 0.06 (0.04-0.46)     | 0.06 (0.03-0.18)    |
| Root dentin at 200 µm| 0.07 (0.04-0.39)       | 0.08 (0.05-1.14)     | 0.07 (0.03-0.24)    |
| Root dentin at 300 µm| 0.08 (0.05-0.80)       | 0.09 (0.05-0.47)     | 0.06 (0.04-0.23)    |

*aDifferent letters indicate statistical significance for endotoxin between the different experimental groups (P < 0.05).
EU/mL = endotoxin unit per milliliters
PUI = passive ultrasonic irrigation, CHNPs = Calcium hydroxide nanoparticles, CH = Calcium hydroxide

4. Discussion

Endotoxin is released during death and lysis of gram-negative bacteria. The concentration of endotoxin may increase after the chemo-mechanical instrumentation[12] and remain in dentin smear layer[13]. It is difficult to eliminate the endotoxin from the root canals and dentin walls because of its irreversibly adherence to the mineralized tissues[14]. Gram-negative bacteria can penetrate into dentinal tubules at about 275 µm while
LPS represented approximately 4 times greater than the bacteria invasion according to its lower molecule weight[11]. The use of 400 EU/mL endotoxin in this study was based on the concentration found in the infected pulp with symptomatic apical periodontitis[15]. The chromogenic LAL test was used to measure endotoxin concentration, is based on the reaction between the clotting enzyme and endotoxin.

The CH medication for seven days can reduce the endotoxin only 4% from chemo-mechanical procedures[12], but, the CH medication in this study greatly reduced the endotoxin because the well-enlargement of the root canal may help the hydroxyl ions to diffuse into deep portions of the dentinal tubules.

The CHNPs solution is an alternative solution that is effective in against the endotoxin. The CHNPs solution used in this study contained nanoparticles of lime hydrate suspended in ethanol. The physical properties of CHNPs, such as the high solubility and stability in ethanol, the lower size of nanoparticles less than 100 nm may be attended to its neutralizing effect of endotoxin. The CHNPs may create more concentration of the hydroxyl ions that can break the fatty acid bonds of Lipid A[2]. The CHNPs were sufficiently penetrated to deep portions of dentinal tubules and increased charge density when reacting with remaining water in the dentin[8]. The nanoparticles solution may be able to diffuse greater than 300 µm depth of dentin. The PUI may also facilitate the penetration of the CHNPs solution because of the acoustic energy of the ultrasonic activation[4]. The well-replenished of solution during PUI may help to removed endotoxin from the root canals. Therefore, the use of CHNPs with PUI for three minutes may be beneficial in one-visit root canal treatment of the infected canals. Further studies are needed to evaluate the effectiveness of irrigation in eliminating endotoxin which adheres to the root dentin.

5. Conclusion
Within the limitations of this in vitro study, the PUI of CHNPs was almost eliminated endotoxin from the root canal and root dentin depth of 300 µm in straight root canals. CHNPs might be potential as an adjunctive irrigation for endotoxin removal.

6. References
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