Original Article

Cytoxicity of root canal antiseptics used in dental practice on L929 fibroblasts: calcium hydroxide powder vs. 2% chlorhexidine solution

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Abstract

Background & Objective: Chlorhexidine solution is one of the widely used mouth antiseptic liquid that prevents teeth tissue damage and also has application as a root canal antiseptic. In this study, cytotoxicity of 2% chlorhexidine solution is compared with another root canal antiseptic, calcium hydroxide powder.

Materials and Methods: Cell cytotoxicity of both chemicals was assessed on cultured L929 fibroblastic cell line for 1, 12, 24, 48 and 72 hours using MTT assay (Methyl tetrazolium bromide assay). Untreated L929 cells were used as a negative control group. MTT results were recorded by ELISA reader and analyzed using one-way ANOVA statistical tests.

Results: Cytotoxicity of studied chemicals showed significant difference in various dilutions and times (1, 12, 24, 48 and 72 h). The highest cytotoxic effect of 2% chlorhexidine solution was observed in concentration of 0.016% for 72 h. Treatment of cells with 0.016% of 2% chlorhexidine liquid and calcium hydroxide powder for 72 hours showed 80% and 45% cytotoxicity, respectively.

Conclusions: Cytotoxicity of calcium hydroxide is significantly less than 2% chlorhexidine liquid and then application of calcium hydroxide powder as root canal antiseptic is recommended.

Key Words: cytotoxicity, calcium hydroxide powder, 2% chlorhexidine liquid, MTT assay, root canal antiseptics

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Introduction

The essential role of microorganisms in the development and sustainability of pulp and periapical diseases has been shown in studies on animal and human models [1-3]. It is difficult to entirely eliminate microorganisms from infected root canal. Numerous protocols using different chemicals or instrumental techniques have been tested to reduce microorganisms in root canal. Concerning root canal space, there is still no strong evidence indicating that mechanical

machines can eliminate the bacteria in root canals [4]. The remaining pulp in the root canal is a nutrient for bacteria. In addition, the remaining tissues deactivate and reduce the antimicrobial properties of canal-filling materials. Therefore, a chemical substance is required to remove tissue from the root canal and to kill the bacteria. Chlorhexidine liquid is widely used for this purpose.

Delany et al [5] reviewed antibacterial effect of 2% chlorhexidine liquid in the infected root canals and observed a significant effect in reducing microorganisms. Many studies have shown that root canal filling materials have four main properties: Antimicrobial activity, non-toxicity to periapical tissues, water solubility and ability to dissolve organic materials. Therefore, a filling material should be able to break down organic materials in root canal.

Meiman and Grossman [6] studied the importance of solubility of root canal filling materials and removal of pulp tissue. Okino et al [7] reported that 2% chlorhexidine liquid was not able to dissolve pulp tissue. White et al [8] evaluated the antimicrobial ability of factors to maintain its effectiveness in the mouth for a maximum period and reported that this ability lasted for a maximum period of 72 hours. This ability depends on the number of chlorhexidine molecules available for reaction with teeth [9].

Toxic effect of chlorhexidine liquid on canine embryonic fibroblast cells revealed that bactericidal concentration is fatal for these cells, while it allows bacteria to survive in its non-toxic concentration [10].
The mechanism leading to toxicity of chlorhexidine is still not clear [11-13]. Chlorhexidine interferes in mitochondrial function and causes cell death [14, 15]. Recently, chlorhexidine liquid has been used in different concentrations as an intracanal washing solution [16-18].

In vitro and in vivo studies have shown that most bacteria isolated from infected root canals are susceptible to calcium hydroxide. When ecological conditions in root canal change during treatment, microbes that survive are those which can tolerate alkalinity, lack of nutrients and increased oxygen level.
In these rare cases, the root canal infection changes from a polymicrobial, anaerobic flora towards a facultative one, and monoinfections occur more frequently [19-22].
Calcium hydroxide powder, as intracanal medicament between appointments, has favorable properties such as dissolving necrosis tissue, high PH (which prohibits dissolving process) and anti-microorganisms properties. However, this material does not have a desirable effect on two species of Entrococcus faecalis and Candida albicans (observed in cases of endodontic failures). Chlorhexidine solution has some desirable properties such as huge anti-microorganism effects and durability; it also affects the two species mentioned above. It has cell cytotoxicity but has no tissue solubility[23, 24].
Considering the mentioned factors, we explored the cytotoxic effects of well known root canal antiseptics calcium hydroxide powder in comparison with 2% chlorhexidine liquid on cultured fibroblast cells of L929.

Materials and Methods

Cell culture

Murine L929 fibroblast cell line was purchased from Iran Pasteur Institute. Frozen cells were cultured in RPMI (GIBCO, USA) enriched by 10% FBS (Fetal Borne serum) and penicillin (100IU/ml) as well as strepromycine (100µg/ml) antibiotics were used to feed cells. Viability of cells was examined by 1% trypan blue dye (Merck, Germany) and viability above 95% was considered as vital. Viable cells (10,000) were seeded into 96-wells plate. Culture plates were incubated at 37ºC and 98% humidity and 5% CO2.

Preparation of materials

Calcium hydroxide powder and 2% chlorhexidine liquid were prepared. They were placed at the bottom of 6-wells plate. Negative control has received complete RPMI medium. Treated plates were incubated for 1, 12, 24, 48 and 72h in temperature of 37˚C, humidity of 98% and CO2 5%.

Cytotoxicity assay

To perform the MTT test, Tetrazolium bromide salt (5mg/mL in PBS) solution was added to each well; they incubated for 4h in culture conditions (37ºC, humidity of 98 % and CO2 5%). To make sure that the samples were stained, they were observed under a microscope. In the next step, 2% chlorhexidine liquid and calcium hydroxide powder were added

immediately. The cells were incubated for 1, 12, 24, 48, and 72 h. Next, their relative cell viability was measured by scanning with an ELISA reader using a 570 nm with reference filter of 620 nm.

Discussion

The essential role of microorganisms in development and perpetuation of pulpal and periapical diseases have clearly been demonstrated in animal models and human studies. Elimination of microorganisms from infected root canals is a complicated task. Numerous measures have been described to reduce number of root canal microorganisms, including the use of various instrumentation techniques, irrigation regimens and intra-canal medicaments [25-27].

Removal of irritants and toxins from root canal is of great importance. In filling root canal, some materials with antibacterial activities are used. Effective toxicity is a concern in choosing these materials. If they come out of the canal, they lead to inflammation and pain.

Calcium hydroxide is the most commonly utilized and studied root canal medication [28]. The main use indications of calcium hydroxide are the stimulation of remineralization, its antibacterial properties and the dissolution of necrotic tissue. Chlorhexidine is also known to inhibit bacterial growth. A microbiological

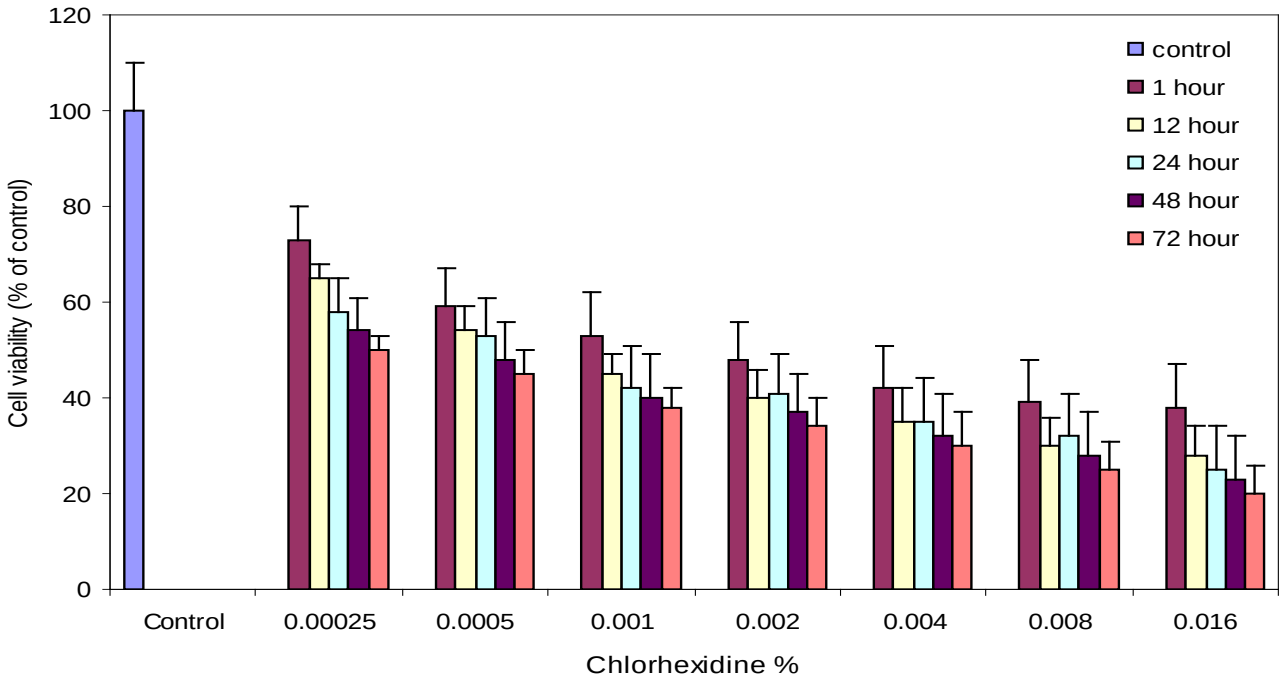

Figure 1: L929 fibroblast cells were cultivated in RPMI medium with the indicated concentrations of 2% chlorhexidine liquid (0.00025, 0.0005, 0.001, 0.002, 0.004, 0.008 and 0.016 %) for 1, 24, 48, and 72 h. Analysis of variance results showed that the effects of dose and time on viable cells are statistically significant (P<0.05).

Results

Cytotoxicity of 2% chlorhexidine liquid:

Average Cell viability of 2% chlorhexidine liquid was tested at concentrations of 0.00025, 0.0005, 0.001, 0.002, 0.004, 0.008, and 0.016% for 1, 12, 24, 48 and 72h using MTT. A significant difference was found between treated and controls cells (one way ANOVA, $p<0.05$).

Cytotoxicity of calcium hydroxide powder:

Cell viability of calcium hydroxide treated cell at concentrations of 0.00025, 0.0005, 0.001, 0.002, 0.004, 0.008, and 0.016% were studied after 1, 12, 24, 48 and 72 h. Significant difference was found between treated and controls.(one way ANOVA, $p<0.05$).

study has shown that some microorganisms are more effectively reduced by calcium hydroxide, while others are more susceptible to chlorhexidine [29]. However, the bacterial advantages of calcium hydroxide and chlorhexidine could have a toxic effect in the periapical tissues. Results from a study on the cytotoxic effect of Chlorhexidine on canine embryonic fibroblasts and *Staphylococcus aureus* showed that bactericidal concentrations of chlorhexidine were lethal to canine embryonic fibroblasts whilst non-cytotoxic concentrations allowed significant bacterial survival. Boyce et al [30] found chlorhexidine (0.05%) uniformly toxic to both cultured human cells and microorganisms. Agarwal *et* al [31] found that Chlorhexidine rapidly disrupts the cell membrane of both crevicular and peripheral blood neutrophils at concentrations above 0.005% within 5 min, indicating that its inhibitory effect on neutrophil function is mostly due to its lytic properties.

Giannelli et al [32] investigated *in vitro* cytotoxicity of chlorhexidine on osteoblastic, endothelial and fibroblastic cell lines. They reported that chlorhexidine affected cell viability in a dose and time-dependent manners. Its toxic effect consisted in the induction of apoptotic and autophagic/necrotic cell deaths.

The mechanisms of the cytotoxicity of chlorhexidine are still unclear and it is important to understand that the cytotoxic effects of chlorhexidine on cell culture are directly dependent on the exposure dose, frequency, duration, and also depend on the composition of the exposure medium.

Cosyn et al [33] concluded that the clinical and microbiological data currently available on the chlorhexidine chip are limited and conflicting, and more research are needed to elucidate the additional value of the chlorhexidine chip when uses as an adjunct to scaling and rooting planning.

Cell culture studies can provide information on the

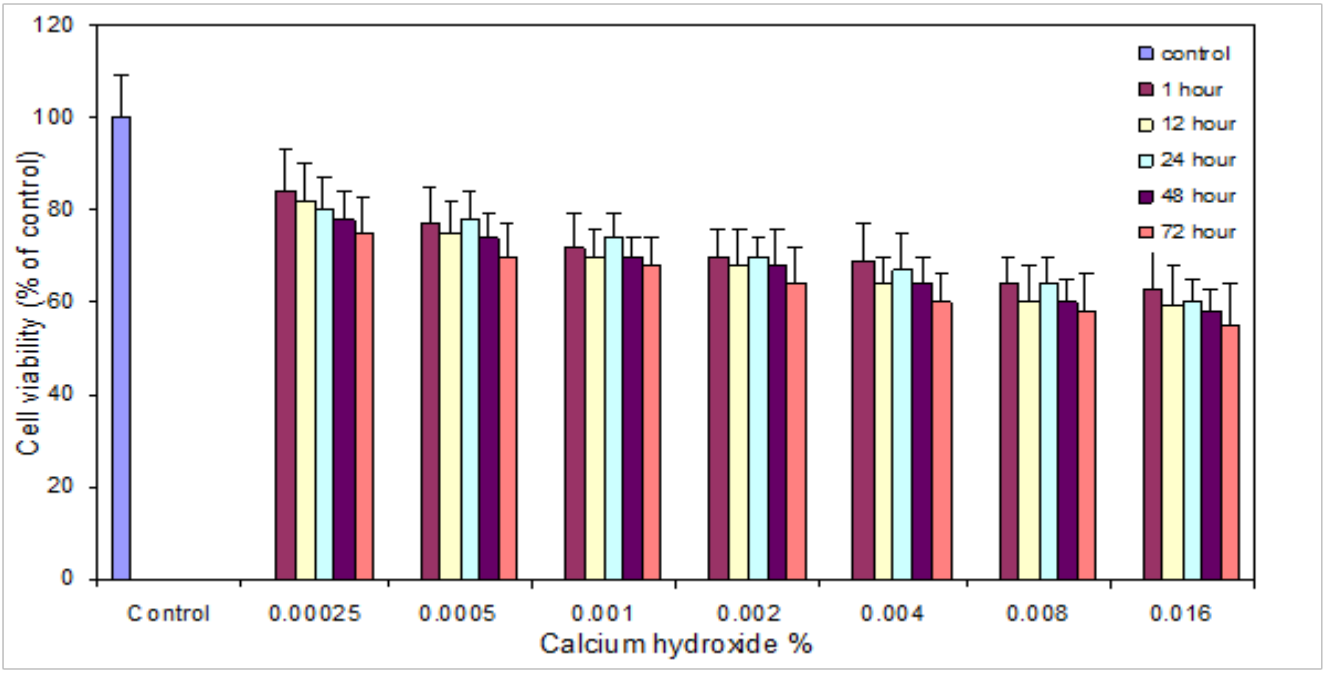

Figure 2: L929 fibroblast cells were cultivated in RPMI medium with the indicated concentrations of calcium hydroxide powder (0.00025, 0.0005, 0.001, 0.002, 0.004, 0.008 and 0.016%) for 1,12, 24, 48, and 72h. Analysis of variance results showed that the effects of dose and time on viable cells are statistically significant (P<0.05).

behaviour of different materials against healthy tissues. Because the periapical tissues can be exposed to intra-canal dressing materials, the possible toxic effect of commercial available calcium hydroxide and chlorhexidine-based gutta-percha points also needs to be evaluated.

By using MTT assays, we showed that the measured cell viability was significantly decreased in the group treated with liquid chlorhexidine 2% (80%) compared with control (Figure1). In this study, our results also showed that Calcium hydroxide inhibits cell growth in L929 fibroblasts cells in a time- and dose-dependent manner. As shown in Figure 2, there was a significant decrease in the cell viability. In summary, our results indicate that liquid chlorhexidine 2% was more effective in reducing cell viability (80%) than Calcium hydroxide (45%). Therefore, Calcium hydroxide is a possible approach for root canal treatment with few side effects.

Conclusion

In summary, our results indicate that liquid chlorhexidine 2% was more effective in reducing cell viability (80%) than Calcium hydroxide (45%). Therefore, Calcium hydroxide is a possible approach for root canal treatment with few side effects.

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