# Abstract

The Bioactive Monomer, CMET, Promotes Odontoblast Differentiation

in 3D - Culture System

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## Introduction

The development of multifunctional adhesive materials possessing remineralization activity, antibacterial activity, anti-biofilm formation activity, and reparative dentin inducibility has long been expected. As is known to all, Vital pulp therapy (VPT), particularly pulp capping, aims to preserve the vitality and function of the dental pulp. The success of such treatments depends on several factors, with biocompatibility being one of the key factors. Biocompatible materials used in endodontics should not cause inflammation, necrosis, or other negative reactions in the pulp tissue. They should also have properties that promote the formation of a dentin bridge or a layer of reparative dentin, which helps protect the pulp and prevent infection. Commonly used biocompatible materials in endodontics include calcium hydroxide (CH), mineral trioxide aggregate (MTA), and various bioceramic materials. These materials have shown good biocompatibility and have been widely used in vital pulp therapy procedures. Nevertheless, its drawbacks have urged the development of further ideal pulp-capping materials. CMET, a calcium salt of 4-methacryloxyethyl trimellitic acid (4-MET), induces dentin remineralization in vitro and increases the shear bond strength, bending strength, and compressive strength of resin-based coating materials [1]. It was also found to inhibit the formation of *Streptococcus mutans* biofilm [2]. Additionally, CMET exhibits low cytotoxicity, high mineralization, and a high differentiation-inducing ability to odontoblast-like cells [3].

The superiority of three-dimensional (3D) cell culture over two-dimensional (2D) cell culture has been increasingly recognized in recent years due to its capacity to better emulate complex physiological cues and support long-term cell viability, making it an attractive option for various biomedical applications [4].

Collagen, specifically Type I collagen, is the primary component of the extracellular matrix in animal connective tissues. It is abundant and biocompatible, making it an ideal choice as a biologic scaffold. Type I collagen can self-assemble and provides a structural framework for cells to attach and interact. Its use as a scaffold in tissue engineering offers great potential for tissue regeneration and repair [5].

Our previous experiments have studied the role of CMET in a 2D environment in vitro, in order to better emulate the complex in vivo environment, this study aims to evaluate the effects of the bioactive monomer, CMET, on odontoblast- proliferation, differentiation, and mineralization in 3D - culture system.

## Materials and Methods

The material, that is CMET in Type I collogen neutral solution (DME –O2H, Koken) were used in these experiments. The CMET were diluted to 0, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6% (w/v) for addition into the medium with Type I collogen neutral.

### Cell culture

Immortalized rat odontoblast-like Mouse dental papilla cells-23 (MDPC-23) cells were generously provided by Professor Jacques E. Nör at the University of Michigan. Cells suspension were mixed with different concentrations of CMET and then inoculated on non-tissue cultured treated plates (Corning, NY, USA). On day one, cover medium was added when collogen became gel. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). All cells were cultured in a 37°C humidified incubator with an atmosphere of 5% $CO_2$ and 95% air. Cell passage 27 was used in the experiments.

### Cell proliferation

MDPC-23 cells ($5\times10^3$ cells/well) were inoculated into 96-well polystyrene plates. Cell viability was performed using the Cell Counting Kit-8 assay (CCK-8; Dojindo, Kumamoto, Japan) on day 4. CCK-8 reagent was added to each well (10 μL/well) and incubated for 1 hour and 30 minutes. The absorbance of lysates was measured at a wavelength of 450nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Alkaline Phosphatase (ALPase) Activity

MDPC-23 cells ($4x10^4$ cells/well) were cultured on 12 wells plates. Mineralization-inducing reagents, 10 mmol $L^{-1}$ glycerol-2-phosphate disodium salt n-hydrate (β-GP, Wako) and 50 μg $mL^{-1}$ L-ascorbic acid phosphate magnesium salt n-hydrate (AA, Wako), and 100nM/mL dexamethasone (Dex, Sigma-Aldrich) were added into the DMEM (5% FBS) on day 5 when cells reached confluence. The following analyses for RT-PCR and mineralization were performed according to the protocols described above. Mineralization inducing capacity was evaluated by ALPase activity. Cells in gel were transferred to a 1.5 mL tube, digested with Type I collagenase (Worthington, Columbus, Ohio, USA) diluted in Hanks' Balanced Salt Solution (HBSS, Gibco, Grand Island, NY, USA) for over one hour until the gel was digested. Cells were collected by centrifugation (1200 rpm, 5 minutes) and lysed with 0.1% Triton X-100 (Sigma-Aldrich) in distilled water, and the lysates were sonicated on ice for 10 minutes, then centrifuged at 12000 g at 4°C for 15 minutes. The supernatant was extracted for ALP activity assay (Wako) and protein quantification (Thermo Fisher Scientific) according to the manufacturer's instructions. Absorbance was determined at 405 and 570nm respectively.

Real-time Reverse-transcription Polymerase Chain Reaction (Real-time RT-PCR)

On day 6, Cells in gel were transferred to a 1.5 mL tube digested with Type I collagenase diluted in HBSS for over one hour until the gel was digested. Cells were collected by centrifugation (1200 rpm, 5 minutes) and total RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA). The mRNA expression of dentine sialophosphoprotein (DSPP), dentine matrix protein 1 (DMP-1) as dentinogenesis-related genes was quantified using primer sets and real-time RT-PCR; reactions were performed using a LightCycler® Nano (Roche, Basel, Switzerland) according to manufacturer's instructions. Target gene expression was normalized to the housekeeping gene rat β-actin. The comparative $2^{-\triangle\triangle CT}$ method was used to calculate relative gene expression.

Alizarin red S staining

Alizarin red S staining was performed on days 7 to detect calcific deposits in the cells. Cells washed with 1×PBS were fixed with 10% formalin neutral buffer solution (Wako) for 20 minutes and then washed again with $dH_2O$. Then alizarin red S solution (ARS, Wako, 1% m/v, pH 4.1) was carefully added and incubated at 37°C for 10 minutes. After that, the monolayer was washed several times with $dH_2O$ to remove the non-specific background stain. Calcific deposition was visualized under phase contrast microscopy. Images of Alizarin red S staining were processed with *Image J* to obtain data.

Selective blockade of mitogen-activated protein kinases (MAPK)

To investigate the effect of CMET on MDPC-23 cells in response to three MAPK inhibitors, Cells suspension were mixed with 0.3% w/v of gel - CMET and then inoculated at an initial density of $4×10^4$ cells/well in non-tissue cultured treated 12-well plates. On day one, cells were covered with the four inhibitors (20 μmol L−1, Cell Signaling Technology, Danvers, MA, USA) separately in DMEM supplemented with 5% FBS, 10mM β-GP, 50μg/mL AA were added into the DMEM (5% FBS) with inhibitors on day 5 when cells reached confluence. 0% w/v of gel - CMET was used as a control group. The following analyses for ALPase activity and mineralization were performed according to the protocols described above.

Statistical Analysis

All experiments were carried out in triplicate, and the results are expressed as the mean± standard deviation. Data were subjected to one-way ANOVA and *post hoc* Tukey's HSD test, and $P < 0.01$ was considered statistically significant.

**Results**

Cell morphology was visualized under phase contrast microscopy. In the 3D environment of type I collagen formation, cells in all groups were successfully attached, spread, and adopted a spindled shape.

From day 2 images, it is obviously that attached cells number is significantly higher in CMET- contain groups especially in 0.4% CMET group than that in the 0% CMET group.

Cell proliferation was evaluated using the CCK-8 assay. In the comparison of different concentrations of gel-CMET, the 0.4% gel-CMET group stimulated the proliferation of MDPC-23 cells ($P < 0.01$).

An ALP activity assay was performed to detect MDPC-23 cell differentiation toward odontogenic lineages. In the comparison of different concentrations of the gel-CMET, the ALP activity was significantly augmented on day 6 in 0.3% gel-CMET group.

Quantitative RT-PCR analysis was performed to investigate the effects of gel- CMET on odontogenic differentiation. 0.3% gel-CMET group strongly enhanced the messenger RNA (mRNA) expression of 2 critical odontogenesis-related markers: dentin sialophosphoprotein (DSPP) and dentin matrix protein-1 (DMP-1) in the comparison of different concentrations of gel-CMET ($P< 0.01$).

To evaluate the effects of the gel -CMET on the promotion of a mature odontoblast phenotype, Alizarin red S staining was conducted on days seven. The results showed that the gel - CMET, significantly promoted the calcium nodule formation of MDPC-23 cells in a concentration-dependent manner in the presence of mineralization-inducing reagents. Images of Alizarin red S staining was processed with *Image J* to obtain data (O.D.= IntDen / Area) which confirmed a significant increase of mineralization in the 0.4% gel-CMET group relative to the control ($P< 0.01$).

After challenging cells with four MAPK inhibitors, ALPase activity was analyzed, as it is considered essential for biomineralization. The results showed that SB202190 neutralized the positive effect of CMET on ALPase activity to a level comparable to that of the control. Alizarin red S staining results showed a similar tendency.

These results were consistent with our previous study regarding 3D cell culture and CMET.

**Discussion**

It is generally believed that many tissues have three-dimensional (3D) spatial features. To represents better cellular characteristics, in this study, type I collagen was used as a 3D substrate material in which mdpc-23 cells could grow in vitro.

According to the results of cck-8, CMET not only exhibits low cytotoxicity, but also promotes cell proliferation at an appropriate concentration. RT-PCR and Alizarin red S staining were conducted to investigate the optimum concentration of CMET in type I collagen on differentiation in MDPC-23 cells. The results indicated 0.3% gel-CMET markedly promoted the odontogenic differentiation, as evidenced by the up-regulation of mRNA of odontogenic differentiation markers such as DSPP and DMP-1 and increased Alizarin red S staining. ALP activity is an essential determinant for evaluating osteoblast and odontoblast initiate differentiation, and it is directly related to tertiary dentine production. The present results demonstrated that the relative ALP activity of the 0.3% gel-CMET group was significantly higher than that of the control group ($P < 0.01$).

In this study, MAPK signaling pathway was hypothesized as the mechanism behind the in vitro effect of CMET on MDPC-23 cells. The MAPK pathway is a well-known signal transduction pathway involved in transmitting signals from cell surface receptors to the cell's nucleus. It plays a crucial role in various cellular processes, including growth, differentiation, and response to external stimuli such as growth factors. Different types of MAPKs have been identified in mammals, including ERK 1/2, JNK 1/2/3, and p38 isoforms (p38α, β, γ, and δ), among others.

To investigate the involvement of the MAPK pathway in the effect of CMET on MDPC-23 cells, three specific MAPK inhibitors were used in the present study: SB202190, SP600125, and PD98059. These inhibitors target different components of the MAPK pathway. SB202190 is a selective inhibitor of p38α and β isoforms, while SP600125 inhibits the phosphorylation of a protein called c-Jun, specifically targeting JNK function. PD98059 blocks the activation of ERK by binding to MAPK kinase 1 and

preventing its activation by upstream protein kinases. To ensure minimal cytotoxicity, the study employed the lowest maximal working concentrations of the three MAPK inhibitors, as recommended by the manufacturer.

**Conclusions**

The results showed that the bioactive monomer, CMET, induced the proliferation and differentiation, and mineralization of odontoblast-like cells in 3D - culture system under appropriate concentrations. CMET is suggested to exhibit excellent biocompatibility and the great potential in dentine regeneration.

**References**


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