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ORIGINAL ARTICLE

An evaluation of the inflammatory response of lipopolysaccharide-treated primary dental pulp cells with regard to calcium silicate-based cements

Wei-Yun Lai[1,2], Chia-Tze Kao[1,2], Chi-Jr Hung[1,2], Tsui-Hsien Huang[1,2,*] and Ming-You Shie[3,*]

This study compared the biological changes of lipopolysaccharide (LPS)-treated dental pulp (DP) cells directly cultured on mineral trioxide aggregate (MTA) and calcium silicate (CS) cements. DP cells were treated with LPS for 24 h. Then, the LPS-treated DP cells were cultured on MTA or CS cements. Cell viability, cell death mechanism and interleukin (IL)-1β expressions were analysed. A one-way analysis of variance was used to evaluate the significance of the differences between the means. A significantly higher IL-1β expression (2.9-fold) was found for LPS-treated cells ($P<0.05$) compared with DP cells without LPS treatment at 24 h. Absorbance values of LPS-treated cells cultured on CS cement were higher than a tissue culture plate. A significant difference ($P<0.05$) in cell viability was observed between cells on CS and MTA cements 24 h after seeding. At 48 h, a high concentration of Si (5 mM) was released from MTA, which induced LPS-treated DP cell apoptosis. The present study demonstrates that CS cement is biocompatible with cultured LPS-treated DP cells. MTA stimulates inflammation in LPS-treated DP cells, which leads to greater IL-1β expression and apoptosis.
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Keywords: calcium silicate cement; dental pulp cell; mineral trioxide aggregate; immune response; interleukin-1β

INTRODUCTION

Mineral trioxide aggregate (MTA) is a root repair material that has several clinical applications in endodontics.[1] MTA is a mixture of 75% Portland cement, 20% Bi_2O_3 and 5% gypsum ($CaSO_4 \cdot 2H_2O$). It received the approval of the US Federal Food and Drug Administration in 1998.[1] Several studies have shown that MTA has better biocompatibility than other materials used in root-end fillings and root repairs.[2–5] It has also been shown to enhance hard-tissue formation.[6] The components of MTA are calcium silicate, tricalcium aluminates, tetracalcium aluminoferrite and gypsum.[7] A new calcium silicate (CS) cement containing CaO, SiO_2, Al_2O_3 and ZnO, which was developed by our laboratory has a shorter setting time (13 min) than MTA (151 min).[8] When hardened with water, CS cement has a similar elemental composition to set MTA. CS cement not only exhibits acceptable osteoconduction effects,[9–10] but also reduces the inflammation of primary dental pulp (DP) cells.[11]

In apicoectomy, the apex's surrounding tissue environment is related to bacteria-induced local metabolic acidosis. The inflammatory responses of different tissues to MTA were evaluated in a BALB/c mouse injection study; when MTA (25 mg per cavity) was injected into normal and pre-treated peritoneal cavities, it induced neutrophil recruitment.[12–13] An MTA implant study using Wistar albino rats showed that inflammatory cells surrounded the implant by the sixtieth day.[14] Moreover, inflamed cell apoptosis was associated with environmental factors.[15] White-coloured MAT (WMAT) mixed with H_2O has a deleterious effect on physical and chemical properties under acidic conditions.[16] The number of ions dissolved from MTA increased under a low pH physiological buffer.[16] More Si ions can be created via dissolution from a silicate-based material; this procedure appears to stimulate cell apoptosis.[17–18] Therefore, the use of lipopolysaccharide (LPS)-treated cells to evaluate material biocompatibility is close to clinical use.

Our previous study demonstrated that CS has better cytocompatibility than MTA.[9] However, little information is available regarding the involvement of MTA or CS cement in the cell behaviour of inflammatory cells or tissue. The present study investigates the inflammatory marker interleukin (IL)-1β and the biological changes of LPS-treated DP cells directly cultured on MTA or CS cements.

MATERIALS AND METHODS

DP cell isolation and culture

The DP cells were freshly derived from caries-free, intact premolars extracted for orthodontic treatment, as previously described.[19] The patients provided informed consent, and approval from the Ethics

[1]School of Dentistry, Chung Shan Medical University, Taichung City, Taiwan; [2]Department of Dentistry, Chung Shan Medical University Hospital, Taichung City, Taiwan and [3]Institute of Oral Science, Chung Shan Medical University, Taichung City, Taiwan
*These authors contributed equally to this work.
Correspondence: Dr TH Huang, School of Dentistry, Chung Shan Medical University, Taichung City 402, Taiwan
E-mail: thh@csmu.edu.tw
Dr MY Shie, Institute of Oral Science, Chung Shan Medical University, Taichung City 402, Taiwan
E-mail: eviltacasi@gmail.com
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Committee of the Chung Shan Medicine University Hospital was obtained (CSMUH No. CS11187). The tooth was split sagittally with a chisel. The pulp tissue was then immersed in a phosphate-buffered saline (PBS; Caisson, North Logan, UT, USA) solution and digested in 0.1% collagenase type I (Sigma-Aldrich, St Louis, MO, USA) for 30 min. After being transferred to a new plate, the cell suspension was cultured in Dulbecco's modified Eagle's medium (DMEM; Caisson, North Logan, UT, USA), supplemented with 20% foetal bovine serum (FBS; Caisson, North Logan, UT, USA), 10 $U \cdot mL^{-1}$ penicillin G solution and 10 $g \cdot L^{-1}$ streptomycin (Caisson, North Logan, UT, USA) in a humidified atmosphere with 5% CO_2 at 37 ℃; the medium was changed every 3 days. The cells were subcultured via successive passaging at a 1 : 3 ratio until they were used for the experiment (passages 3–8). Then, the DP cells were cultured on tissue culture plates and pre-treated with 200 $\mu g \cdot L^{-1}$ LPS (Sigma-Aldrich, St Louis, MO, USA) in DMEM for 12 and 24 h.

IL-1β expression

Western blot was used to evaluate IL-1β protein expression in LPS-treated DP cells. After the incubation period, the DP cells were lysed in a buffer and centrifuged at 16 000*g* for 30 min at 4 ℃. The protein concentration of the lysates was measured using the DC Protein Assay kit (Bio-Rad, Richmond, CA, USA). These protein samples (25 μg) were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and incubated with primary antibodies against IL-1β (GTX22105, 1 : 3 000; GeneTex, San Antonio, TX, USA) and β-actin (GTX300041, 1 : 5 000; GeneTex, San Antonio, TX, USA). Membranes were washed with PBS-T (PBS containing 0.1% Tween 20) and incubated using the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h. After the incubation period, the membranes were washed with PBS-T and incubated on an enhanced chemiluminescent substrate. The stained bands were scanned and quantified using a densitometer (Syngene Bioimaging System, Syngene, Frederick, MD, USA) and Scion Image software (Scion Corp., Frederick, MD, USA).

Calcium silicate-based specimen preparation

White-coloured ProRoot MTA (Dentsply, Tulsa, OK, USA) and CS (composition: 65% CaO, 25% SiO_2, 5% Al_2O_3 and 5% ZnO) cements[8] were used in present study. In brief, appropriate amounts of as-received SiO_2, CaO, Al_2O_3 and ZnO powders were mixed at 1 000 $r \cdot min^{-1}$ for 10 min using a hybrid-defoaming mixer (ARE-250; Thinky, Tokyo, Japan). The oxide mixtures were then sintered at 1 400 ℃ for 2 h using a high-temperature furnace. The sintered granules were then ball-milled in ethyl alcohol using a centrifugal ball mill (S 100; Retsch, Hann, Germany) for 6 h. The CS powder (0.6 g) was mixed using a liquid/powder ratio of 0.35 $mL \cdot g^{-1}$. After mixing, the cement fully covered each well of the 24-well plate (GeneDireX, Las Vegas, NV, USA) to a thickness of 2 mm, and the plate was stored in an incubator at 100% relative humidity and 37 ℃ for 1 day of hydration. According to the MTA manufacturer's instructions, a liquid/powder ratio of 0.3 $mL \cdot g^{-1}$ was used to mix the cement. Before the experiments, all specimens were sterilised via soaking in a 75% ethanol solution and exposure to ultraviolet light for 1 h.

Cytotoxicity

LPS-treated DP cell suspensions (at a density of 10^4 cells per mL) were directly seeded over each specimen and incubated at 37 ℃ in a 5% CO_2 atmosphere; the medium was changed every 3 days. After different culturing times, cell cytotoxicity was evaluated using the PrestoBlue assay (Invitrogen, Grand Island, NY, USA). The reagent PrestoBlue was used for real-time and repeated monitoring of cell proliferation; this method is based on the detection of mitochondrial activity. At the end of the culture period, the medium was discarded and the wells were washed with PBS. Each well was filled with a 1 : 9 solution of PrestoBlue in fresh DMEM and incubated at 37 ℃ for 30 min. The solution in each well was transferred to a new 96-well plate. The plates were analysed using a multiwell spectrophotometer (Hitachi, Tokyo, Japan) at 570 nm with a reference wavelength of 600 nm. Cells cultured on a tissue culture plate were used as a control. The results were obtained in triplicate from six separate experiments for each test.

Ion concentrations

Si and Ca ion concentrations from the DMEM (after being cultured with LPS-treated DP cells on MTA and CS cements) were analysed using an inductively coupled plasma-atomic emission spectrometer (OPT 1MA 3000DV; Perkin-Elmer, Shelton, CT, USA) at 12, 24 and 48 h. Six samples were measured for each data point.

Apoptosis and necrosis analysis

Annexin V/propidium iodide (PI) staining confirmed that a significant proportion of treated cells displayed phosphatidylserine externalisation during early phase apoptosis (Ann^+, PI^-). The numbers of cells that were viable (Ann^-, PI^-), exhibited late apoptosis (Ann^+, PI^+) and necrosis (Ann^-, PI^+) were determined accordingly.[1,20] At 12, 24 and 48 h, floating and adherent cells were collected, centrifuged and fixed in cold ethyl alcohol at −20 ℃ overnight. The adherent cells were detached using trypsin-EDTA (Caisson, North Logan, UT, USA). Then, cell samples were collected, centrifuged and stained with PI and annexin V–fluorescein isothiocyanate (Invitrogen, Grand Island, NY, USA) in 1 mL of PBS in the dark at 4 ℃. The resulting cell suspension was analysed using a flow cytometer (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ, USA). The sample was evaluated using Win MDI 2.8 software (Scripps Research Institute, La Jolla, CA, USA). The average of six assays was recorded.

Data analyses

A one-way analysis of variance was used to evaluate the significance of the mean differences between the experiment groups and the control. Scheffe's multiple comparison test was used to determine the significance of the deviations in the data for each specimen. In all cases, the results were considered significant at a P value of <0.05.

RESULTS

LPS-induced DP cell inflammation

The effects of LPS on DP cell IL-1β expression are shown in Figure 1. IL-1β expression increased with increasing DP cell culture time ($P<0.05$). The 24-h group showed a 1.7-fold higher IL-1β expression ($P<0.05$) than the 12-h group.

DP cell viability

The viabilities of LPS-treated DP cells cultured on MTA or CS cements are shown in Figure 2. LPS-treated cell viability was not significantly different ($P>0.05$) among the three groups after 12 h of culturing. A significant difference ($P<0.05$) in the DP cell viability on CS and MTA cements was observed at 24 and 48 h after seeding. The DP cell viability of the MTA group was the lowest at 48 h ($P<0.05$).

Ion concentration

Si and Ca ion concentrations in DMEM (after being cultured with LPS-treated DP cells) on MTA and CS cements for various time

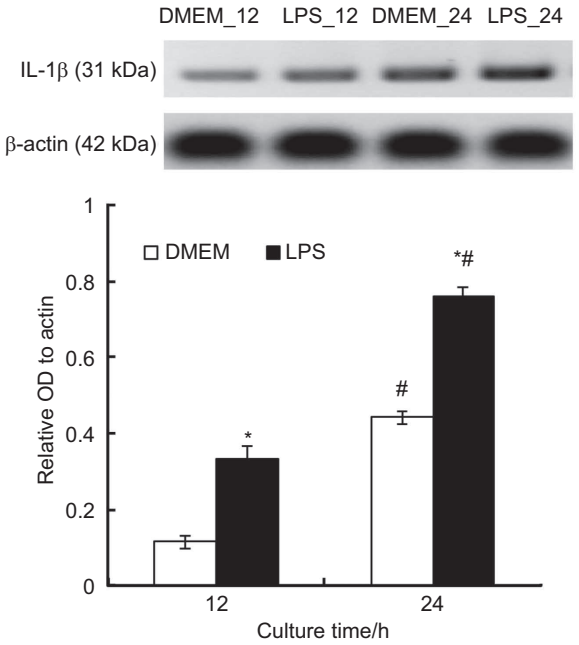

Figure 1 Comparing the IL-1β expression of DP cells treated with or without LPS (200 μg·L^{-1}) for 12 and 24 h. *P<0.05, compared with DMEM. #P<0.05, compared with 12 h. DMEM, Dulbecco's modified Eagle's medium; DP, dental pulp; IL, interleukin; LPS, lipopolysaccharide; OD, optical density.

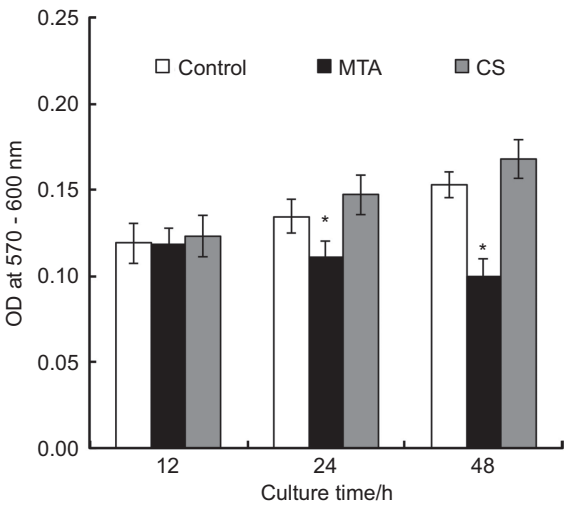

Figure 2 PrestoBlue assay for LPS-treated DP cell viability on MTA or CS cements at various time points. *P<0.05, compared with the control. CS, calcium silicate; DP, dental pulp; LPS, lipopolysaccharide; MTA, mineral trioxide aggregate; OD, optical density.

periods are shown in Figure 3. After 12 h, the Si concentrations in the MTA group (4.6±0.2) mmol·L^{-1} were significant higher than those in the CS group ((3.0±0.1) mmol·L^{-1}; P<0.05). Si concentrations increased with incubation time (Figure 3a). The Ca ion concentration of the medium increased after 12 h of incubation and then decreased to approximately 0.9 mmol·L^{-1} after 48 h, which was lower than the baseline Ca concentration of DMEM (1.8 mmol·L^{-1}; Figure 3b).

Cell death analysis

The numbers of LPS-treated DP cells on control and CS substrates did not significantly differ at any time point (Figure 4). A significant reduction was observed with regard to the living cells in the MTA group, which dropped to 70.27% at 48 h from 93.16% at 12 h (P<0.05). Compared with LPS-treated cells, the percentage of Ann

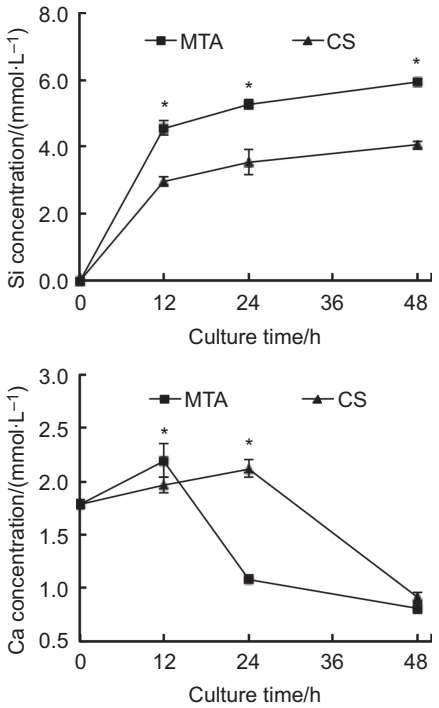

Figure 3 Si and Ca ion concentrations of LPS-treated DP cells cultured on MTA and CS cements, respectively, at various time points. *P<0.05, compared with CS. CS, calcium silicate; DP, dental pulp; LPS, lipopolysaccharide; MTA, mineral trioxide aggregate.

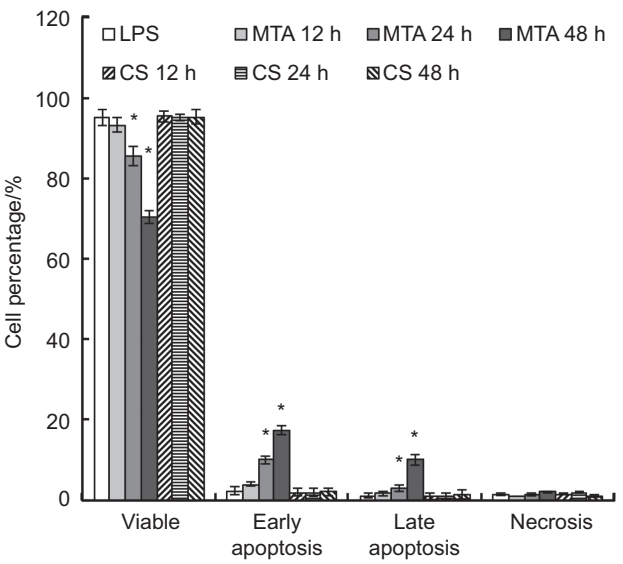

Figure 4 Quantitative analysis results of the LPS-treated DP cells cultured on MTA or CS cements at various time points. *P<0.05, compared with the LPS-group. CS, calcium silicate; DP, dental pulp; LPS, lipopolysaccharide; MTA, mineral trioxide aggregate.

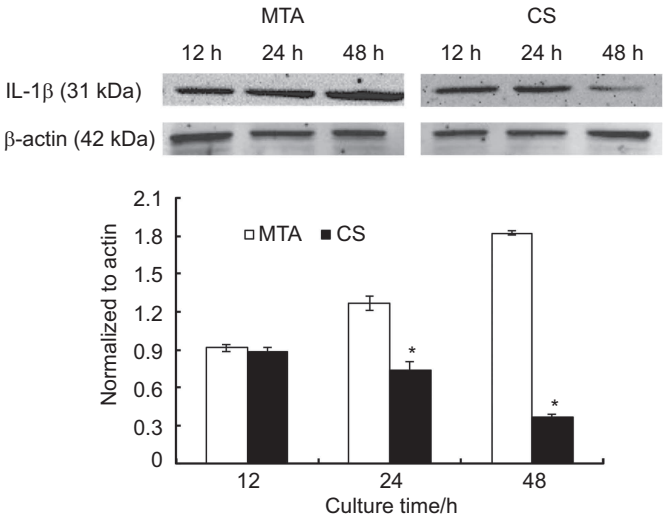

Figure 5 IL-1β expression of LPS-treated DP cells cultured on MTA or CS cements. *$P<0.05$, compared with MTA. CS, calcium silicate; DP, dental pulp; LPS, lipopolysaccharide; MTA, mineral trioxide aggregate.

V-positive cells increased significantly ($P<0.05$) after being cultured on MTA for 24 h (3.8-fold) and 48 h (8.0-fold). In contrast, no differences were observed between the proportions of necrotic cell deaths for cells on MTA or CS cements at any time point ($P>0.05$).

IL-1β expression

At 12 h, IL-1β expressions did not significantly differ ($P>0.05$) between cells on MTA and CS cements (Figure 5). The IL-1β expression of the MTA group increased with culturing time. In contrast, IL-1β expression decreased after culturing on the CS cement for 24 and 48 h. The IL-1β expression level for the CS group was significantly lower ($P<0.05$) than that of the MTA group at both 24 and 48 h.

DISCUSSION

Ideal root-end filling materials should possess acceptable biocompatibility. Cell exposure to certain root-end filling materials results in immunotoxic effects.[1,21] Biomaterials are foreign to the host body, and the magnitude and duration of the inflammatory process directly affect biocompatibility.[2–4,10,22] The pH value of the environment surrounding the materials can change from neutral to acidic due to inflamed tissue-induced local acidosis.[6,23] Apoptosis due to the inflammatory cell itself is associated with environmental factors.[7,15] Therefore, the mechanism associated with the root-end filling material effects on inflammatory tissue should be clarified.

The physical and chemical properties of MTA and CS cements are similar.[8–9,11] Low solubility is a necessary characteristic for a root-end filling material with regard to successful endodontic therapy. Ca^{2+} release from MTA was faster than that from CS cement at 12 h. In addition, the initial increase of Ca ion concentration corresponded to the dissolution of $Ca(OH)_2$ and an increase in the pH of the environment.[9,24,25] Changes in extracellular pH are likely to induce cell death because they change the potential across the cell membrane, and ion exchange systems could be inhibited. A high Ca^{2+} concentration can induce apoptosis and DNA fragmentation.[10,26,27] This effect might result in a build-up of intracellular pH and protein denaturation.[12,28]

LPS is an outer membrane component of a Gram-negative bacteria that induces several inflammations, such as matrix metalloproteinase (MMP)-2 and MMP-9, and many cytokines (IL-1β, IL-6 and so on) in inflamed cells, thereby leading to the destruction of host tissues.[14,29,30] IL-1β production in DP cells can be stimulated using LPS,[14,29] and IL-1β is highly expressed in inflamed pulp tissues.[15,31] Bacterial components might also play an important role in pulpitis.[15,29] Therefore, the present study stimulated DP cell inflammation with LPS for 12 and 24 h, and compared IL-1β expression in LPS-treat DP cells with that of untreated cells. IL-1β formation increased with culture time. The increase in IL-1β molecule formation in DP cells indicates the presence of inflammation.[17,18,32]

The viability of LPS-treated DP cells cultured on CS cement was greater than that of cells cultured on MTA for all time points.[8,11] Interestingly, the number of LPS-treated DP cells cultured on CS cement increased with culturing time. In contrast, MTA gradually reduced LPS-treated DP cell viability up until 48 h. MTA might have released Si ions into the medium, thereby leading to LPS-treated DP cell death. After cell culturing on MTA for 24 and 48 h, the dissolved Si concentration in DMEM was higher than 5 $mmol \cdot L^{-1}$, enabling the induction of cell apoptosis.[18] Bioactive silicate-based substrates (which release Si ions) can enhance new hard tissue formation.[33] Si ion concentration is a key factor in cell behaviour.[17,34] The dissolution of silanol (Si–OH) formed an amorphous SiO_2 phase that increased the Si ion concentration of the culture medium.[35] The medium might have contained an excess of osmolytes (inorganic ions) after cell culturing on MTA, and cells can be stimulated to apoptosis in hyperosmolality.[36] Si ion concentration might regulate apoptosis, proliferation and other cell behaviours.[18] However, the high number of Si ions released from MTA caused hyperosmoticity in the medium, which induced IL-1β expression, possibly via the p38 pathway.[37]

IL-1β expression was high in LPS-treated DP cells, and this expression increased with time among cells cultured on MTA; furthermore, this expression was greater than that of cells on CS cement after culturing for 24 and 48 h. MTA might have induced the acute inflammatory response via the calcium oxide dissolved in the body's fluids,[8] which can increase the pH surrounding the tissue and stimulate IL-1 and inducible nitric oxide synthase (iNOS) expression.[38] Similarly, an MTA implant study using Wistar albino rats showed that more inflammatory cells surrounded the implant at the sixtieth day.[14] Angelus MTA caused moderate reactions at day 7, and these reactions decreased with time.[39] The CS cement caused LPS-treated DP cells to moderately react after day 1; however, the inflammation marker decreased with time.[11] These results consistently indicated that an appropriate Si concentration released from CS cement effectively supports the proliferation of DP cells and reduces the biological response of cells through the production of inflammation proteins.

In conclusion, the present study demonstrated that MTA stimulates LPS-treated DP cells such that IL-1β expression and apoptosis increase. In contrast, CS cement is biocompatible and enhanced LPS-treated DP cells' proliferation. Therefore, we think MTA is suitable for endodontic use in clinical practice.

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