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Evaluating cytotoxicity of Thai white portland cement in cell culture using MTT assay author

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Abstract:

Objective: To evaluate the cytotoxicity of two formulations of Thai white Portland cement and Mineral Trioxide Aggregate on human periodontal ligament cells (PDL) using MTT assay.

Materials and Methods: Two formulations of TWPC and WMTA were tested with human PDL cells, cultured in a 24-transwell culture plate. Each test material was mixed with sterile distilled water at a powder:liquid ratio equal to 1 g: 0.35 mL. A 0.2 gram of mixed material was inserted into each chamber insert. No material was put in the chamber insert of the control group. The effect of leachable toxic substances to cells was evaluated after 72 hours of diffusion through 0.45 μm porous membrane. Cytotoxic effect was assessed using the MTT assay. The optical density values of the solution were read by spectrophotometer at a wavelength of 540 nm. The testing was repeated five times.

Results: The percentages of cell viability of WMTA, TWPC1 and TWPC2 groups, when tested with PDL cells were 109.43, 110.67 and 108.42 respectively. No statistically significant differences were found among percentages of cell viability between WMTA and TWPC groups ($p>0.05$) and were not different from the control group as well ($p>0.05$).

Conclusion: Two formulations of TWPC and WMTA were not toxic to human PDL cells at 72 hours exposure.

Key words: cytotoxicity, MTA, Thai white portland cement

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การประเมินพิษของพอร์ตแลนด์ซีเมนต์ขาวไทยต่อเซลล์เพาะเลี้ยงด้วยวิธีเอ็มทีที

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บทคัดย่อ

วัตถุประสงค์: เพื่อประเมินความเป็นพิษของพอร์ตแลนด์ซีเมนต์ขาวไทยสองสูตรและเอ็มทีเอขาวต่อเซลล์เพาะเลี้ยงเอ็นยึดปริทันต์มนุษย์ด้วยวิธีเอ็มทีที

วัสดุอุปกรณ์และวิธีการศึกษา: นำพอร์ตแลนด์ซีเมนต์ขาวไทยสองสูตรและเอ็มทีเอขาวมาทดสอบกับเซลล์เอ็นยึดปริทันต์มนุษย์ที่เพาะเลี้ยงในถาดเพาะเลี้ยงทรานส์เวลล์ 24 หลุม วัสดุทดสอบแต่ละชนิดจะผสมในสัดส่วนผง 1 กรัม ต่อน้ำกลั่น 0.35 มล. นำวัสดุทดสอบที่ผสมแล้วมาจำนวน 0.2 กรัม ใส่ในแชมเบอร์อินเซิร์ท สำหรับกลุ่มควบคุมไม่ใส่วัสดุลงในแชมเบอร์อินเซิร์ท ประเมินความเป็นพิษของวัสดุที่ใช้ทดสอบภายหลังซึมผ่านเมมเบรนแบบรูพรุน 0.45 ไมโครเมตรไปสู่เซลล์เป็นเวลา 72 ชั่วโมง ตรวจสอบความเป็นพิษของวัสดุทดสอบโดยวิธีวิเคราะห์เอ็มทีทีโดยนำไปอ่านค่าดูดกลืนแสงด้วยเครื่องสเปคโตรโฟโตมิเตอร์ที่ความยาวคลื่น 540 นาโนเมตร และทำการทดสอบซ้ำอีกห้าครั้ง

ผลการศึกษา: พบว่าเมื่อนำพอร์ตแลนด์ซีเมนต์ขาวไทยสองสูตรและเอ็มทีเอขาวที่นำมาทดสอบกับเซลล์เอ็นยึดปริทันต์มนุษย์ พบเซลล์มีชีวิตร้อยละ109.43 110.67 และ 108.42 ตามลำดับ และไม่พบความแตกต่างของร้อยละของเซลล์ที่มีชีวิตในกลุ่มพอร์ตแลนด์ซีเมนต์ขาวไทยทั้งสองสูตรและเอ็มทีเอขาวอย่างมีนัยสำคัญ ($p>0.05$) และสารทดสอบทั้งหมดพบจำนวนของเซลล์ที่มีชีวิตไม่แตกต่างจากกลุ่มควบคุม ($p>0.05$)

บทสรุป: พอร์ตแลนด์ซีเมนต์ขาวไทยสองสูตรและเอ็มทีเอขาวไม่มีความเป็นพิษต่อเซลล์เอ็นยึดปริทันต์มนุษย์

รหัสคำ: ความเป็นพิษ, พอร์ตแลนด์ซีเมนต์ขาวไทย, เอ็มทีเอขาว

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Introduction

Endodontic surgery is commonly performed to eliminate irritants from root canal and to seal the apical portion of the root canal. In some situations, root perforation occurs: it may cause failure of root canal treatment and result in necessity to repair by a biocompatible material to maintain healthy tissue. Several materials have been suggested for endodontic surgery and root perforation such as amalgam, zinc oxide eugenol cement, glass ionomer and composite resin[1]. However, main disadvantages of these materials, including microleakage, toxicity and moisture sensitivity, may decrease the percentage of success rate in surgical and perforated cases.[2]

Mineral trioxide aggregate (MTA) is a current material for root-end filling and perforation repair because of its superiority over other materials, including sealing ability[3], antimicrobial properties[4] and biocompatibility[5-6]. MTA has also been used as an alternative material for endodontic applications such as capping material in mechanically exposed pulp[7], root perforation repair[8] and pulpotomy[9]. ProRoot MTA (Dentsply Endodontics, Tulsa, OK, USA) is composed of 75% Portland cement (PC), 5% gypsum and 20% bismuth oxide. The patent states that MTA is a Type I PC with added bismuth oxide for dental radiological diagnosis[10]. Gray MTA (GMTA) has been available and white MTA (WMTA) is more recently produced by exclusion of iron compounds. It contained smaller particles than that of GMTA, and is accounted for its improvement in clinical properties such as esthetic concern.

Comparative studies between MTA and PC have shown similar compositions of MTA and PC, except for bismuth oxide which is purposely added to MTA for radiopacity[11-12]. The X-Ray diffraction analysis also showed that they were composed of tricalcium silicate, dicalcium silicate, tricalcium aluminate and tetracalcium aluminoferrite[12]. Funteas *et al.*[13] reported that there were no significant difference in 14 elements of MTA and PC using ICP-ES. Islam *et al.*[14] compared the physical properties, e.g. pH, radiopacity, setting time, solubility, dimensional change and compressive strength between MTA and PC. The result showed that physical properties of MTA and PC were similar. However, the radiopacity of MTA was higher than that of PC. Scanning electron microscope examination of the particle size of MTA and PC showed that PC was composed of particles with a wide range of sizes whereas MTA showed uniform particle size[15].

Several studies have compared the biological effects of MTA and PC. Both MTA and PC have been shown to be biocompatible[16-18]. De Deus *et al.*[16] evaluated toxicity of Pro-Root MTA®, MTA Angelus® and PC by MTT assay which showed a similar cytotoxic effect that decreased gradually with time. Subcutaneous connective tissue reactions of PC and MTA in rats showed that they were biocompatible, and no difference was found between 7 and 60 days[17]. Sakai et al[18] compared the clinical and radiographic effectiveness of MTA and Portland cement as a pulpotomy dressing agent in carious primary teeth. All pulpotomised teeth were clinically and radiographically successful at 6, 12, 18 and 24-month follow-up appointments. No statistically significant difference regarding dentine bridge formation was found between MTA and Portland cement. Based on these results, the potential of PC has been considered as an alternative material to MTA for dental applications.

In recent times, the chemical compositions and physicochemical properties of Thai white Portland cement (TWPC; Research unit, Faculty of Dentistry, Mahidol University, BKK, Thailand) were investigated[19]. It appeared that the main compounds found in the TWPC and WMTA were similar, except bismuth oxide in WMTA. In a study of TWPC as an alternative material

to WMTA in endodontic applications, toxicity testing must be conducted to assure its safety for human use. Therefore, the objective of this study was to evaluate cytotoxicity of two formulations of TWPC in cell culture using MTT assay.

Materials and methods

Preparation of culture cells

Human periodontal ligament (PDL) cells were obtained as described by Vajrabhaya *et al.*[20] Briefly, PDL cells of human lower third molars were obtained from the Oral Surgery Department of Mahidol University. The teeth must be neither carious nor periodontal involved, and extraction was performed atraumatically. Immediately after extraction, the teeth were placed in tissue culture tubes containing 10 mL of Dulbecco's modification of Eagle's medium (DMEM; Gibco BRL, Grand Island Biological Co,Grand Island, NY, USA) supplemented with 10% fetal calf serum and antibiotics (Penicillin G 200 μg/mL + streptomycin 200 μg/mL + fungizone 2 μg/mL) in order to prevent contamination. Then, the tissue culture tubes were placed on ice for cell preservation and immediately transferred to the laboratory.

PDL tissue at the middle of the root was gently removed with a scalpel, and was transferred to a 2.5 cm diameter sterile Petri dish contained 1 mLl of DMEM. The Petri dish was maintained at 37 °C, 5% CO2 and 100 % humidity in an incubator. The cell culture was examined daily under an inverted microscope (Nikon Model TMS, Kanagawa, Japan) to assess the fibroblast-like cells profusely proliferating from the explant and to observe any sign of contamination. After a confluent monolayer was obtained, cells were subcultured into a 50 mL tissue culture flask (Costar, Corning Life Sciences, Acton, MA, USA) by trypsinization. The medium was changed every other day. After reaching confluency, first passage cells were trypsinized and subcultured until sufficient cells were obtained. Passage 4 to 10 of the PDL cells were used in this experiment.

Test materials preparation

The study materials were ProRoot white MTA (WMTA; Dentsply Tulsa Dental, Tulsa, Okla, USA.) and Thai white Portland cement (TWPC; Research unit, Faculty of Dentistry, Mahidol University, BKK, Thailand). Two formulations with different ratios of TWPC powder and bismuth oxide powder (Schariau Chemic SL, Spain) were prepared as formulation 1 ($TWPC_1$) and 2 ($TWPC_2$). All experimental materials were disinfected by ultraviolet light for 24 hours before starting the experiment. WMTA was mixed with distilled water at a powder:liquid ratio equal to 1 g: 0.35 mL on the sterile glass slab to produce a homogenous paste according to the manufacturer's instructions. The TWPCs were mixed in the same manner as WMTA.

Testing procedure

The subcultured human PDL cell density was adjusted to 10^5 cells/mL. The 200 μL of cell suspension was seeded into a 24- transwell culture plate (Transwell Clear, Corning, NY, USA, 6.5 mm diameter, 0.45 μm pore size) and 800 μL of culture medium was added to each well. The transwell culture plate was placed in the incubator for 24 hours to obtain a monolayer cell growth and cell attachment. The culture medium from each well was removed, and cells were washed with 1 mL of sterile phosphate buffered saline (PBS) solution twice, then one mL of culture medium was replenished in each well. Each freshly mixed material of 0.2 g was inserted into the bottom of the chamber insert (Fig.1). The 24 transwell culture plate was divided into four parts with three wells each for containing MTA, $TWPC_1$, $TWPC_2$ and control group. Three wells were included for each

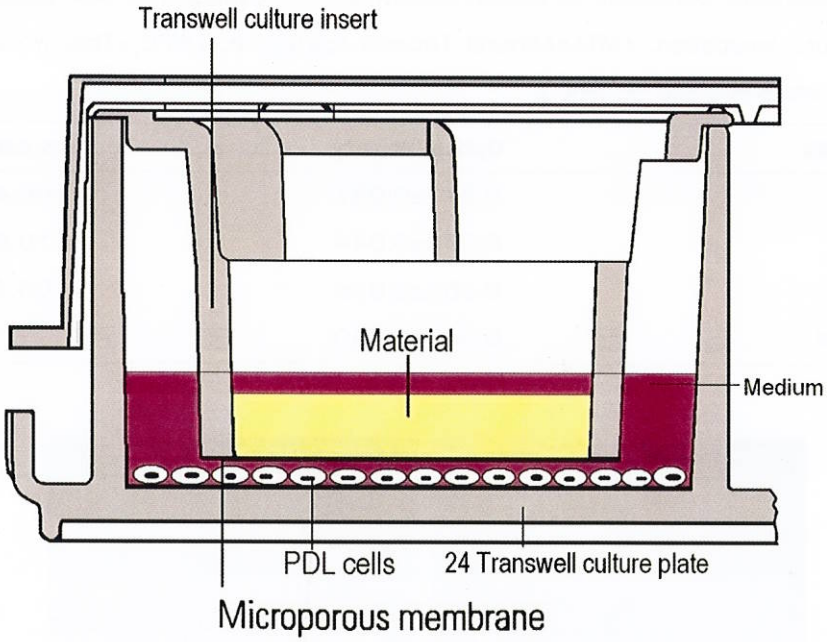

Fig. 1 Scheme of Transwell culture system

experimental material. The control group contained the transwell culture insert without materials. The chamber inserts with all groups were transferred immediately to the transwell culture plate and incubated for 72 hours. During incubation, cells were exposed to the leachable components from the test material. After 72 hours exposure, chamber inserts were removed from the transwell culture plate. The culture medium in each well was removed and cells were washed with 1 mL of sterile PBS solution twice. Then 500 µL of 50% MTT in complete medium solution (DMEM supplemented with fetal calf serum and antibiotics) was added to each well, and the plate was placed in a 37°C incubator for 3 hours. After incubation, the MTT solution in the well was discarded, and the well was washed twice with PBS solution. Then 500 µL of dimethyl sulfoxide was added to each well to dissolve formazan from cells. The plate was shaken for 30 minutes to achieve uniform color. Two hundred microliters of solution from each well were drawn and placed into a 96 well tissue culture plate. The optical density values of the solution in 96 well tissue culture plate were read using a Micro plate reader (BIO-TEK Instrument Inc, VT, USA) at a wavelength of 540 nm. The testing was repeated five times.

Statistical analysis

Statistical difference of optical density values among the four groups was performed with non-parametric multiple comparisons by Kruskal-Wallis test at 95% level significance. The percentage of cell viability in each experimental material was calculated as percentages of the control by the equation below.

$$\text{Percentage of cell viability} = \frac{\text{OD of test material} - \text{OD of DMSO}}{\text{OD of control} - \text{OD of DMSO}} \times 100$$

Results

The means and standard deviation of optical density after contacting cells for 72 hours with leachable substances from the experimental groups and

Table 1 Means and standard deviations of optical density and percentages of cell viability of test materials after 72 hours incubation. (MTA=Mineral Trioxide Aggregate, $TWPC_1$=Thai white Portland cement$_1$, $TWPC_2$= Thai white Portland cement$_2$)

Materials	Optical density	% cell viability
MTA	0.371±0.037	109.43±12.63
$TWPC_1$	0.373±0.044	110.67±14.69
$TWPC_2$	0.367±0.036	108.42±12.04
Control	0.343±0.050	100

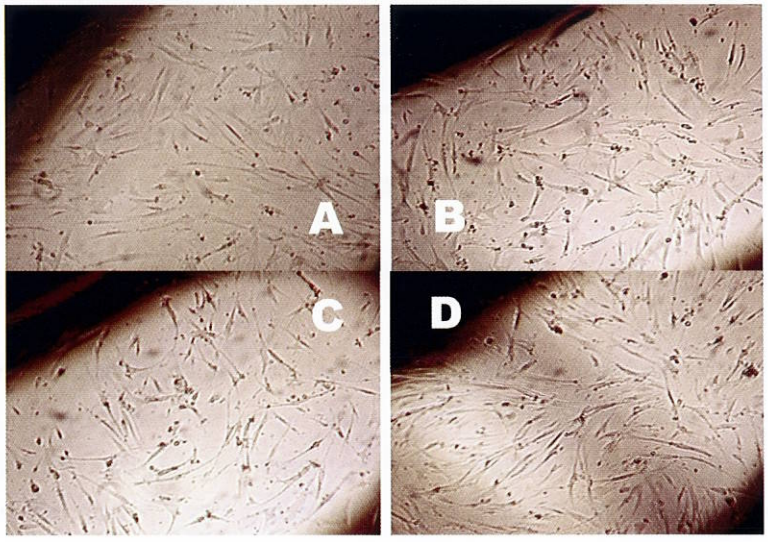

Fig. 2 Cell appearance under inverted microscope (4X) before testing with different materials: (A) Control (B) WMTA (C)$TWPC_1$ (D)$TWPC_2$

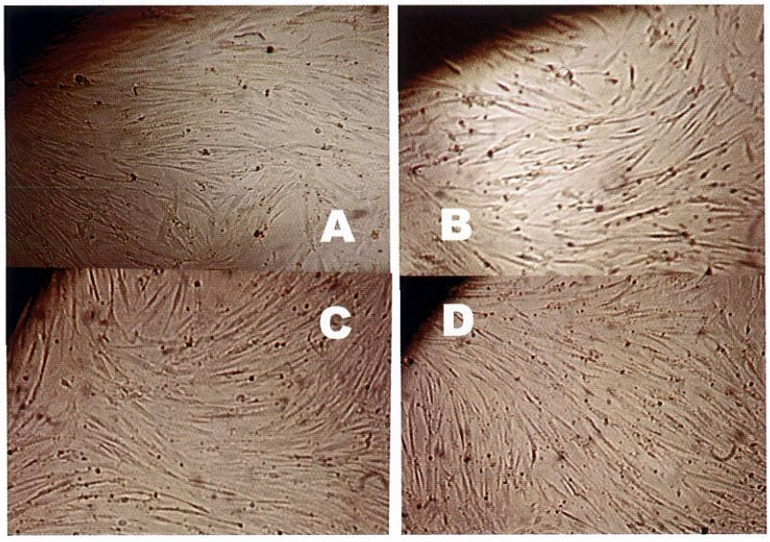

Fig. 3 Cell appearance under inverted microscope (4X) after testing with different materials: (A) Control (B) WMTA (C)$TWPC_1$ (D)$TWPC_2$

percentages of cell viability are shown in Table 1. There were no statistically significant difference in optical density among the control and experimental groups were found ($p>0.2$).

Cell growth and morphology were assessed under an inverted microscope. Before exposure to material, cells were monolayer and had a spindle-shaped appearance with central nucleus (Fig.2). After 72 hours incubation period, cells were spreading and forming to a confluent monolayer of spindle-shaped cells over the bottom of the culture dish. Cell density in the control group and experimental groups at the end of the experiment were increased (Fig.3).

Discussion

Cytotoxicity testing by using cell culture is one of the most favorable in vitro techniques. The advantages of this method are that it is easy to perform, less expensive, and reduces the number of animals that need to be tested. A transwell culture system as described by Safavi *et al.*[21] which used in this study has advantages. Firstly, it allows the diffusion of materials or leachable compounds through a porous membrane as it simulates the clinical situation, where toxic substances leach directly into the surrounding periodontal tissue. Secondly, it continuously allows for leachable compounds to reach the cells over time. The quantity of the material in this study was considered on the basis of the approximate amount used clinically. PDL cells, which are the major cells in wound healing after root tip resection, were chosen to simulate the clinical environment.

In toxicity tests of dental materials by cell culture, the materials can be tested either in freshly mixed state or set state. The freshly mixed MTA was found to be more cytotoxic than in the set state, and the toxicity decreased rapidly after material setting[22]. It is possible that the leachable compounds from test material could diffuse to the cells during the setting process. For this reason our studies used freshly mixed samples to evaluate cytotoxicity of TWPC. The result in this study showed that freshly mixed MTA and TWPC were not toxic to cultured cells. Our findings are agreement with Yasuda *et al*[23], who tested the cytotoxicity of freshly mixed MTA on rat dental pulp cells by using the MTT assay method, and found that freshly mixed MTA was not toxic to those cells.

In this study, the percentage of cell viability was assessed by MTT method as described by Mosmann[24]. This assay determines the capacity of mitochondrial dehydrogenese enzymes of viable cells to convert the yellow tetrazolium salt (3-[4, 5-dimethyldiazol-2-yl]-2,5-diphenyl tetrazolium bromide/ MTT) to insoluble formazan crystals. The advantage of this method is simplicity, speed, precision and it requires no radioisotope. This assay determines the remaining vital cells after contact with test materials for 3 days. The result showed that the percentages of cell viability in TWPC groups were not significantly different from that of MTA group. The result was similar to previous studies, although the test method was different. Camilleri *et al.*[25] compared the biocompatibility of MTA and Portland cement using extracts from both materials, and cytotoxicity was assessed using the MTT assay. The result showed that both materials had similar biocompatibility and cell activity significantly increased when compared with control medium ($p<0.05$). Supported by SEM analysis, Abdullah *et al.*[26] reported that cells in contact with Portland cement showed good biocompatibility and cell confluence. These results imply that TWPC, which has similar constituents to MTA, is a non-toxic material and that TWPC could be considered as an alternative material to WMTA for root-end filling and perforation repair. Some reports hypothesized that biocompatibility of MTA was derived from calcium

hydroxide formation as a by-product of the hydration reaction of MTA[27]. Others reported that the biocompatibility of MTA was attributed to the formation of hydroxyapatite-like appearance between MTA and tooth structure[28]. In a previous study, Abdullah *et al*[26] observed that PC promoted the proliferation of SaOS-2 cells in vitro and stimulated a biological response in these cells through the production of cytokines and a bone-specific protein. The expression of cytokines in the presence of MTA and PC suggests that not only is it very biocompatible, but also it may have the potential to promote bone healing.

PC toxicity is from elements that may contaminate the material during the manufacturing process or are present in the raw material. The elements that are potentially toxic are heavy metals and arsenic. ISO 9917-1 29, entitled Dental Water-based Cements, has a limit of arsenic level (<2 mg/kg) for PC. One study of arsenic in TWPC found that the arsenic concentrations in MTA and TWPC were 0.64 and 1.50 mg/kg and were below the limit set in the ISO standard[30]. From this result it can be concluded that TWPC is not contraindicated for dental use in terms of the presence of arsenic.

The amount of heavy metal elements in MTA and PC has been previously reported[13, 15, 19]. MTA and PC contained similar heavy metal levels[19]. The heavy metal contamination in a material can probably affect the biological properties of material. However, the excellent biocompatibility of MTA and PC has been demonstrated in several studies[16-18]. It can be concluded that the small amount of arsenic and metal elements in TWPC was too little to be critical in a cytotoxicity study, as there was no measurable toxicity to human PDL cells after 72 hours exposure.

The different amount of bismuth oxide added in TWPCs showed no statistically significant effect on cell response ($p>0.05$). In contrast, a biocompatibility study of pure bismuth oxide demonstrated no cell growth over bismuth oxide [31] and bismuth oxide was not a biocompatible material. In this study, normal cell proliferation appeared when a small amount of bismuth oxide was added to the TWPCs, and showed no cytotoxic effect. However, if the amount of bismuth oxide in TWPCs increased, its toxicity may become a concern.

Conclusion

Two formulations of Thai white Portland cement and MTA were not toxic to human PDL cells at 72 hours exposure.

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