In vitro investigation of the cytotoxic, apoptotic and genotoxic effects of pulp capping materials on L929 mouse fibroblast cells

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ABSTRACT: This study evaluated the *in vitro* cytotoxic, apoptotic and genotoxic effects of MTA, TheraCal and Dermabond (2-octyl cyanoacrylate) on L929 mouse fibroblast cells. Culture medium was exposed to the materials for 24 hours, 1 week and 2 weeks. Cells were seeded in 96 well plates with 5×10^3 cells/100 μL media per well for MTT assay, 6 well plates with 2×10^5 cells /2 mL media per well for comet assay,12-well plates with 2×10^5 cells/well for apoptosis assay and cells were exposed to the material eluates. The cell viability, genotoxicity and apoptotic status were evaluated by MTT, comet and apoptosis assays respectively at three different times (24 hours, 1 week and 2 weeks). There was no statistically significant difference ($p > 0.05$) between MTA and Dermabond at 24 hours, 1 week and 2 weeks exposures. However, TheraCal significantly reduced the cell viability at 1-week exposure to 66.70% ($p < 0.05$). TheraCal caused DNA damage at all exposures and there was a statistically significant difference ($p < 0.05$). According to flow cytometry results, TheraCal at 24-hour and 1-week showed significantly higher cytotoxic effects compared to MTA and Dermabond. No significant differences were detected between MTA and Dermabond. As observed from the current study it can be concluded that MTA and Dermabond are biocompatible capping materials in vital endodontic treatments. Since TheraCal exhibited cytotoxic and genotoxic effects caution should be exercised when it is being used.

KEYWORDS: L929 cells; cytotoxicity; genotoxicity; apoptosis; mta; theracal; dermabond.

1. INTRODUCTION

According to Hanning [9], preventive dental practices are becoming widespread all over the world. Nevertheless, tooth loss is still occurring due to cavities. Tooth loss can lead to malocclusions, nutritional disorders caused by chewing problems, psychological problems and speech disorders. For these reasons, preventive dental practices are needed to protect the integrity of the oral tissues.

The consequences of pulp exposure from caries can be severe, with pain and infection. The medical problems associated with treating pulp exposures often results in requiring either extraction or root canal therapy. The root canal therapy and tooth restoration procedures are costly and require several appointments which can be time-consuming. Therefore, vital pulp therapy is indispensable to maintain tooth vitality by provoking the remaining pulp tissue to regenerate the dentin-pulp complex. Direct pulp capping is whereby the exposed pulp is covered with a protective pulp capping material over the site of exposure, which enhances pulp healing and induces reparative dentin [11]. This is done in an attempt to maintain pulp vitality and avoid the more extensive treatment dictated by extraction or endodontic therapy [18].

In vivo and *in vitro* tests are performed to evaluate if the materials used in the pulp cap give good biocompatibility results. Biocompatibility refers to the quality of not being toxic or injurious to biological systems. Most materials exert some toxic effect when they are fresh and the effect is reduced with time as the concentration of leachable components decreases [23]. Therefore biocompatibility of capping materials is of primary importance. The criteria used to test the biocompatibility is to see whether the material is genotoxic

and cytotoxic or not. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium), apoptosis assay by flow cytometry using annexin V staining method and single gel (comet) assays are performed. The MTT assay is a colorimetric assay that is used to measure enzyme activities that are responsible for the reduction of MTT to formazan dyes, resulting in purple color. MTT assay is also responsible for cell viability and cell proliferation assessment. Cytotoxicity of potential medicinal agents and toxic materials can also be determined by MTT assay since those agents activate or inhibit cell viability and growth [16]. Flow cytometry assesses cell death patterns by determining membrane permeability. This assay is based on the principle that normal cells are hydrophobic due to the phosphatidylserine in the inner membrane. When apoptosis occurs the inner membrane flips to become the outer membrane thereby exposing the phosphatidylserine. Annexin V then detects the exposed phosphatidylserine and necrotic cells are stained by iodide stains, that have leaky DNA contents to identify apoptotic and necrotic cells [12]. The basic principle of the single gel (comet) assay is that DNA fragments migrate in an agarose matrix under electrophoresis. When viewed under a microscope, cells have a comet appearance, with a head which is the nuclear region and a tail. The tail contains DNA fragments or strands migrating toward the anode [6]. In the single gel (comet) assay process, cell damage is evaluated by applying the theory that the amount of DNA damage is indicated by the amount of DNA that leaves the nucleus when a charge is applied to a cell. Comet assay is one of the suitable methods to investigate the genotoxicity of compounds used in dental practice according to Ribeiro and coworkers [22].

Calcium hydroxide, Mineral Trioxide Aggregate (MTA) and resin-modified calcium silicate (TheraCal) are used for the treatment as capping materials and they are preferred the most due to their low toxicity. TheraCal is a calcium silicate-based material that consists of resin monomers as the main component (45%), including bisphenol A-glycidyl methacrylate and urethane dimethacrylate [17]. *In vivo* and *in vitro* studies have been done on these materials, with MTA having good biocompatibility results [16]. Although MTA is a good capping material various problems occur in vital endodontic treatments due to its disadvantages such as long setting time [24]. Therefore, the use of calcium silicate-based materials with a short curing time has increased. Dermabond has been used in medicine and dentistry but there are not so many studies on their biocompatibility. Different cyanoacrylate derivatives have been studied for their use as direct pulp capping materials. According to [3,4,7] cyanoacrylate derivates can be safely used in sutures, as well as for pulp capping, and retro-filling material in endodontic surgeries. However, there is limited literature regarding the biocompatibility of Dermabond (2-Octyl Cyanoacrylate) as a pulp capping material. Therefore this study aimed to compare the cytotoxic and genotoxic effects of Dermabond (2-Octyl Cyanoacrylate) for direct pulp capping with other capping materials in L929 (mouse fibroblasts) cell lines by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) and single-cell gel (comet) assays *in vitro*. Apoptosis by flow cytometry was also performed to determine the mechanisms of the potential effects of the materials. Calcium hydroxide (Kerr life material) was used as a positive control and DMEM was used as a negative control.

2. RESULTS

Figure 1 represents the results of the MTT assay of the materials. The genotoxic effects of the materials are reported in Table 1 and Figure 2. The values were given as mean ± standard error (SEM). Viable cell percentages and mean tail intensities were determined on MTT and Comet assay respectively. Figure 3 shows the images of damaged and non-damaged DNA. Apoptotic effects of the materials on cell viability are reported in Table 2 and Figure 4. The figures represent cell populations % as follows: nonapoptotic live (7-AAD negative, Annexin V negative), non-apoptotic dead (7-AAD positive, Annexin V negative), apoptotic live (7-AAD negative, Annexin V positive), and late apoptotic/dead (7-AAD positive, Annexin V positive) cells.

2.1. Cytotoxicity of the materials on L929 cells using MTT assay

The data obtained from the analysis showed no statistically significant difference (p>0.05) for MTA and Dermabond at all exposures. However, TheraCal reduced cell viability at 1-week exposure (p<0.05). 24 hours and 2 weeks showed no cytotoxicity, there was no statistically significant difference (p>0.05) for TheraCal. The positive control was compared to all the materials. Cytotoxicity data indicated that there was no significant difference when MTA and Dermabond were compared to the positive control. A significant difference was observed when TheraCal was compared to the positive control.
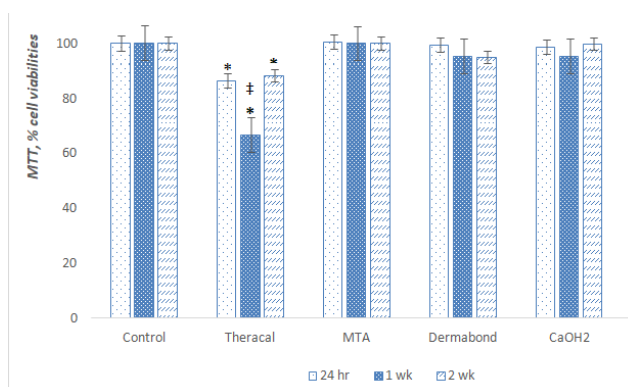
Figure 1. Cytotoxic effects of the materials on L929 cells viability for 24 hours, 1 week and 2 weeks. *$p<0.05$, compared to the negative control. ‡$p<0.05$, 1-week cell viability was compared to 24-hour and 2-week cell viability. Experiments were performed in triplicate and the results were given as mean ± SEM. (Repeated Measures ANOVA).

2.2. Genotoxicity of the materials on L929 cells using comet assay

The genotoxic effects of the materials were evaluated by the Comet assay. Dermabond and MTA did not cause DNA damage at all exposures. TheraCal caused DNA damage at all exposures and there was a statistically significant difference ($p<0.05$).

Genotoxicity data indicated that there was no significant difference when MTA and Dermabond were compared to the positive control. A significant difference was observed when TheraCal was compared to the positive control. Images of comet assay results of non-damaged and damaged DNAs are presented in Figure 3.

Table 1. Genotoxic effects (mean ± standard error values); n=100 following exposure of L929 cells to the materials.

	24-hour	1-week	2-week
Control	2.0939 ± 0.4783	0.5087 ± 0.5087	2.6199 ± 2.6199
TheraCal	8.5014 ± 0.8501*	9.4030± 0.9403*	6.6491 ± 0.6649*‡
MTA	3.3920 ± 0.8441	2.7330 ± 0.5628	3.1246 ± 1.0430
Dermabond	3.2052 ± 0.6151	0.9503 ± 0.6001	3.3913 ± 0.7289
Ca(OH)₂	1.3737 ± 0.2895	4.0550 ± 0.3773	2.3566 ± 0.7708
H₂O₂		44.4831 ± 3.0213*	

*$p<0.05$, compared to untreated cells (negative control). ‡$p<0.05$, 2-week tail intensity was compared to 24-hour and 1-week tail intensities. (Repeated Measures ANOVA).

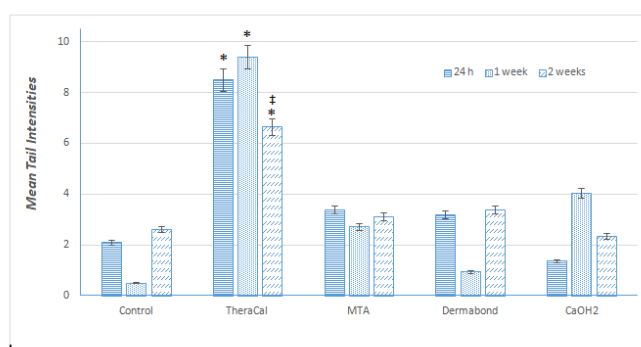**Figure 2.** Genotoxic effects of the materials on L929 cells viability for 24 hours, 1 week and 2 weeks. Experiments were performed in triplicate and the values were given as the mean tail intensities ± standard error (n=100). (Repeated Measures ANOVA). *$p<0.05$, compared to untreated cells (negative control). ‡$p<0.05$, 2-week tail intensity was compared to 24-hour and 1-week tail intensities.
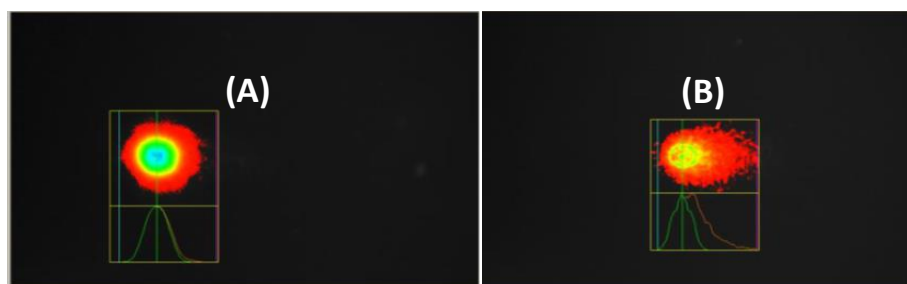
Figure 3. (a) Non-damaged DNA (MTA at 1 week); (b) Damaged DNA (TheraCal at 1 week).

2.3. Apoptotic effects following exposure of L929 cells to the materials

MTA and Dermabond recorded high viability values; 100% and the lowest viability values were observed in TheraCal at 1-week exposure with 67.70%. TheraCal at 24 hours and 1 week showed significantly higher cytotoxic effects compared to MTA and Dermabond. No cytotoxic effecs were observed in TheraCal at 2-week exposure. No significant differences between MTA and Dermabond were observed.

Table 2. Apoptotic effects following exposure of L929 cells to the materials.

	%Gated				
	Live	Early Apoptotic	Late Apoptotic/Dead	Dead	Total Apoptotic
Control 24-hour	100.00	0.00	0.00	0.00	0.00
Control 1-week	99.65	0.35	0.00	0.00	0.35
Control 2-week	98.10	1.90	0.00	0.00	1.90
TheraCal 24-hour	87.75	5.55	5.05	1.65	10.60
TheraCal 1-week	67.70	16.45	15.30	0.55	31.75
TheraCal 2-week	100.00	0.00	0.00	0.00	0.00
MTA 24-hour	100.00	0.00	0.00	0.00	0.00
MTA 1-week	100.00	0.10	0.00	0.00	0.10
MTA 2-week	99.90	0.00	0.00	0.00	0.00
Dermabond 24-hour	100.00	0.00	0.00	0.00	0.00
Dermabond 1-week	100.00	0.00	0.00	0.00	0.00
Dermabond 2-week	99.85	0.15	0.00	0.00	0.15
$Ca(OH)_2$ 24-hour	99.95	0.05	0.00	0.00	0.05
$Ca(OH)_2$ 1-week	99.40	0.60	0.00	0.00	0.60
$Ca(OH)_2$ 2-week	100.00	0.00	0.00	0.00	0.00

3. DISCUSSION

Materials used in dentistry are in contact with oral tissues for long periods. Therefore, it is necessary to evaluate their effects on genetic tissues to minimize risks to patients. When selecting a material for vital pulp therapy biocompatibility is an important property to be considered because of the materials' direct contact with vital tissues. *In vivo* and *in vitro* tests are performed to evaluate the biocompatibility of the materials. *In vitro* study was employed in our present study. *In vitro* studies are simple, inexpensive to perform. It gives a significant amount of information and can be performed under controlled conditions [21].

In this study, MTT and apoptosis assays were used to evaluate the cytotoxicity of these materials. MTT assay is a gold standard for cytotoxicity testing owing to its simplicity, speed and precision. Comet assay was then used to evaluate the genotoxicity. Comet assay is sensitive to detect low levels of DNA damage, inexpensive, rapid and flexible (fresh or frozen samples can be used).

The MTT results showed that neither MTA nor Dermabond was cytotoxic to the cells at 24-hour, 1- week and 2-week exposure. However, cell viability reduced significantly (66.70%) ($p < 0.05$) for TheraCal at 1-week exposure. The result at 1-week exposure for TheraCal from our study supports a study by Collado-Gonzalez *et al* [15] whereby the cell viability was very low or almost absent for TheraCal eluates. Stem cells were extracted from human exfoliated primary teeth and exposed to TheraCal for 24, 48 and 72 hours in that study. Cell viability was also determined by MTT assay. MTA results from our study endorse the study carried by Lessa *et al* [14] where both white MTA and Bio-MTA presented low cytotoxic effects on odontoblast-like cells (MDPC-23). Their immersion time of the materials in the culture medium was 24 hours and 7 days.

Cyanoacrylates are increasingly used in medicine and dentistry. They have a chemical formula $(CH_2=C(CN)COOR$ where R can be any alkyl group ranging from methyl to decyl. Ahn *et al* [1]) stated that cyanoacrylates have found wide application in the field of dentistry due to their better biocompatibility. In endodontics, cyanoacrylates are used as pulp capping material, as a retrograde filling material and as a dental sealant, in the early stages of dental caries management, to name a few. Park *et al* [10] carried out a study on rabbit fibroblasts. Different forms of cyanoacrylates were used. A neutral red cell toxicity assay was used in the study and exposure was done by extraction. It was observed that Dermabond provided higher cell viability compared to other cyanoacrylates. Long-chain cyanoacrylates are less toxic than short chains both *in vitro* and *in vivo* [5]. This is also confirmed by the results of our present study.

Lee *et al* [13] investigated the cytotoxicity of new pre-polymerized cyanoacrylate-based tissue (PACA) and tissue adhesive Dermabond. A direct and indirect contact test *in vitro* cell culture was used. The immersion time of the materials in the culture medium was 24, 48, 72 hours and cell viability were evaluated by WST assay. Cell viability increased with time. Dermabond showed (50.8, 66.1 and 95.3%) cell viability for 24, 48,72h respectively. For the indirect method, L929 cells were exposed to elution of PACA and Dermabond for time courses. The indirect contact method showed no cytotoxicity for both PACA and Dermabond. In our study 24 hours, 1 week and 2weeks evaluations were done with the extraction method and the cell viabilities were found to be 99.3, 95.21 and 94.87% respectively.

Neda *et al* [1]evaluated the genotoxicity of MTA on L929 mouse fibroblasts cells with serial ascending concentrations (0 to 1.000 µg/ml). They reported that damage of cells by MTA was only observed at the highest concentration (1.000 µg/ml). Several studies assessed the genotoxic effects of MTA [8,20,27]. Their data indicated that MTA does not have genotoxic effects on mouse lymphoma or even when exposed to human peripheral lymphocytes. These results were in agreement with our findings although exposure durations were different.

Genotoxicity of TheraCal was assessed on Human fibroblasts with concentrations ranging from 0-1000 µg/ml [26]. The comet assay revealed that TheraCal did not cause DNA in any concentration. These results were in contrast to our findings which may be attributed to the use of different cells and exposure durations. Literature regarding the genotoxic effects of TheraCal is limited.

According to the apoptosis results of the present study, TheraCal exhibited higher cytotoxic effects than MTA and Dermabond while low cytotoxic effects and high cell viability were observed in MTA and Dermabond. No apoptosis for TheraCal at 2-week exposure, cells were viable. Cells adapted to the conditions resulting in defensive responses. The cells defensive effects overwhelmed the adverse effects, that's why there is no apoptosis at 2 weeks. An adaptive situation was created [19].

Cell viability in TheraCal at 2-week from flow cytometry was observed to be higher than that in MTT. 100% cell viability was observed in flow cytometry whereby 88.20% in MTT at 2-week. Additionally, cell viability values that were found from apoptosis results in Dermabond at 1-week and 2-week were observed to be higher than those in MTT results. From this data, it can be claimed that MTT results and apoptosis results are not the same throughout. This difference might be a result of the different principles between MTT and flow cytometry. MTT gives cell viability information by measuring mitochondrial metabolic activity whilst flow cytometry assesses cell death patterns by determining membrane permeability [2].

Cytotoxicity results obtained in our study showed that there was no significant difference for MTA, Dermabond at all exposures (24 hours, 1 week, 2 weeks). No statistically significant difference was noted between MTA and Dermabond in terms of genotoxicity at all exposures. TheraCal caused DNA damage at all exposures. The positive control was compared to all the materials. The cytotoxicity and genotoxicity data indicated that there was no significant difference when MTA and Dermabond were compared to the positive control. A significant difference was observed when TheraCal was compared to the positive control.

4. CONCLUSION

MTA and Dermabond exhibited good compatibility. According to the experimental conditions and the methodology used in the present study, it may be concluded that MTA and Dermabond have low or no cytotoxic effects on L929 cells. Therefore, MTA can be used in vital endodontic treatments and Dermabond can be an alternative to pulp capping materials considering that it does not have cytotoxic and genotoxic effects. TheraCal showed cytotoxic and genotoxic effects on L929 cells therefore caution should be exercised when it is being used.
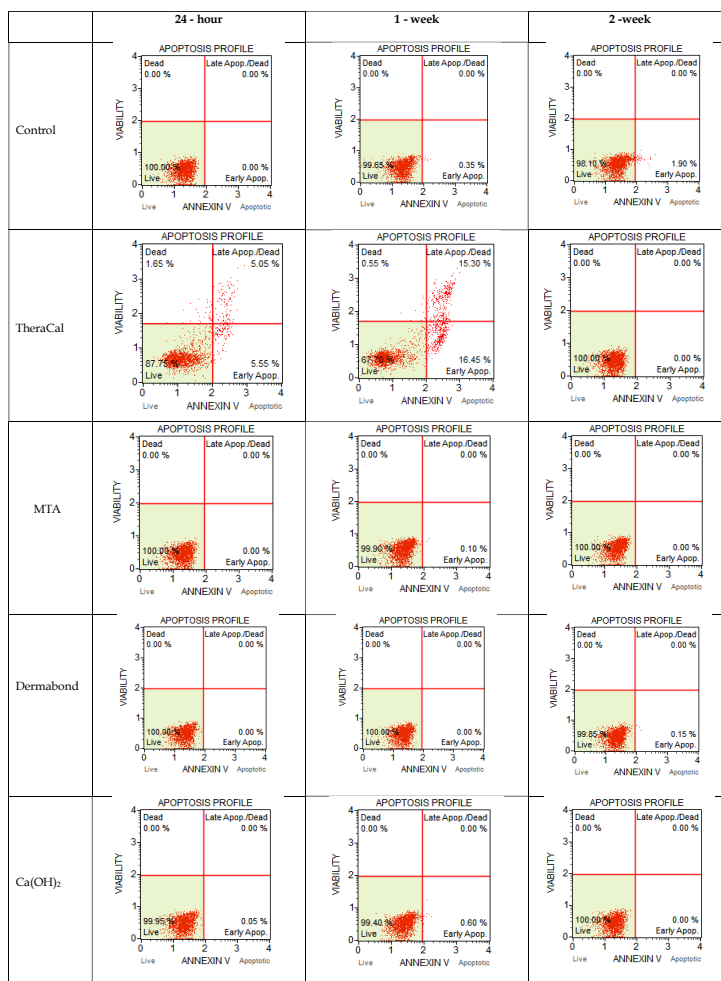
Figure 4. The apoptotic cell population % results of the materials on L929 cells for 24 hours, 1 week and 2 weeks by Muse Cell Analyzer. The figures represent cell populations % as follows: nonapoptotic live (7-AAD negative, Annexin V negative), non-apoptotic dead (7-AAD positive, Annexin V negative), apoptotic live (7-AAD negative, Annexin V positive), and late apoptotic/dead (7-AAD positive, Annexin V positive) cells.

5. MATERIALS AND METHODS

5.1. Cell line and reagents

The L929 mouse fibroblast cell line was obtained from NCTC CLONE 929, ATCC, Germany. The materials; TheraCal, Mineral Trioxide Aggregate, Dermabond and Kerr Life were provided by Bisco Inc USA, Ethicon Inc USA, Cerkamed Poland and Kerr Inc USA respectively. Dulbecco's Modified Eagles Medium (DMEM) was purchased from Gibco USA. The MTT and other cell culture materials were purchased from Sigma-Aldrich, USA and Biological Industries, Israel. Low melting point agarose (LMPA) was obtained from Sigma-Aldrich, USA. Other chemicals such as ethylene diaminetetraacetic acid disodium salt (Na_2EDTA), t-octylphenoxy polyethoxyethanol (Triton X-100), dimethyl sulfoxide (DMSO), Tris (hydroxymethyl) aminomethane (Trizma base), sodium lauroylsarcosinate (sarkosyl) and ethidium bromide were purchased from Sigma-Aldrich, USA.

5.2. Cell culture

The L929 cells were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin. Cultures were supplied with fresh media and incubated in a 37°C incubator (Heal Force HF240, China) with 5% CO_2 and 95% humidity. Trypsin was used to detach confluent cells. Aliquots of separated cells were then passaged. Cell counting and viability were determined using the trypan blue dye exclusion method. The cell suspension was mixed with 0.4% trypan blue (Biological Industries, Israel) in a cryo-vial at a ratio of 1: 1. The mixture was loaded onto a slide. Cells were counted using the automated cell counter (Bio-Rad TC 20, Singapore).

5.3. Preparation of materials

The materials used in the study were prepared in a sterile working cabinet (Esco, Class II Biological Safety Cabinet, Singapore), taking into account the manufacturer's recommendations. The sample size of the materials was determined by aiming to have a surface/medium ratio of 0.5-6 cm²/ml. The materials were placed in the 24-plate and the base was completely covered.

5.3.1. Preparation of MTA (PPH CERKAMED, Stalowa Wola, Poland)

MTA and distilled water were mixed with a sterile metal spatula on sterile glass according to the manufacturer's instructions. The process was continued until the mixture became homogeneous. The obtained mixture was placed in 24-well plates and condensed. The moistened mixture was allowed to harden.

5.3.2. Preparation of Dermabond (Ethicon, Inc., Cincinnati, OH, U.S.A)

This material has a ready-to-use formulation. The material was placed in 24-well plates in a sterile environment.

5.3.3. Preparation of TheraCal (BISCO, Inc., Schaumburg, IL, U.S.A.)

The material was applied evenly and homogeneously to the bottom of the 24-well plates according to the manufacturer's recommendations. A 1 mm thick material applied was polymerized for 20 seconds with a led light source (Woodpecker LED-B Light device, Guilin Woodpecker Medical Industry, Ltd, Guangxi, China). Thereafter the material was applied to the polymerized layer again at a thickness of 1 mm and polymerized for 20 seconds.

5.3.4. Preparation of Kerr Life (Kerr Corp., Orange, CA, U.S.A)

The base to catalyst ratio of 1:1 was mixed on sterile glass with a sterile metal spatula according to the manufacturer's instructions. This process was continued until the mixture became homogeneous. The obtained mixture was placed in 24-well plates and condensed. The moistened mixture was allowed to harden.

All samples placed in 24-well plates were kept at 37 °C, with 95% humidity for 24 hours to harden. A volume of 2 mL DMEM was added to each well on the samples to obtain extracts to be used in cytotoxicity and genotoxicity analysis. To obtain the extracts, the 24-well plate was kept in the oven at 37°C for 24 hours, 1 week and 2 weeks until the cells were applied. After the exposure durations, DMEM which was exposed to the capping materials was passed through filters with a pore size of 0.22 µm.

Application of test chemicals to cells: According to ISO 10993 [25], there are 3 (three) *In vitro* test models used in dental treatment (Figure 5):

1. Direct contact (Direct Method
2. Indirect contact with a barrier (indirect method)
3. Extraction Method (Cells are exposed to extracts from the biomaterials); In our research extraction method was used.
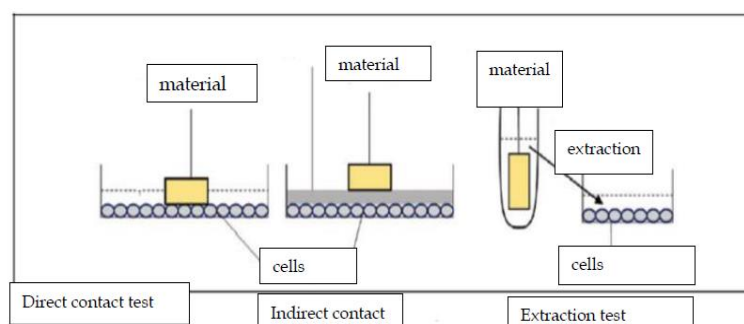

Figure 5. *In vitro* test methods in dental treatment.

5.4. MTT assay

The L929 cells were seeded in 96 well tissue culture plates with 5x10³ cells/100 µL media per well. After 24 hours, the media was aspirated and the cells were exposed to the materials. Cell-free media was placed in

columns 1 and 2, and the cell solution with DMEM was placed in the 3rd column. TheraCal LC was placed in columns 4 and 5, MTA in columns 6 and 7, Dermabond in columns 8 and 9, CaOH$_2$ in columns 10 and 11. Cell-free media was placed in the 12th column. The plates were incubated for 24 hours. After 24 hours the media was aspirated and replaced with new media containing DMEM and MTT solution. A volume of 200 µL solution was added to each well. The new media was prepared by adding 3 ml of MTT solution to 27 ml of DMEM. The plates were incubated for 3 hours and thereafter the formazan precipitate was dissolved in DMSO solution. A volume of 200 µL of DMSO was added to each well. The microplate (Heidolph Titramax 101, Germany) was gently shaken for 10 minutes and the absorbance at 540 nm was measured using a Multi-plate reader (Thermo Scientific Mutiskan Co, Finland). The same procedure was applied to 24 hours, 1 week and 2 weeks of culture wells exposure to the materials tested. Each experiment was performed in triplicate and test results were expressed as the mean percentages of viable cells compared with the negative controls.

5.5. Single cell gel electrophoresis (SCGE, comet) assay

The L929 cells were seeded in a 6 well plate with 2 x 10^5 cells /2 mL media per well. One plate was used as a control. Cells were incubated for 24 hours. After 24 hours of incubation, culture media was replaced with new media exposed to the materials. Thereafter media was removed and trypsin was immediately added with subsequent 3 minutes incubation and the stop medium was added. The media was transferred to centrifuge tubes and centrifuged (Hettrich Rotina 90, Germany) for 6 minutes at 1000 rpm. The supernatant was removed and the cells were washed with PBS.

A volume of 100 µL 0.5% LMPA heated at 37°C was mixed with 50 µL cell suspension. 3-4 drops of the mixture were placed on each slide and covered with coverslips. The slides were previously immersed in a 1% NMA solution. Slides were placed on an iced metal flat surface for 5 minutes followed by the removal of coverslips and the slides were immediately immersed in the fresh lysing solution and placed in the fridge for 1 hour.

After an hour the slides were washed with distilled water and then placed on a horizontal electrophoresis platform and covered with electrophoresis solution which consisted of 300 Mm NaOH, 1 mM Na$_2$EDTA and left for 20 minutes before the current was applied. The electrophoresis solution was 0.5 – 1 cm above the slides. The electric field was then applied for 20 minutes at 25 V and 300 mA.

Slides were rinsed in distilled water for 5 minutes and then in the neutralizing solution for 15 minutes. Thereafter slides were rinsed in distilled water again for 5 minutes. Slides were then exposed to 50%, 70% and 100% ethanol for 5 minutes. Slides were allowed to air dry at room temperature in slide boxes.

For comet analysis 100 nuclei were randomly selected from 2 replicate slides. Ethidium bromide (20 µg/ml) was used as the fluorescent dye. The dye was added to the slides dropwise (approximately 60 µL). The nuclei were examined and photographed through a fluorescence microscope (Leica PM 1000, Germany). The percentage of DNA in the comet's tail, which is an estimation of DNA damage, was analyzed using Comet Assay IV.

5.6. Apoptosis assay

The Muse® Annexin V & Dead Cell Kit was used to determine the apoptotic effects of the materials on L929 cells according to the manufacturer's instructions. Cells were seeded in 12-well plates (2x10^5 cells/well) cultured for 24 hours with DMEM cell culture medium. One column was used as a control. After the incubation period, the DMEM culture medium was discarded and a volume of 1ml culture medium containing the material eluates was added to each well. Media in the control was not discarded. The plates were incubated for 24h. After the completion of the incubation period, the culture medium was removed from the wells and added into centrifuge tubes. A volume of 500 µl was added into each well to detach the cells and incubated for 6 minutes.

After incubation, the cell solution was pipetted into centrifuge cells using a pastor pipette and centrifuged for 5 minutes at 4000 rpm. After centrifuge, the supernatant was removed and 1 ml of DMEM was added and resuspended. A volume of 100 µl of the cell solution was added into an eppendorf followed by the addition of 100 µl of the reagent in the dark. This was left in the dark for 20 minutes at room temperature. It was then kept in the refrigerator at -20 °C. Cells were then analyzed as live, early apoptotic, late apoptotic and dead by Muse Cell Analyzer (Millipore, Germany).

5.7. Statistical analysis

Statistical evaluations were made using the SPSS program (SPSS Windows Release 22.0). Repeated Measures ANOVA test was used for all assays. Comet test results were evaluated by using Tail Intensity values of all the 100 selected nuclei for each sample. The level of statistical significance was set at 5%.

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