J Appl Biomed
DOI: 10.32725/jab.2024.002
journal homepage: http://jab.zsf.jcu.cz

Journal of Applied Biomedicine

Original research article

Anti-inflammatory effect of luteoloside against methylglyoxal induced human dental pulp cells

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Abstract

Purpose: The aim of this study was to investigate whether luteoloside, a flavonoid, could protect human dental pulp cells (HDPCs) against inflammation and oxidative stress induced by methylglyoxal (MGO), one of the advanced glycated end products (AGE) substances.

Methods: HDPCs were stimulated with MGO and treated with luteoloside. MTT assay was used to determine cell viability. Protein expression was measured via western blotting. Reactive oxygen species (ROS) were measured with a Muse Cell Analyzer. Alkaline phosphatase activity (ALP) and Alizarin red staining were used for mineralization assay.

Results: Luteoloside down-regulated the expression of inflammatory molecules such as ICAM-1, VCAM-1, TNF-α, IL-1β, MMP-2, MMP-9, and COX-2 in MGO-induced HDPCs without showing any cytotoxicity. It attenuated ROS formation and enhanced osteogenic differentiation such as ALP activity and Alizarin red staining in MGO-induced HDPCs. Overall, luteoloside showed protective actions against inflammation and oxidative stress in HDPCs induced by MGO through its anti-inflammatory, anti-oxidative, and osteogenic activities by down-regulating p-JNK in the MAPK pathway.

Conclusion: These results suggest that luteoloside might be a potential adjunctive therapeutic agent for treating pulpal pathological conditions in patients with diabetes mellitus.

Keywords: Advanced glycated end product; Diabetes mellitus; Mineralization; Osteogenesis; Oxidative stress; Pulpitis

Highlights:

- Advanced glycated end products like methylglyoxal are associated with chronic diabetic complications as well as pulpitis and periodontitis.
- Luteoloside is known for its anti-inflammatory and osteogenic activity.
- No studies have been performed on the effect of methylglyoxal on human dental pulp cells.
- Methylglyoxal induced inflammation in human dental pulp cells.
- Luteoloside showed protective action in methylglyoxal induced human dental pulp cells.

Introduction

Diabetes mellitus (DM) has been associated with multiple oral diseases, including pulpitis and periodontitis (Lima et al., 2013). It is recognized as a major health problem that puts economic and social burden on patients as well as the health-care system. Patients with DM undergo multiple oral alterations such as pathogenic infections, delayed wound healing, tooth loss, and xerostomia (Kim et al., 2011; Rohani, 2019). Patients with DM are also prone to developing pulpal and periapical pathologies (Fouad and Burleson, 2003). DM, in particular, can increase inflammatory responses in pulpitis. It is accompanied by treatment complications.

Complications from prolonged diabetic condition come from the accumulation of advanced glycated end products (AGEs) (Sabanayagam et al., 2009). Chronically elevated levels of blood glucose can contribute to accelerated generation of largely irreversible AGEs formed by non-enzymatic reactions between glucose derived metabolites (glyoxal, methylglyoxal, and 3-deoxyglucosone), and amino groups of intra and extra cellular proteins. AGEs are thought to be responsible for major diabetic complications. Intracellular AGEs play an important role as stimuli by activating intracellular signaling pathways and modifying the function of intracellular proteins (Brownlee, 1995; Negre-Salvayre et al., 2009).

Dental pulp is a heterogeneous connective tissue with cellular, neural, and vascular components (Gronthos et al., 2002). It contains undifferentiated mesenchymal cells and cells of the immune complex, which can constantly work to maintain vitality of the tooth. Enhanced formation of AGEs in DM is linked to the pathogenesis of various diseases (Singh et al.,

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http://doi.org/10.32725/jab.2024.002
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Submitted: 2023-07-14 • Accepted: 2024-01-12 • Prepublished online: 2024-02-14
J Appl Biomed 22/1: 33–39 • EISSN 1214-0287 • ISSN 1214-021X

2014). Recently, AGEs have been predicted to be associated with DM-related periodontitis (Zanini et al., 2017). Methylglyoxal (MGO) is a potent glycating agent that can react rapidly with intracellular molecules to form advanced glycated end products. It is endogenously produced by our body via different glycolytic pathways.

Luteoloside, also known as cynaroside, is a plant derived flavonoid. Several studies have shown that it has anti-inflammatory, anti-cancer, and chondroprotective-osteogenic effects (Lee et al., 2020). It has been shown that luteoloside can inhibit the proliferation and metastasis of hepatocellular carcinoma cells and effectively scavenge free radicals (Fan et al., 2014). Other protective actions include attenuation of neuroinflammation in focal cerebral ischemia and hypolipidemic effects (Li et al., 2019; Sun et al., 2021). However, the effect of luteoloside on AGE-induced pulpal tissue is unknown. Thus, the present study aimed to determine the effect of MGO, one of the AGEs, on human dental pulp cells and the possible protective action of luteoloside against such effect of MGO.

Materials and methods

Materials

Antibodies against Intra-Cellular Adhesion Molecules-1 (ICAM-1), Vascular Cell Adhesion Molecules-1 (VCAM-1), and Interlukin-1 beta (IL-1β) were purchased from Santa Cruz (Santa Cruz, CA, USA). Antibodies against Tumor Necrosis Factor alpha (TNF-α), Matrix Metalloprotease-2 (MMP-2), Matrix Metalloprotease-9 (MMP-9), and Cycloxygenase-2 (COX-2) were purchased from Cell Signaling Technologies (Beverly, MA, USA). Luteoloside was purchased from Cayman Chemical (Ann Arbor, MI, USA). Methylglyoxal (MGO) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Actin was purchased from Bioworld Technology (Louis Park, MN, USA).

HDPCs culture

HDPCs were isolated and cultured as reported previously (Kim et al., 2012). Briefly, dental pulp tissue from a freshly extracted third molar was separated by splitting with a hammer and minced into small pieces. After digesting with 3 mg/ml collagenase type II (Sigma-Aldrich, St. Louis, MO, USA) and 0.5 mg/ml trypsin (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at 37 °C, it was seeded into a 25 mm^2 cell culture bottle with Dulbecco's Modified Eagle's Medium (DMEM; Gibco Life Technologies, NY, USA) supplemented with 10% Fetal Bovine Serum (FBS, Gibco Life Technologies, NY, USA). Cells were grown in a humidified incubator at 37 °C with 5% CO_2. Once 80% confluence was reached, cells migrating from the tissue were collected followed by subculture. Cells of third to eight passages were used in this experiment. Cells were treated with indicated MGO concentration for 2 h followed by media change. Fresh media was replenished every 3 days along with MGO treatment for sustained inflammatory state. In combined treatment with MGO and luteoloside, cells were pre-treated with luteoloside for 1 h followed by MGO treatment, repeated along with media change every 3 days.

Cell viability

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to determine the viability of cultured pulp cells. Briefly, pulp cells were seeded into 24-well plates at a seeding density of 1×10^5 cells per well. Once confluence was reached, cells were treated with different concentrations of MGO for 2 h followed by fresh media change or incubation with luteoloside for 24, 48, and 72 h. After the indicated incubation time, culture medium containing MTT (5 mg/ml) was added to each well and incubated at 37 °C with 5% CO_2 for 3 h. Dimethyl sulfoxide (DMSO) was added to dissolve the Formazan crystals. The absorbance was measured at 570 nm using an Enzyme Linked Immunosorbent Assay (ELISA) reader (Synergy 2, Biotek, Winooski, VT, USA).

Western blot analysis

HDPCs were seeded into 100 mm culture plates at a density of 5×10^6 cells/plate and cultured until confluence was reached. Cells were then treated with indicated drugs for specified time durations. Lysis buffer containing 150 μM NaCl, 5 mM EDTA, 50 mM Tris HCl (pH 8.0), 1% NP 40, 1 mM aprotinin, 0.1 mM leupeptin, and 1 mM pepstatin was used to prepare cell lysates. Protein quantification was done with a Bradford Method. Cell lysates were centrifuged, and supernatants were collected. An aliquot of 20 μg proteins from each sample was separated by 8–10% sodium dodecyl sulfate polyacrylamide gels under denaturing conditions and electro blotted onto nitrocellulose membranes. Membranes were incubated with 5% non-fat dry milk for 1 h at room temperature, followed by incubation with a specific primary antibody (diluted 1 : 5000) at 4 °C overnight. These membranes were washed with PBS containing 0.1% Tween-20 three times on a lab shaker, followed by incubation with horseradish peroxide conjugated secondary antibody (diluted 1 : 3000) at room temperature for 1 h. Signals were developed using an enhanced chemiluminescent detection reagent (Amersham Pharmacia Biotech, London, UK) following the manufacturer's protocol, and captured using LAS 400 mini (Fuji Film, Tokyo, Japan) digital western blot imaging system. Membranes were re-probed with anti-actin antibody to verify equal loading of proteins. The Image QuanT TL 1D gel analysis program from band detection software (GE Healthcare Bio-Science, Uppsala, Sweden) was used to analyze the protein expression level. The quantification was done with ImageJ (National Institutes of Health, Bethesda, MD, USA) software.

Alizarin red staining

For osteogenic differentiation of pulp cells, osteogenic media (OM) was used which contains DMEM with 10% FBS supplemented with 100 nM of dexamethasone, 10 mM of β-glycerophosphate, and 50 μg/ml of L-ascorbic acid, media changed every three days. After 14 days of differentiation, the cells were washed with PBS, air dried, and fixed in 95% ethanol ice cold at –20 °C for 30 min. Then 40 mM of Alizarin Red S (pH 4.2) was used to stain cells for 1 h at room temperature. Cells were extensively washed for five times with deionized water and rinsed with PBS devoid of magnesium and calcium for 15 min.

Alkaline phosphatase activity

At 3, 7, and 14 days, samples with conditioned medium were collected. Pulp cells were sonicated with a cell disrupter after being scraped into ice cold PBS and then put into an ice-cold bath. SensoLyte pNPP (p-nitrophenyl phosphate) ALP Assay Kit (anaSpec, Fremont, CA, USA) was used to determine ALP activity in the supernatant. Absorbance was measured with an ELISA reader (Synergy 2, Bio-Tek, USA) at 405 nm. The pH of the assay was 9.4.

Determination of ROS generation

ROS generation by human dental pulp cells was measured with a fluorescence-based Muse Oxidative Stress Kit (Luminex Corporation, Austin, TX, USA) using a Muse Cell Analyzer

(Luminex Corporation, Austin, TX, USA) following the manufacturer's protocol. Briefly, pulp cells were treated with luteoloside for 1 h prior to MGO treatment and incubated for 2 h. Samples with 1×10^7 cells/ml were prepared in 1× assay buffer followed by treatment with an oxidative stress reagent based on dihydroethidium (DHE) which was used to detect ROS oxidized with superoxide anion, producing a DNA binding fluorophore ethidium bromide that could interact with DNA, resulting in a red fluorescence.

Statistical analysis

All results of the control and experimental groups were analyzed independently. Results were expressed as the mean ± standard deviation of three independent experiments. SPSS 15.0 software (SPSS, Chicago, IL, USA) was used to perform statistical analysis. An independent two sample *t*-test at a significance level of $p < 0.05$ was used to examine the differences in variables under different experimental conditions to ascertain their statistical significance.

Results

Effects of MGO on cellular cytotoxicity and inflammatory molecules

HDPCs were treated with indicated concentrations of MGO. The cytotoxicity of MGO to HPDCs was determined by MTT assay. Treatment with MGO at concentrations up to 500 μM for 24 h did not show any cytotoxicity as there was no significant change in cell viability. However, treatment with MGO for 48 h showed significant cytotoxicity compared to the control. Thus, this study used a concentration of 400 μM for MGO because 500 μM of MGO showed a detrimental effect on cell viability (Fig. 1A).

Next, the effects of MGO on expression levels of inflammatory molecules were analyzed by western blotting. MGO increased the expression of inflammatory molecules including ICAM-1, VCAM-1, TNF-α, IL-1β, and COX-2 in HDPCs in a time-dependent manner. Expression levels of these molecules were increased significantly from day 6 to day 15 after treatment. Expression levels of collagenase-related molecules such as MMP-2 and MMP-9 were also increased by MGO in a time-dependent manner (Fig. 1B). Expression levels of inflammatory molecules induced by MGO are graphically illustrated in Fig. 1C.

Anti-inflammatory effects of luteoloside in MGO-induced HDPCs

Luteoloside at concentrations of 1 to 30 μM showed no toxicity to cells. On the other hand, all concentrations of luteoloside increased cell viability compared to the control at 72 h after treatment (Fig. 2A). Therefore, this experiment used 5 and 10 μM of luteoloside to analyze its effect and mechanism.

Luteoloside showed anti-inflammatory properties in MGO-induced HDPCs. MGO-induced increased expression levels of ICAM-1, VCAM-1, TNF-, IL-1β, and COX-2 were at-

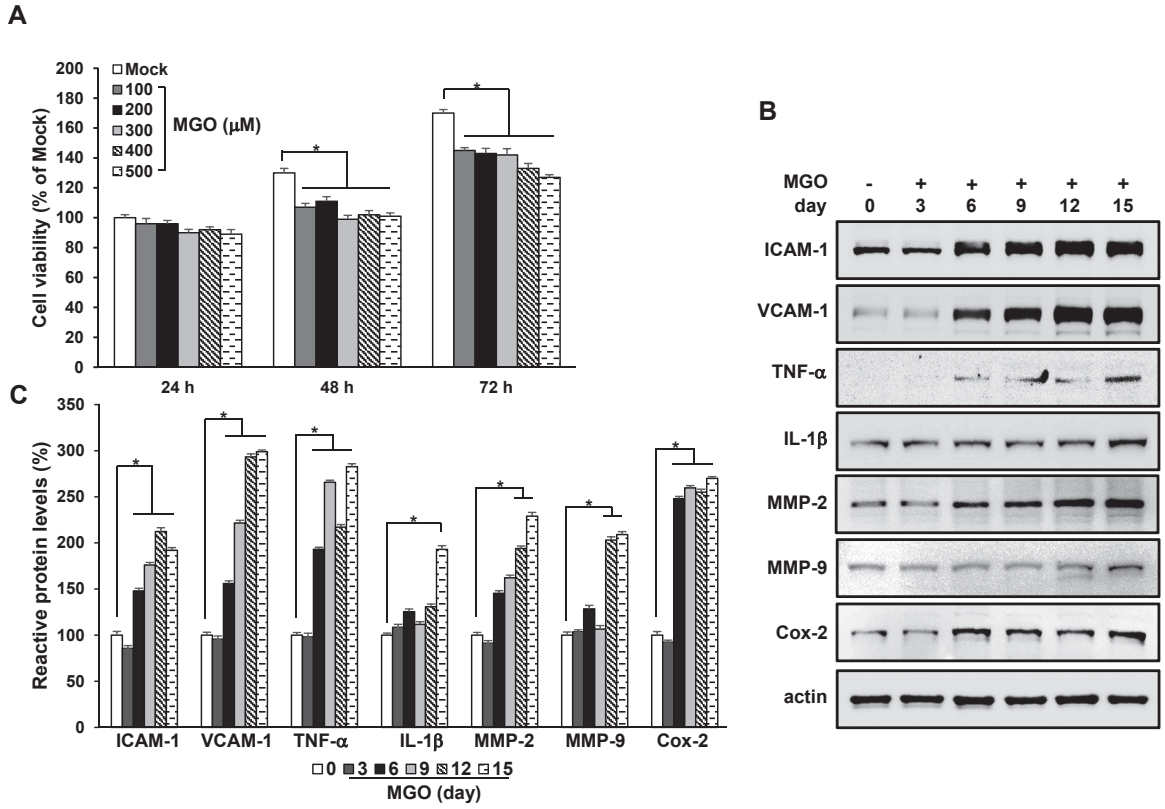

Fig. 1. Effects of MGO on cell viability and inflammatory molecules. HDPCs were treated with MGO at a concentration of 100 to 500 μM for 2 h and the medium was changed. Cell viability was determined by the MTT assay (**A**). To determine inflammatory molecules, cells were treated with MGO at a concentration of 400 μM for 2 h followed by fresh media change, repeated every 3 days and determined by western blotting (**B**). Blots were reprobed with anti-actin antibody (actin was used as a loading control). Graphical representation of western blotting (**C**). Results are shown as mean ± SD of three independent experiments and the symbol * indicates a significant difference between the mock and MGO groups ($p < 0.05$).

tenuated by luteoloside at 3, 7, and 15 days after treatment. Luteoloside also down-regulated the expression of MMP-2 and MMP-9 (Fig. 2B). Inhibitory effects of luteoloside on MGO-induced inflammatory molecules are shown graphically in Fig. 2C.

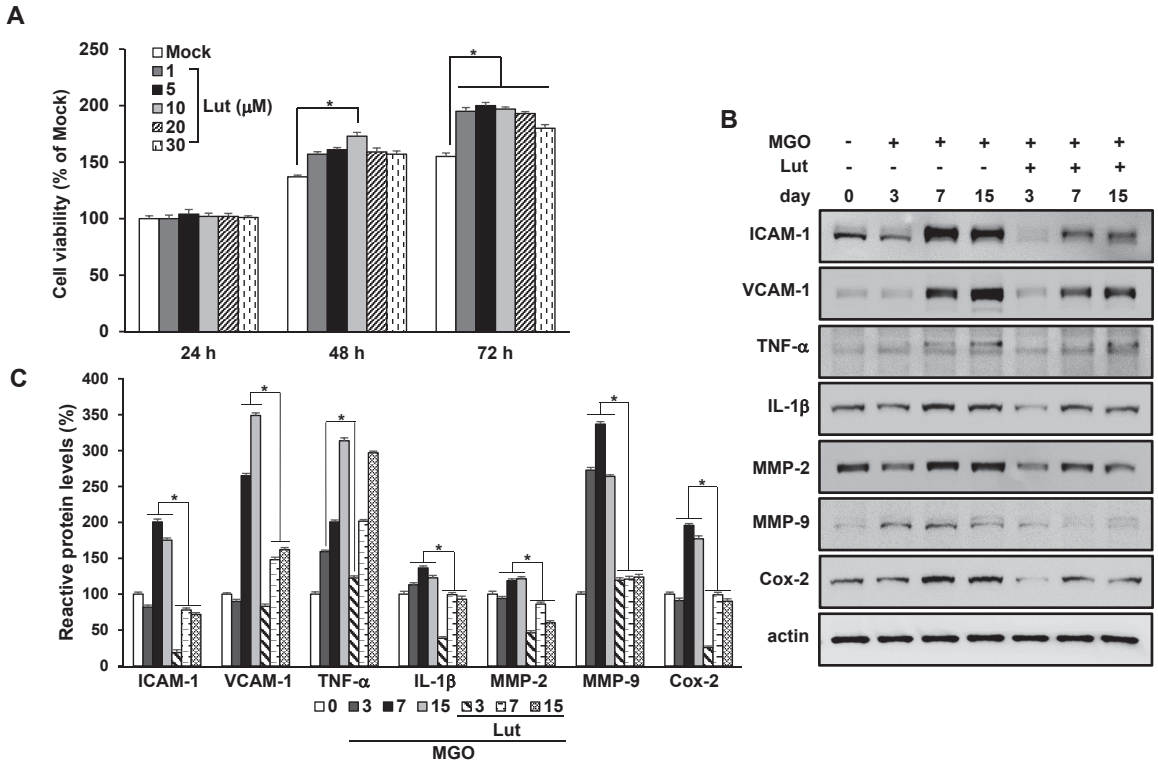

Fig. 2. Effects of luteoloside on cell viability and MGO-induced inflammatory molecules. HDPCs were treated with luteoloside at a concentration of 1 to 30 μM for 24, 48, and 72 h (**A**). To determine inhibition of inflammatory molecules by luteoloside, cells were pretreated with luteoloside (10 μM) for 1 h followed by MGO stimulation for 2 h. The medium was then changed and luteoloside (10 μM) was added for indicated time periods (days). Expression levels of inflammatory molecules was determined by western blotting (**B**). Blots were reprobed with anti-actin antibody. Results are presented as mean ± SD of three independent experiments. Graphical representation of western blotting (**C**). The symbol * indicates a significant difference between the MGO and Lut + MGO groups ($p < 0.05$).

Luteoloside regulates the p-JNK pathway in MGO-induced HDPCs

To determine the involvement of luteoloside in signal pathway, expression levels of ERK, JNK, and p-38 in MAPK pathways were analyzed. All three signaling molecules were activated by MGO. However, luteoloside only down-regulated the expression level of p-JNK activated by MGO (Fig. 3A). The activation of other kinases of MAPK pathway, including ERK and p-38, was not affected by luteoloside treatment in MGO-induced HDPCs. This result indicated that luteoloside could exert an anti-inflammatory effect in HDPCs mediated by p-JNK in the MAPK pathway (Fig. 3). A graphical representation for the inhibition of luteoloside on MGO-induced p-JNK activation is shown in Fig. 3B.

Luteoloside inhibits ROS production and induces odontoblast mineralization and differentiation in MGO-induced HDPCs

ROS in HDPCs were analyzed at 24 h after treatment with MGO (400 μM). MGO significantly increased ROS production, whereas luteoloside at 5 and 10 μM significantly decreased such MGO-induced ROS production (Fig. 4A). A graphical representation of the result showed that MGO treatment increased the production of ROS, while such increase was attenuated in the group treated with MGO and luteoloside (Fig. 4B).

Alkaline phosphatase activity was gradually increased in HDPCs grown in osteogenic media (OM). However, MGO treatment significantly reduced such ALP activity increased by OM (Fig. 4C). Treatment with luteoloside alone did not affect ALP activity in HDPCs grown in OM. The reduced ALP activity by MGO was restored by luteoloside (Fig. 4C). Mineralization was determined based on Alizarin red staining and ALP assay. Luteoloside at a concentration of 10 μM increased Alizarin red staining compared to the control and the MGO group at day 14 (Fig. 4D).

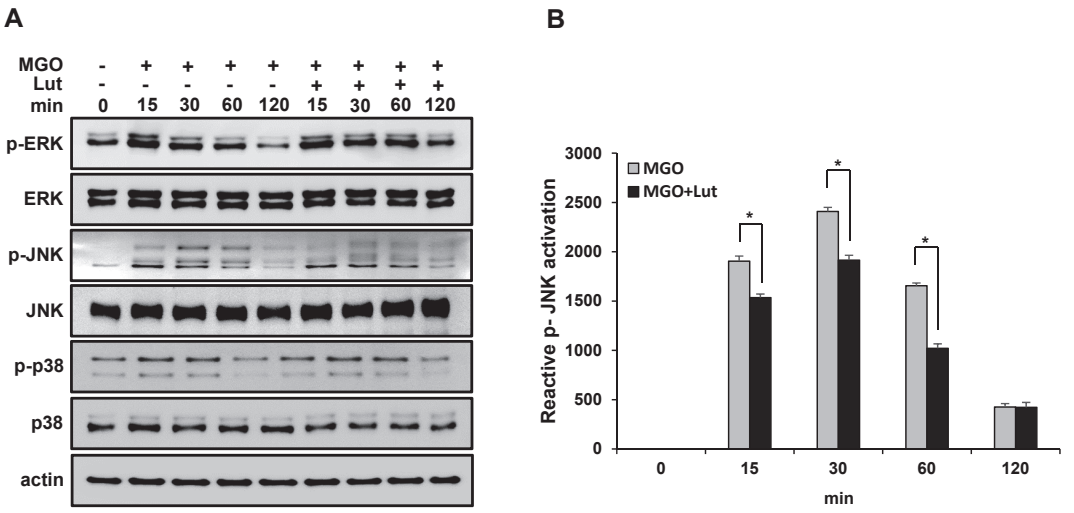

Fig. 3. Effect of luteoloside on MAPK pathway in MGO-induced HDPCs. HDPCs were treated with 10 μM of luteoloside. After 1 h of incubation, cells were treated with 400 μM of MGO for 15, 30, 60, and 120 min. Activation of MAPK pathway was analyzed by western blotting (**A**). Results are presented as mean ± SD of three independent experiments. Graphical representation of western blot result (**B**). The symbol * indicates a significant difference between the MGO and Lut + MGO groups ($p < 0.05$).

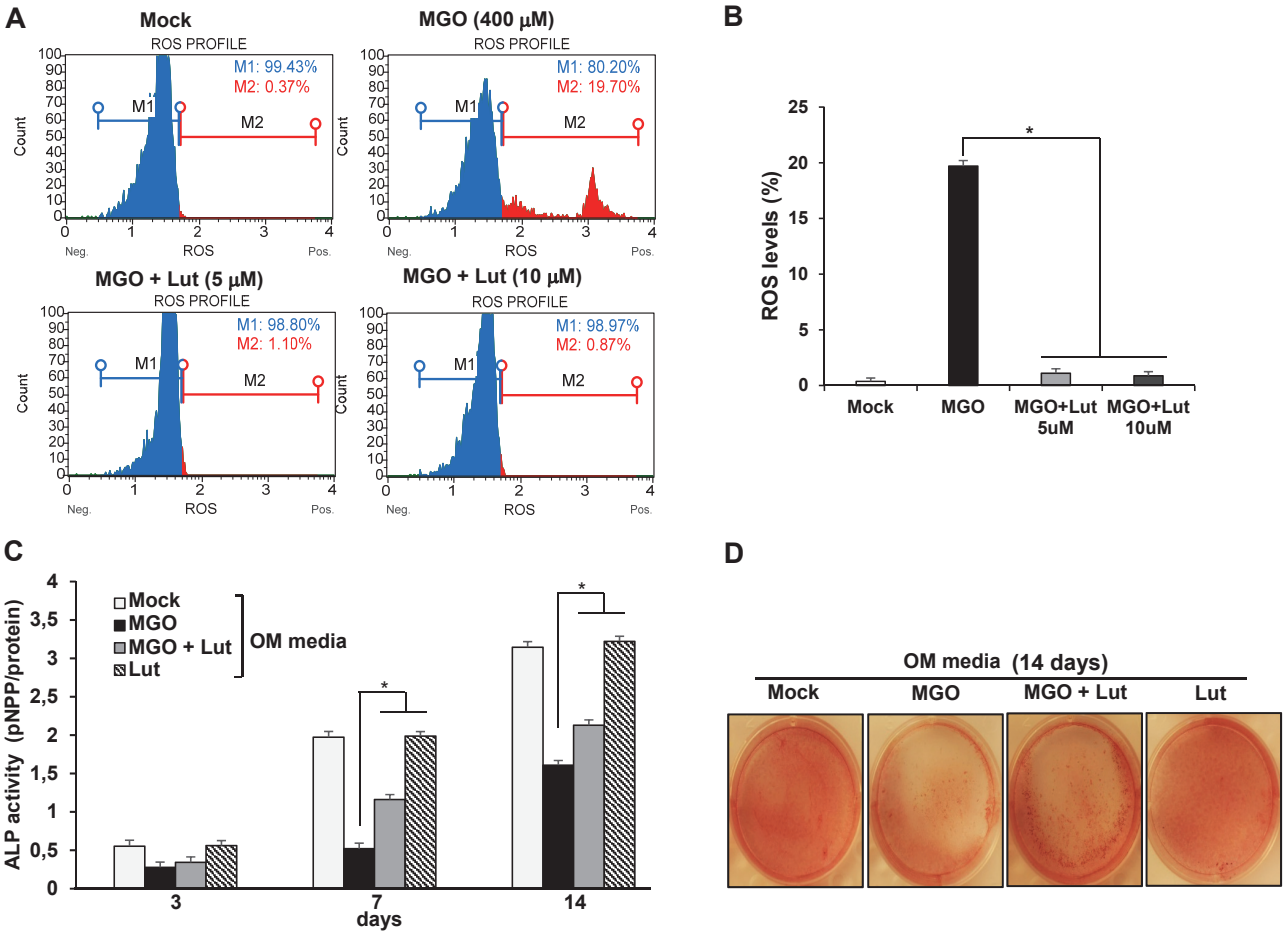

Fig. 4. Effect of luteoloside on ROS generation, ALP activity and mineralization in MGO-induced HDPCs. HDPCs treated with MGO (400 μM) and luteoloside (5 and 10 μM) for 24 h. The ROS formation was analyzed with a Muse cell analyzer (**A**) and graphical representation of ROS formation (**B**). Results are presented as mean ± SD of three independent experiments. Cells were cultured in osteogenic media as described in the methods section and ALP activity was measured at 3, 7, and 14 days (**C**). Mineralization was determined by Alizarin Red Staining at 14 days (**D**). Each value is reported as mean ± standard deviation of three independent experiments and the symbol * indicates a significant difference between the MGO and Lut + MGO groups ($p < 0.05$).

Discussion

Diabetes mellitus (DM) is associated with multiple health-related issues. Hyperglycemia and insulin resistance are factors that can lead to long-term complications. A sustained hyperglycemic state can induce the formation of AGEs. Diabetes, a major hyperglycemic state, is accompanied by an elevated level of glycated end products that can lead to a glycated stress state. Pulpitis is an inflammatory disease of the dental pulp tissue with localized accumulation of inflammatory mediators, cytokines, and chemokines. Uncontrolled high blood glucose levels can aggravate inflammatory conditions such as DM (Hert et al., 2014). DM can directly affect the integrity of structural components of dental pulp by thickening the basement membrane of dental pulp and impairing collateral circulation. AGEs have been implicated as one of the causes (Lima et al., 2013; Zanini et al., 2017). Flavonoids are a class of non-nitrogen-based biological compound that are extensively present in plants. Luteoloside is a plant-derived flavonoid that has shown a number of positive effects *in vitro*. Studies have shown anti-inflammatory action in a variety of experimental settings (Lee et al., 2020). However, its effect on HDPCs stimulated by MGO is yet to be reported.

It is speculated that human dental pulp tissue is deleteriously affected by the presence of AGEs. To assess whether MGO has any effect on HDPCs, these cells were stimulated with a sub-lethal concentration of MGO for the indicated time period. AGEs are associated with chronic diabetes where patients remain in hyperglycemic state for a sustained and longer period. To mimic this state, the cells were cultured in the presence of MGO for a longer duration of 15 days. Media was replenished every 3 days along with MGO treatment for 2 h, followed by a fresh media change to ensure a sustained inflammatory state induced by MGO. This study observed that MGO, one of the AGEs, adversely affected cell viability and increased expression levels of inflammatory molecules in HDPCs. MGO is inevitably produced as a byproduct of glucose breakdown. It is essentially metabolized via the glyoxalase pathway, which is a cellular defense mechanism against MGO toxicity (Kalapos et al., 2022). MGO, an intracellular electrophilic compound, can react with cellular components such as protein, DNA, and lipids, causing cellular dysfunction. The finding of decreased cell viability suggests that characteristics of MGO can affect the survival of HDPCs. MGO-induced cellular dysfunction showed that collagen-degrading molecules (such as MMP-2 and MMP-9), inflammatory cytokines (such as TNF-α, and IL-1β), and inflammatory molecules (such as COX-2, ICAM-1, VCAM-1) were all upregulated in HDPCs. This result suggests that excessive production of MGO in high glucose conditions can trigger inflammatory responses in tissues, leading to a variety of diseases. It also explains that overproduction of AGEs is associated with the development of various diseases (Singh et al., 2014). Although there is little research on the effects of MGO on dental pulp cells, it has been confirmed that MGO is a pathological factor in dental pulp cells (Monache et al., 2021).

Luteoloside is known for its potential health benefits in improving insulin sensitivity and reducing the risk of chronic diseases such as diabetes (Zang et al., 2016). Its antioxidant and anti-inflammatory properties progressively increased the growth of HDPCs over time in culture. In particular, it significantly reduced the expression of inflammatory molecules induced by MGO. Some studies have reported that luteoloside can inhibit the production of pro-inflammatory molecules such as TNF-α, IL-6, and IL-1β (Lee et al., 2020; Wang et al., 2018). In this study, luteoloside showed broad anti-inflammatory activities including inhibiting inflammatory molecules and tissue-degrading enzymes in HDPCs. To explore possible mechanisms for these actions of luteoloside, this study tested whether its antioxidant and anti-inflammatory properties were closely related to MAPKs. In this study, all MAPKs were activated by MGO. However, only JNK activated by MGO was inhibited by luteoloside. Downregulation of JNK by luteoloside suggests that JNK among MAPKs might play an important role in the anti-inflammatory action of luteoloside in MGO-induced HDPCs. The JNK pathway is a signaling pathway involved in many cellular processes, including inflammation. It is a complex pathway depending on specific context and tissues (Lee et al., 2021). It is known that the MAPKs pathway is involved in the inflammatory response of HDPCs and that the control of these MAPKs is effective in inhibiting inflammation (Choi et al., 2013; Paudel et al., 2014). This study demonstrates that luteoloside can exert anti-inflammatory properties by inhibiting the activity of MAPKs.

ROS play an important role in various physiological processes, including host defense against pathogens and cellular signaling pathways. However, excessive ROS production can lead to oxidative stress, which can contribute to the development of various diseases such as inflammation. In recent years, there has been a growing interest in the potential anti-inflammatory effects of ROS inhibitors (Yahfoufi et al., 2018). In the present study, luteoloside was shown to inhibit the production of ROS, which in turn led to anti-inflammatory effects.

AGEs are known to restrain osteogenic differentiation of periodontal ligament stem cells (Guo et al., 2019). HDPCs are known to contain multipotent stem cells which can differentiate into osteoblast and odontoblast like cells with osteogenic activities. The osteogenic activity of HDPCs was down-regulated by treatment with MGO. Luteoloside has been reported to be able to inhibit lipopolysaccharide induced osteolysis and RANKL induced osteoclastogenesis (Song et al., 2018). This osteoprotective action of luteoloside was also verified by the results of the present study. The osteogenic activity of HDPCs was assessed by ALP activity and Alizarin red staining. It was found that luteoloside enhanced the osteogenic activity of HDPCs even in the presesnce of MGO.

Conclusion

In this study, the effect of advanced glycated end product Methylglyoxal has been studied for the first time for its potential effect on human dental pulp cells. The results demonstrated negative effects of AGEs such as MGO on HDPCs. MGO induced inflammation and inhibited osteogenic differentiation of HDPCs. Luteoloside down-regulated the inflammation induced in HDPCs and also enhanced osteogenesis by reducing the pathogenicity of MGO. In conclusion, luteoloside might be an effective adjunct to treat pulpitis in persistent diabetic conditions where AGEs are formed.

Funding
This work was supported by a grant from the National Research Foundation (NRF) of Korea, funded by the Korean government (MSIP) (NRF-2021R1F1A1049585).

Ethical aspects and conflict of interest
The authors have no conflict of interest to declare.

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