Cytotoxicity of Root Canal Sealers Containing Calcium Hydroxide

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• Abstract

The purpose of this study was to investigate the possibility to reduce the toxicity of oil based root canal sealers containing calcium hydroxide using MTT & agar overlay assays. Thus some formulations of traditional root canal sealers were replaced with oil-soluble solvents and experimental root canal sealers manufactured.

In MTT assay, Cell viability of all experimental sealers in addition with oil soluble solvents were observed significantly higher than both control groups, especially according to replace zinc and/or calcium ion components. Also agar overlay assay was appeared moderate to no cell responses into modifying both zinc and/or calcium ion components and oil soluble solvent weight.

Authors found the reducing effect of cell toxicity through significant role of oil soluble solvent factor into root canal sealer containing calcium hydroxide.

• Key word : cytotoxicity, endodontic sealer, calcium hydroxide, oil based, MTT, agar overlay

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Introduction

Root Canal Sealers with calcium hydroxide as the principle component enable to elaborate dense filling of deep portions within the root canal and exert biologic effects at the tissue adjacent to the periapical area. But it is difficult and impossible to make a single application of calcium hydroxide powder on anatomical structures such as severely irregular and narrow root canals. Calcium hydroxide powder has the physicochemical disadvantage of being radiolucent and easily dissolves at the periapical area and is not viscous and does not adhere nor flow. So distilled water or silicone oil is used as a carrier and when distilled water (water soluble carrier) is used as the carrier it is odorless and can be easily removed from the canal while the silicone oil (oil soluble carrier) carrier has good flow and does not evaporate after mixing, extending the application time.

Shin et al.[1] reported that there was no difference in microleakage among calcium hydroxide powder, water soluble carrier and oil soluble carrier. Harris and Wendt[2] applied oil soluble and water soluble agents containing steroid for 1 week inside the root canal and conducted a microleakage experiment using dye, which did not show any statistically significant differences but showed more microleakage compared to the group without intracanal medication. Theoretically, water soluble agents are more easily removed than oil soluble agents but this is not the case. Without developing an efficient method of removal, it is suggested that no water soluble or oil soluble agents be used inside the canal for canal filling. Rivera et al.[3] reported that when the thickness of the agent inside the canal increases so does the amount of calcium hydroxide and the mean amount of calcium hydroxide was less for oil soluble agents (30㎎) compared to water soluble agents (36㎎). At the apical third, oil soluble agents showed elaborate filling without bubbles (50%) and tended to overfill more than water soluble agents (86% and 61% each). Oil soluble agents reach the apical third but water soluble agents do not. Depending on the clinical situation in which the apical patency is not maintained because of dentinal debris occluding the apex, the overfilling of oil soluble agents was somewhat decreased. Among the oil soluble agents, Vitapex showed microleakage after 2 days (0.067 ± 0.072㎜) using dye penetration in a study by Shin et al.[1] and Lim[4] reported that on the first day (mean 1.6 ㎜) dye penetration increased according to duration and on the seventh day (mean 9.2 ㎜) significant dye leakage was observed. Porkaew et al.[5] reported in vivo that apical microleakage was significantly decreased compared to the control without root canal filling material and there was a large amount of apical microleakage at the second week (3.21 ± 3.91 ㎜). A minimal amount of initial microleakage is a temporary phenomenon but the effects of microleakage of calcium hydroxide agents remaining inside the canal should be continually observed.

An animal experiment using oil soluble agents by Kawakami et al.[6] showed by using ^{45}Ca that in mice the calcium ions of Vitapex are moved into the bone through blood and a portion is excreted from the digestive organs. Another portion of calcium goes through two different calcification processes of normal bone formation and heterotropic (dystrophic and matrix vesicle) calcification. Lim et al.[7] reported that the running direction of apical fibers at the apex of canine teeth filled with Vitapex after 1 week were in good condition and the inflammation was decreased at 6 weeks compared to 3 weeks.

Finally, Yoon et al.[8] conducted apexification on 9 teeth of 7 patients for 21 months and observed them radiographically and clinically for more than 6 months. They reported a certain amount of intracanal resorption, gradual resorption of Vitapex outside the apical foramen and gradual healing of the periapical lesion. Thomas et al.[9] conducted a one-visit clinical study conducting root canal treatment with iodoform paste on 36 teeth with pulp necrosis of thirty-two 3-9 year-old children. The results showed a 94.4% success rate through clinical and radiographic observation after 3 months.

In this study, Oil soluble root canal filling material containing calcium hydroxide was experimentally manufactured and MTT assay and agar-overlay method was conducted to analyze and report the relation between the solvent of oil soluble component from root canal filling material and its cytotoxicity.

Material and Method

1. Material

In this study, an experimentally manufactured oil soluble root canal filling material containing calcium hydroxide (Exp*) (Table 1) was used as the experimental group and

Table 1. Components of root canal sealer for experimentally manufactured*.

Components (%)	Exp 1	Exp 2	Exp 3
Calcium hydroxide	32	32	32
Iodoform	40	50	40
Silicone oil	20	15	20
Others			
Zn compound	8	3	3
Ca compound			5

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the conventional oil soluble agent, Vitapex (Cont 1, Lot No.; RB01, Neo Dental Chemical Product Co. Ltd, Tokyo, Japan) and Metapex (Cont 2, Lot No.; 0211050132, Meta Biomed Co. Ltd, Cheongju, Korea) was used as the control to compare the cytotoxicity of each group.

2. Experimental Method

1) MTT assay

Specimens from the experimental group and control group used in the MTT assay were eluted in distilled water at 37℃ for 24 hours at a rate of 1g/5㎖ and filtrated through a 0.2㎛ syringe filter (Felman Science, USA). Rat L929 fibroblasts were cultured in 10% (vol/vol) Eagle's minimal essential medium (MEM) with fetal calf serum. After cell concentration was diluted to 1×10^5/㎖ and 0.18㎖ was placed in each well of a 96-well plate for 24 hours at 37℃, in 5% carbon dioxide.

On the last day of culture, the media of each well was removed and twice the amount of media was mixed with a 1:1 mixture of experimental and control group effluent then cultured for 24 hours. MTT dye solution was prepared by mixing phosphate buffered saline with 3-(4,5-dimethylthiazol-2-yl)-2, 5-dimethyltetrazolium bromide (MTT, 98%, molecular weight 414, $C_{18}H_{16}BrN_5S$, Sigma, USA) powder then filtrating. 100㎕ of MTT was added to each well containing experimental and control group effluent that had been cultured for 24 hours. The plates were well shaken for 3 hours so the live cells and MTT solution could react to form MTT formazan.

The MEM media containing MTT solution was removed and 180㎕ of DMSO was added then shaken for 30 minutes. A Microplate Reader (BIO-RAD, AC, USA) was used to measure the absorbance at 570nm wavelength.

The measured absorbance of the experimental and control groups were compared to negative control with only MEM media to calculate cell survival rate. (Table 2)

Table 2. Definition and classification of cytotoxicity scores based on relative growth Rate(RGR)*.

Classification	Score	RGR (%)*
None	0	≥100
Weak	1	75~99
Moderate	2	50~74
Marked	3	25~49
Strong	4	1~24
Extreme	5	0

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2) Agar overlay method

A specimen of 5 mm diameter and 1 mm thickness from the experimental and control groups were manufactured and sterilized with ethylene oxide gas before the experiment. L-929 cell suspension (3×10^5/㎖) was produced according to the conventional method and 10 ㎖ was poured onto a 90 mm diameter Petri dish then cultured for 24 hours. After checking whether the culture was in a single layer, media was removed from the Petri dish and 10㎖ of 45~50℃ Eagle's Agar medium was added. The Eagle's Agar medium left at room temperature for 30 minutes and 10㎖ of neutral red vital stain solution was added to the center of the solid and left for 30 minutes so the dye could spread over the entire surface. Thereafter the residual dye solution was removed and the specimen (contain with positive and negative control), were closely adhered to agar as quickly as possible, then cultured for 24 hours at 37℃, in 5% carbon dioxide. The Petri dish was put onto white paper and the Zone index was calculated by observing the depigmentation range. The Lysis index was determined by calculating the ratio of dead cells within the depigmented area with a phase-contrast microscope (CK2, Olympus, Japan). These were indicated as Zone index and Lysis index independently and the cell response index (Response index = Zone index / Lysis index) was drawn from the mean value of the specimen. (Table 3) PVC was used as a positive control and Polyethylene was used as a negative control.

Table 3. Response index and cytotoxicity.

Cytotoxicity	Response index
None	0/0
Mild	1/1-1/5, 2/1
Moderate	2/2-2/5, 3/1-3/5, 4/1-4/3
Severe	4/4-4/5, 5/1-5/5

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3) Statistical Analysis

The level of significance of the comparisons between each material and control was analyzed with ANOVA at 95% reliability. SAS 6.12 system [Tukey's Studentized Range (HSD)] was used for post hoc studies.

Results

1. Cell survival rate of the experimental group with root canal filling material containing calcium hydroxide and control group measured by MTT assay. (Table 4).

The relative optical density was determined by conducting MTT assay on oil soluble root canal filling paste containing calcium hydroxide extracted with 3㎖ distilled water. The cell survival rate according to optical density was highest for Exp 1 and decreased in the order of Exp 2, Exp 3, and control ($P<0.05$). The control group, Cont 1 and Cont 2, showed low cell survival rate and no statistically significant difference between the groups ($P \geq 0.05$).

2. Response index [Zone index (Figure 1) / Lysis index (Figure 2)] of the experimental group with root canal filling material containing calcium hydroxide and the control group were measured by Agar-overlay method. (Table 5).

Table 4. The value of cell viability measured at experimental and control group.

	Exp 1	Exp 2	Exp 3	Con 1	Con 2
1	101.9±224.4	72.5±10.8	40.5±7.7	9.9±2.8	7.9±0.3
2	100.0±20.0	80.9±6.2	22.6±9.2	13.4±4.8	8.2±0.5
3	72.3±19.1	80.6±8.9	25.5±6.9		8.9±0.8
4		25.2±8.1			
Mean±SD	95.2±23.7	77.8±9.5	27.5±10.0	11.6±4.2	8.3±0.7
Score	1	1	3	4	4

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Table 5. Results of agar overlay test.

Zone/lysis index	Exp 1	Exp 2	Exp 3	Cont 1	Cont 2
1	4/3	3/3	2/2	2/5	2/5
2	4/1	1/2	2/0	2/5	2/5
3	3/3	3/1	3/3		
4	3/3	0/0	0/0		
Cell toxicity	moderate	mild	mild	moderate	moderate

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The cell response index was calculated by determining the zone index and lysis index of each specimen by culturing L-929 fibroblast suspension and oil soluble root canal filling paste specimen containing calcium hydroxide for 24 hours. There was no depigmentation or lysis of the negative control from the experimental group (Exp 1,2,3), while the positive control showed 11.5~14 ㎜ of depigmentation and 62.1~87.9% of cell lysis even with 5 mm specimens.

The response index of cytotoxicity (depigmentation length/lysis percentage) of the experimental group was highest in Exp 1 group with a moderate level and Exp 2 group (5.0±4.9/24.8±19.9) and Exp 3 group (3.5±3.1/22.6±27.6)

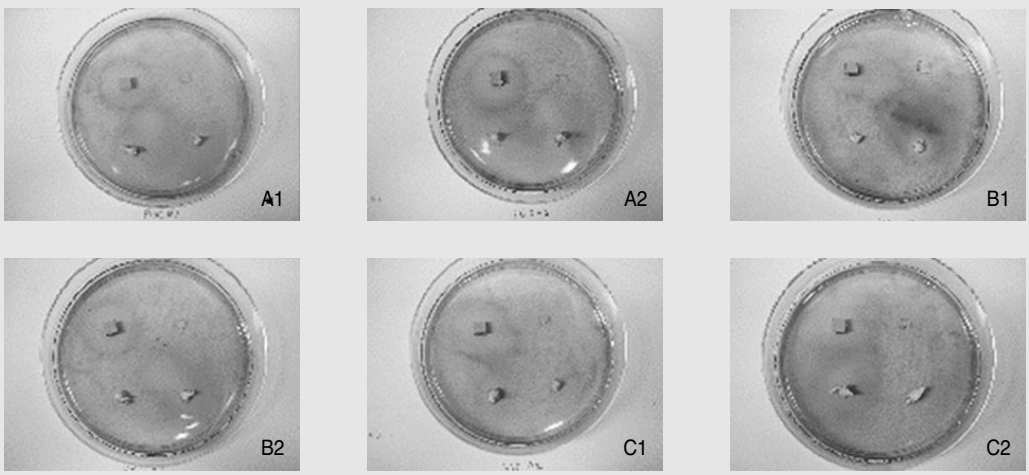

Figure 1. Decoloration index photographs of agar overlay test.
A: Exp 1 groups (A1~A2), B: Exp 2 groups (B1~B2), C: Exp 3 groups (C1~C2)

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showed similar mild levels. Especially zone index 0 grade was observed in Exp 2 and 3 groups and lysis index 0 grade without any cell lysis was seen in the Exp 2 and 3 groups. This shows that the experimental group has shorter depigmentation length and lower lysis percentage compared to the positive control.

But Cont 1 group (1.9±0.1/93.0±4.6) showed 1.8 and 2.0 ㎜ depigmentation length and 6.2 and 92.7% lysis percentage, and Cont 2 group (2.4±0.1/96.6±4.8) showed 2.3 and 2.5 ㎜ depigmentation length and 93.2 and 100% lysis percentage. The moderate cell response index shown by these control groups is similar to the depigmentation length and lysis percentage of the positive controls.

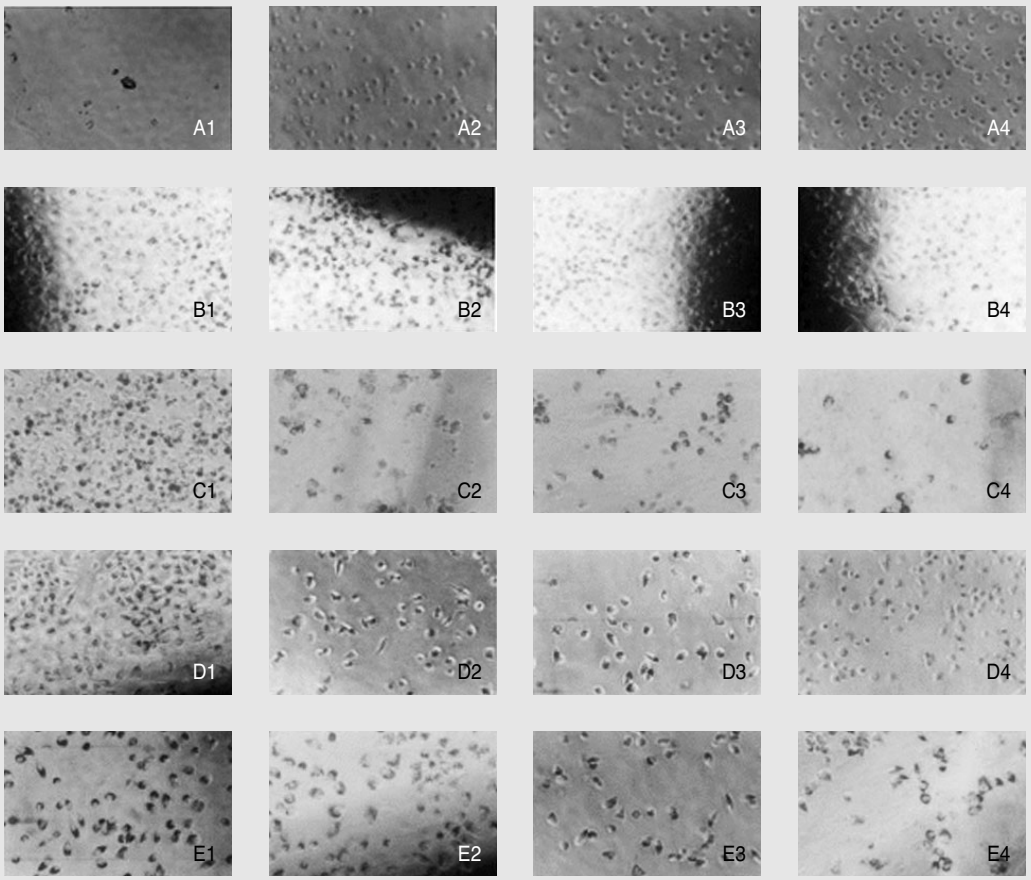

Figure 2. Lysis index photographs of agar overlay test.
A: positive control groups (A1~A4), B: negative groups (B1~B4), C: Exp 1 groups (C1~C4), D: Exp 2 groups (D1~D4), E: Exp 3 groups (E1~E4)

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Discussion

Root canal filling material containing calcium hydroxide is composed of calcium hydroxide, iodoform, silicone oil and other substances. The main component, calcium hydroxide, was first introduced by Hermann in 1920 as a root canal filling material and therapeutic agent. It has a slightly bitter taste and the pH is very high (12.5) causing protein dissolution and bacterial destruction when coming in direct contact with pulp tissue. Calcium hydroxide has the biochemical effect of inducing and initiating mineralization, dentinal bridge formation, pulp and root canal disinfection, and dissolution of pulp necrotic tissue. Calcium hydroxide is used for cavity lining, indirect pulp capping, direct pulp

capping, pulpotomy, long-lasting temporary disinfectant, disinfectant for infected root canal and periapical lesion treatment and apical closure, prevention of idiopathic exarticulation or compensatory root resorption of transplanted teeth, restoration of iatrogenic perforation and treatment of horizontal root fracture. Root canal sealers containing calcium hydroxide have bioactivity that accelerate healing of periapical tissue and induce apical foramen closure by dentine accumulation and are used as temporary root canal filling material with superior antimicrobial effect[10]. The human tooth dentine contains 30% calcium, but the cervical area of the crown has 20%, the middle third has 15% and the apical third has 10%[11], so the experimental root canal filling material has over 30% calcium hydroxide.

Walkhoff in 1928 observed that iodoform as a root filling material (KRI-1) did not set until it was totally absorbed from the periapical tissue and showed chemical activity. In canine studies, the overfilled material that was pushed out into the periapical tissue was quickly removed and granulomatous tissue was replaced into normal tissue accelerating apical healing. Iodoform paste has strong sterilizing power and is non-irritating, radio-opaque, does not contract, and is insoluble[12]. The radio-opacity is similar to dentine and the absorption rate is higher than zinc-oxide eugenol. Absorbable characteristics are not necessary for permanent filling of permanent teeth, but it is helpful for the ideal filling of primary teeth. It is a useful material when apexogenesis that produce the apical stop at the apical area is impossible for primary teeth with complex canal structures and severe dilacerations.

Finally silicone oil is an odorless, clean, colorless solution that is water insoluble but dissolves in chloroform, tetrachlorocarbon, ethyl alcohol and paraffin oil and shows low surface tension and lubrication effect[13].

Pasted root canal filling materials are ointment form mixtures that are put in conventional root canal carrier syringes for easy clinical use in root canal filling. Among these, the commercially developed oil soluble root canal filling material containing calcium hydroxide is generally used. In this study, various solvents that dissolve oil from components of the conventional commercial oil soluble calcium hydroxide root canal filling material such as calcium hydroxide, iodoform, and silicone oil were added to experimentally manufactured an oil soluble root canal filling material with good flow.

Oil soluble calcium hydroxide paste has superior flow that allows the material to outflow from the apical foramen when it is filled near to the foramen. The short and long term effects on the root canal and periapical area from direct contact with the applied medication have been reported. Soares et al.[14] observed chronic inflammation around the periapical tissue regardless of the root canal filling material used, bone resorption of the apical area and new hard tissue formation on the apical canal wall of the open apical foramen of completed roots were observed, but it was impossible to decide whether there was healing loss or delay of the periapical tissue. This study is an in vitro experiment conducted to investigate the etiology that affects cell toxicity, and for this, various solvents that dissolve oil components were added to manufactured an experimental oil soluble root canal filling material containing calcium hydroxide. MTT assay and agar overlay method were used.

The cytotoxicity test are affected by many factors such as cell type, physical property of material, method of contact between cell and material and method of measuring cell damage. The method of extracting material (time, temperature, pattern), surface area of specimen and volume ratio of extraction media, contact type of cell and material cause error[15] making it difficult to compare study results reported by various scientists that conducted studies under different conditions such as cell type, method of cell-material contact, and exposure time[16] that cause changes in measurement values. Considering the fact that for every cytotoxicity test, sufficient contact between the cultured cell and experiment material should be allowed, the material should be made into a solution immediately after mixing and put into culture media, or the solidified specimen should be directly put into the culture media for observation. Most dental materials are not water soluble so a permeable intermediate media is necessary to indirectly obtain sufficient contact between the specimen and cell or culture media[17]. When using extract solution, the pH and components extracted from the solution should be analyzed to elucidate the cytotoxic component and activity-accelerating factor. Many studies use hardened solids or their extracts but there is no consensus on the physical properties of the materials to be inspected. In studies with solid dental material, the method of solution extraction may cause different results so it is reasonable to prepare the material as it is clinically used, intraorally, for cytotoxicity evaluation[15]. In this study L292 fibroblasts cultures were

used for agar overlay method that measures changes in the penetration of cell membrane and colorimetric assay using tetrazolium dye (MTT) for cytotoxicity evaluation. For MTT assay, a experimental solution of root canal filling material was eluted for 24 hours from specimens of various cell lines and cells primarily cultured in the oral cavity were used. In agar overlay method, the material spreads through the agar layer[18] before expressing toxicity so the experimental semisolid paste material does not directly contact the cells resulting in satisfactory correlation. After culturing for 24 hours, the remaining toxic substance dissolved from the specimen causes depigmentation within the effusion zone. When the concentration of toxic substance is high the substance goes through the agar media to cause cell lysis within the effusion zone. The experiment is performed quickly and clearly and the results are reproducible within the same laboratory but it is difficult to reproduce the response index in a random experiment between different laboratories[15].

On the other hand, Ciapetti et al.[19] reported that in a study using more than 2 types of cytotoxicity tests the results are often contradictory so it is recommended to use more than one method to prevent invalid resulting from errors.

Using more than one method, Lee et al.[20] conducted cytotoxicity tests including the cell multiplication and survival rate test, agar overlay test, and Milliporer filter technique to compare the differences among 4 types of root canal filling material. This study reflected the difference in mechanism according to the toxic substance of each material that causes changes, and a collective evaluation based on the cytotoxicity test method of the material is recommended. Since the clinical contact surface between non-overfilled periapical tissue and root canal filling material is small, in vitro test results do not totally match clinically applied results but it is attested that relative cytotoxicity tests of experimental material may help in granting clinical meaning. Youn et al.[21] tested the cytotoxicity of 4 types of dental composites using the MTT assay and agar overlay method expecting that composite resin would contain more resin monomer compared to compomer or glass ionomer being more harmful to cells, but MTT assay results showed that there was no difference and only the agar overlay method showed higher response indices and moderate cytotoxicity. Im[22] measured the cytotoxicity of 7 types of dental retrograde filling material using MTT and LD test showing there was a difference in toxicity expression. The results of the MTT and LD test were identical for ZOE.

The cytotoxicity test results of filling material are dependent on the material preparation method, solidification degree, and filling method of cytotoxicity. There has been an effort to identify the toxicity-causing substance that identically expresses toxicity following various experimental methods. Choi et al.[23] reported that the cytotoxicity of 6 types of dental retrograde filling material measured with radiochromium release method, MTT assay, and LD test of L929 fibroblast showed differences. The reason for this discrepancy was thought to be the difference in each experimental method, and differences caused in the material amount, mixture ratio, and lack of standard ratio in extracting the filling material into solution during the procedure of preparing extracts of each experimental material.

In this study, Exp 1, 2, and 3 groups showed significantly lower cytotoxicity in the MTT assay compared to the control group of Vitapex and Metapex ($P<0.05$). Especially in Exp 1 group, in which conventional products was replaced with zinc ion compound contained oil soluble solvent, the cell survival rate ($95.2 \pm 23.7\%$) was significantly higher than the control group(Vitapex : 11.6 ± 4.2, Metapex : $8.3 \pm 0.7\%$) ($P<0.05$). The MTT measurement value of Exp 2 group($77.8 \pm 9.5\%$), which had less zinc ion contents contained oil soluble solvent than Exp 1, showed a slight but statistically significant lower cell survival rate compared to Exp 1 ($P<0.05$). And the cell survival rate of Exp 3 that had trace zinc ion compound and a large amount of calcium ion compound replaced with oil soluble solvent showed a significantly lower cell survival rate ($27.5 \pm 10.0\%$) ($P<0.05$). But the cell survival rate of the two commercial oil soluble paste products in the control group, Vitapex and Metapex, did not show any significant differences and had the lowest value ($P \geq 0.05$). In this study, the addition of zinc or calcium oil soluble solvent increased the cell survival rate compared to controls. Among the experimental groups the reducing zinc ion solvent and addition of trace zinc lowered the cell survival rate and, replacing zinc ion solvent with calcium ion and increasing the amount of calcium ion solvent lowered cell survival rate.

The agar overlay method results showed that compared to the control group composed of commercial oil soluble paste product, the cell response index of Exp 1 was showed moderate to highest toxicity among the experimental groups. Exp 2, which has less solvent and trace zinc added

to Exp 1 and Exp 3 with calcium ion solvent added to Exp 2, both showed a mild toxicity. This is expressed as irregular cell response indices between 0-3 levels which are low cytotoxicity to non-toxic. The control group, Vitapex and Metapex, both showed a level 5 lysis index among cell response indices, which means moderate toxicity. The cell response index results show that by adding oil soluble solvent and decreasing the amount of zinc ion oil soluble solvent rather than replacing the calcium ion solvent, the cell response indices may be lowered to no response and it will be possible to improve root canal filling material until it is non-toxic.

By comparing the cytotoxicity results of MTT assay and agar overlay method, Exp 1 shows mild toxicity in MTT assay while moderate toxicity was expressed in the agar overlay method. The high optical density in the MTT assay may have been caused by the relatively high oil soluble solvent content and further studies should be done to elucidate relevance.

Conclusion

1. The cell survival rate using the MTT assay was significantly higher for the Exp 1 group (95.2±23.7%) compared to the control groups (Vitapex : 11.6±4.2, Metapex : 8.3±0.7%). ($P<0.05$)
2. MTT values of Exp 2 (77.8±9.5%) with additional zinc ion to Exp 1 were slightly but statistically significantly lower than Exp 1. ($P<0.05$)
3. The cell survival rate of Exp 3 (27.5±10.0%) in which the zinc ion of Exp 2 is replaced with calcium ion was significantly lower. ($P<0.05$)
4. The cell toxicity response indices of the agar overlay method were moderate for Exp 1 and mild for Exp 2 and 3.
5. The cell response index of Exp 1 in which oil soluble solvent was replaced and added was moderate showing similar results with the control group.
6. The cell response indices of Exp 2 and Exp 3 groups that had additional zinc and calcium ions were mild.

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