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Stimulatory Role of Magnesium Chloride in Expression of Dentin Matrix Proteins

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Abstract

Magnesium-based biomaterials might provide an innovative therapeutic potential to substantially enhance regeneration of dental tissues. In previous work, magnesium oxide (MgO) has been studied for its potential ability to enhance cell attachment, proliferation rate and dentin matrix protein expression of human dental pulp cells (HDPCs). However, to date, dentinogenic effect of magnesium chloride (MgCl2) on cell viability and expression of extracellular matrix proteins in HDPCs has not been investigated. This study was designed to compare the stimulatory effect of different concentrations of MgCl2 on dentinogenesis of HDPCs. HDPCs were cultured with 0.5 mM, 1 mM, 2 mM, 4 mM, 8 mM concentrations of supplemental MgCl2, 0 mM as negative control group. Stimulatory effect of MgCl2 was assessed by evaluating cell viability, and expression of dentin matrix proteins: dentin sialoprotein (DSP), dentin matrix protein1 (DMP-1), dentin sialophosphoprotein (DSPP) and type I collagen (COL-I). Statistical analysis was carried by Multi-Way Analysis of Variance (ANOVA) with Wilks' lambda test. Supplemental MgCl2 concentration groups between 0.5 mM-4 mM elicited a significantly higher expression of DSP and DMP-1, while 0.5 mM-2 mM supplemental MgCl2 concentrations showed highest stimulatory effect on cell viability and, expression of DSPP, and COL-I, compared to the negative control group at all-time points ($P<0.0001$). However, 8 mM MgCl2 group had an inhibitory effect on HDPCs with significant lower cell viability and expression levels of DSP, DMP-1, DSPP, and COL-I compared to the control ($P<0.0001$). In conclusion, optimal (0.5 mM-2 mM) supplemental MgCl2 concentration groups significantly upregulated odontogenic differentiation with enhanced expression of dentin matrix proteins. This is the first study to reveal the dentinogenic effect of MgCl2 on dentin matrix protein expression in HDPCs. Magnesium-containing biomaterials may serve as a potential material for pulp repair and dentin regeneration.

Key words: Magnesium • Human dental pulp cells • Dentin matrix proteins • Dentinogenesis • Regeneration

Introduction

Dental pulp tissue can be injured as the result of deep caries lesions and accidental trauma used during the restoration of teeth affected by carious lesions. Thus, protection of the pulp by applying capping agents directly or indirectly on pulp tissue is called vital pulp therapy (VPT). The nature and specificity of the mechanisms by which the amputated dentin-pulp interface is therapeutically healed determine the properties of the barrier at this site and play a critical role in the outcome of vital pulp therapy. Healing of the dentin-pulp complex proceeds either by natural repair which results in defensive hard-tissue formation, or therapeutically regulated dentin regeneration which aims to reconstitute the normal tissue architecture at the dental pulp periphery. In recent years, with the progress of regenerative and molecular approaches, it is known that the efficacy of direct and indirect pulp capping might be affected by biomaterials and their biological properties. Calcium hydroxide [Ca (OH)2] and mineral trioxide aggregate (MTA) have long been considered the pulp capping materials of choice. However, the application of these materials showed induction of fibrodentin/reparative dentin formation, often at the expense of the underlying pulp tissue. An alternative pre-clinical model aiming to reconstitute normal tissue architecture directly at the dentin-pulp interface should be designed on the basis of the direct induction of odontoblast-like cell differentiation and reparative dentin formation. A more in-depth knowledge of the cascade of cellular and molecular events underlying dentinogenesis is crucial. Further optimization of new biological approaches leading to a targeted interaction between tooth tissue and bioactive molecules for VPT is needed. Magnesium (Mg2+) is an essential inorganic component, where it participates in numerous biological processes allowing osteogenesis and mineralization of mesenchymal stem cells (MSCs) [1]. The balanced combination of Mg2+ with different elements such as calcium and phosphate demonstrated osteoinductive effects through a specific interaction between fibronectin and integrin α5β1. Numerous reports have demonstrated that Mg2+ provides scaffolds with structural characteristics similar to those of bone, allowing anchorage and osteogenesis of progenitor cells [2]. Mg2+-enriched microenvironment has also been reported to promote odontogenic differentiation with enhanced expression of runt-related transcription factor 2 (RUNX2), DMP-1, and DSP in dental pulp stem cells (DPSCs) [3].

In previously published work, MgO significantly increased HDPCs adhesion, proliferation, odontogenic differentiation with upregulation of expression of dentin matrix proteins: DSP, DMP-1, DSPP, COL-I [4]. Despite the influence of MgO on several cellular parameters involved in dentinogenesis. However, the optimal concentration of MgO at which it produced its effect was within a narrow window. This was possibly related to the considerable insoluble MgO nature. Also, MgO dissociation when immersed in various physiological fluids under standard cell culture conditions is mostly limited because of its poor solubility.

Nevertheless, MgCl2 as an ionic compound has higher solubility than MgO, yielding a greater average of dissociated Mg2+ ions. In this study, MgCl2 was selected as a suitable source of Mg2+ ions to induce Mg2+ mediated dentinogenesis. It was hypothesized that the use of MgCl2 may initiate the delivery of more Mg2+ ions, which could have a more enhanced effect on

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dentin matrix protein expression than the limited effect of MgO. Therefore, the introduction of MgCl2 as a biologically based developed strategy, to replace damaged structures, stimulating molecular pathways, leading to tissue repair or regeneration. In the present study, the main target was to determine the biological effect of MgCl2 in terms of 1- To evaluate the stimulatory effect of different concentrations of MgCl2 in terms of cell viability 2- and to determine the optimal concentration that would deliver sufficient Mg2+ ions stimulating expression of extracellular matrix-related proteins: DSP, DMP-1, DSPP and COL-I. Exploring the applicability of MgCl2 as a bioactive molecule in VPT to promote a better biological response. This is the first report providing the insight on the role of MgCl2 on an enhanced extracellular matrix protein expression of HDPCs in vitro.

Materials and Methods

Magnesium chloride preparation

Magnesium chloride hexahydrate (MgCl2) Thermo Fisher Scientific, Cambridge, MA, USA) was dissolved in deionized water and five stock solutions were prepared at concentrations of 5 mM, 10 mM, 20 mM, 40 mM and 80 mM respectively. Each concentration was subsequently filtered under sterile condition in the biological hood.

Establishment of HDPCs

Human dental pulp explants were collected from young and systemically heathy patients between the age group of 15-25 years requiring third molar or orthodontic premolar extractions. Patients were given informed consent before the extraction procedure at the oral surgery clinic at Boston University. This study was approved under Boston University IRB approval H-33173. Human dental pulp cells (HDPCs) were isolated. Teeth were sectioned with a #7 chandler bi-bevel bone chisel until the pulp tissue in the pulp chamber was exposed. The pulp pieces were removed with sterile instruments and placed immediately into a 12.5 cm2 culture flask (Thermo Fisher Scientific). Culture medium consisted of 10% fetal bovine serum (FBS) (R and D Systems), 1X Penicillin antibiotic (100 U/mL), and 1X Streptomycin (100 ug/mL) (Gibco), Amphotericin B anti-fungal (0.25 ug/ml) in Eagle's Basal Medium (BME) (Gibco). All tissues were maintained at 37°C, in a standard 5% CO2 incubator, saturated humidity and cultured up to the second passage. Culture media was changed every 72 hours. Nearly confluent cells were trypsinized with 0.05% Trypsin (Gibco). Re-suspended cells were then aspirated and collected in a sterile 15 mL disposable tube placed in the TJ-6 Beckman Centrifuge at 1000 rpm for 5 minutes. After centrifugation, a pellet of cells was formed. The cells were then counted using a hemocytometer and utilized in the experiment. Characterization of dentinogenic phenotype of the cells was confirmed by expression of dentinogenic markers induced by vit D3 stimulation. Human dental pulp cells were transferred to 24 well plates (Thermo Fisher Scientific) and grown in the culture medium supplemented with 0 mM (control), 0.5 mM, 1 mM, 2 mM, 4 mM and 8 mM supplemental MgCl2 concentrations respectively. For differentiation studies, culture media was replaced with pre-inductive dentinogenic media at the following time intervals: 4, 7, and 11 days. Dentinogenic media was consisted of the following: 10% charcoal stripped fetal bovine serum (FBS) (Life Technologies), 100 IU/ml Penicillin (Gibco), 100 µg/m streptomycin (Gibco), 10-8M Menadione (Sigma Aldrich), 10 mM β-Glycerophosphate (Sigma Aldrich), 0.05 mg/mL L-ascorbic acid (Sigma Aldrich), and 2 mM L-glutamine (Gibco). The next day, cells were cultured in pre-inductive dentinogenic media with the addition of 10 nM Vitamin D3(172 g/mol) (Sigma Aldrich) for two additional days before the predetermined time intervals. Supernatant fluid was collected on days 7, 10, and 14. Dentin sialoprotein (DSP), dentin matrix protein 1 (DMP-1), dentin sialophosphoprotein (DSPP), and collagen type I (COL-I) were measured in the collected supernatants. From measurements obtained from proliferation data in a previously published study [5], the cell numbers for each time interval 7, 10 and 14 days were used to determine the values of extracellular protein levels on a per million cell base formula.

Determination of HDPCs viability

HDPCs were seeded at a density of 300 cells per well in 100 µL of cell culture media of a 96-well plate. Cells were incubated for 16 hr at 37° C, 5% CO2. Cellular viability was measured via 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) Ab211091 Cell Cytotoxicity Assay Kit (Abcam). After 16 h, 100 µL of cell culture media containing supplemental MgCl2 in the concentrations of 0.5 mM, 1 mM, 2 mM, 4 mM, 8 mM, 0 mM as negative control, were added to the cells. Culture medium for the both the control and experimental groups was changed by a fresh one every 3 days. Cell viability was assessed by measuring mitochondrial succinic dehydrogenase (SDH) activity at 16 hr, 3, 7, 10 and 14 days. At each required time point, growth media was removed, 50 µL of serum free media and 50 µL of 3-[4,5-dimethylthiazole-2-yl]-2,5- diphenyltetrazolium bromide (MTT) reagent was added to each well. For background control wells 50 µL of MTT reagent and 50 µL cell culture media (no cells) was added. The plates were incubated at 37°C for 3 hrs. After incubation, the MTT reagent supplemented media was removed. 150 µL of MTT solvent was added into each well. The mitochondria in viable cells were able to transform the tetrazolium into a purple-colored compound called formazan. The absorbance reading of the produced purple formazan of each group was recorded at wavelengths 590 nm using the microplate reader. The data then were plugged into the following equation to calculate the percentage of cytotoxicity for each group: % Cytotoxicity=100 × (Control-Sample)/Control

Assessment of HDPCs Dentin Sialoprotein (DSP) expression

Dentin Sialoprotein Xpress Bio TM ELISA Kit (Human) was used to determine expression levels of secreted dentin sialoprotein (DSP). In a 96-well microplate (Thermo Fisher), five replicate wells were labeled, based on each MgCl2 concentration (0.5 mM, 1 mM, 2 mM, 4 mM, 8 mM and 0 mM as the negative control group) and time intervals at 7, 10 and 14 days. Test samples were diluted (2x) with sample dilution buffer. Then, 100 µL of the standards, controls, and the diluted test samples were pipetted into thewells, incubated at 37°C for 90 minutes. 100 µL of the biotin conjugated detection antibody was added to the appropriate standard, blank and test sample wells. The plate was then incubated for 60 minutes at 37°C. Thereafter, 100 µL horse radish peroxidase (HRP) Streptavidin was added into each well and incubated for 30 minutes. 90 µL TMB substrate was then added. The mixture was incubated for 10-20 minutes at 37°C. Finally, the reaction was terminated by adding 50 µL stop solution to each well. The intensity of the solution color in yellow was measured spectrophotometrically at 450 nm in a micro-plate reader (TECAN, Infinite 1000 Pro). DSP expression was calculated by a standard curve and normalized on a per million cell base formula.

Assessment of HDPCs Dentin Matrix Protein (DMP-1) expression

The sample supernatants collected at days 7, 10 and 14 were tested using dentin matrix acidic phosphoprotein 1 (DMP-1) XpressBio TM ELISA Kit (Human. The standard solutions were prepared in concentrations of 7.8 pg/mL, 15.625 pg/mL, 31.25 pg/mL, 62.5 pg/mL, 125 pg/mL and 250 pg/mL. Sample dilution buffer was aliquoted and added into the standard and the control (blank) wells. 100 µL of the diluted (2x) test samples were added into the 96 well plates (Thermo Fisher). 100 µL of standards, controls, and test samples were pipetted into the wells and incubated at 37°C for 90 minutes. 100 µL of the biotin conjugated detection antibody was added to the appropriate standard, blank and test sample wells. Thereafter, 100 µL HRP-Streptavidin conjugate (SABC) working solution was added into each well and incubated for 30 minutes. 90 µL TMB substrate was added into each well and incubated at 37°C in the dark for 15-30 min. Finally, the reaction was terminated by adding 50 µL stop solution. Following, the completion of the assay, the intensity of the solution color in yellow was then measured spectrophotometrically at 450 nm in a micro-plate reader (TECAN, Infinite 1000 Pro). DMP-1 concentration was calculated by a standard curve and normalized to DMP-1 expression measured per million

of cells.

Assessment of HDPCs Dentin Sialphosphoprotein (DSPP) expression

Dentin Sialophosho protein (DSPP) CusabioTM ELISA Kit (Human) was used to evaluate the expression levels of secreted DSPP. In a 96-well microplate (Thermo Fisher Scientific), five replicate wells were labeled. Sample supernatants were collected at days 7, 10 and 14. Standards were prepared. 100 µL of all samples (standards, test samples and controls) were pipetted into each well. Plates were incubated for 2 hours at 37°C. 100 µL of biotin- antibody (1x) was added to the appropriate wells. Plates were then incubated for 60 minutes at 37°C, decanted and washed. Thereafter, 100 µL of avidin conjugated Horseradish Peroxidase (HRP) (1x) was added into each well. The microtiter plate was incubated for 60 minutes at 37°C. 90 µL TMB substrate was then added into each well. The plate was incubated for 15-30 min at 37°C. Finally, the reaction was terminated by adding 50 µL stop solution to each well. Following, the completion of the assay, the microplate reader (TECAN, Infinite 1000 Pro) was set at a wavelength of 450 nm to identify the intensity of color (O.D) versus the respective DSPP concentration. DSPP expression was calculated by a standard curve to obtain the concentration measured per million of cells.

Assessment of HDPCs Type I Collagen (COL-I) protein expression

To assess the expression level of secreted type I collagen (COL-I), Xpress Bio TM ELISA Kit (Human) was used. Sample supernatants at days 7, 10 and 14 were diluted (2x). 100 µL of the diluted test samples, standards, and controls were added into a 96 well black plate with a clear bottom (Thermo Scientific). Test samples were incubated at 37°C for 90 minutes. 100 µL of the biotin conjugated detection antibody was added to the standard, blank, and test sample wells. The plate was then incubated for 60 minutes at 37°C. Thereafter, 100 µL HRP-Streptavidin was added into each well, incubated for 30 minutes. 90 µL TMB substrate was then added into each well, the plate was incubated in the dark for 10-20 minutes. Finally, the reaction was terminated by adding 50 µL stop solution into each well. Following, the completion of the assay, the intensity of color was measured spectrophotometrically at 450 nm in a micro-plate reader (TECAN, Infinite 1000 Pro). COL-I concentration was calculated by a standard curve and normalized to COL-I expression on a per million cell base formula

Statistical analysis

The means and standard deviations (SD) of HDPCs cell viability data at 16 hours, 3, 7, 10 and 14 days, and the levels of dentinogenic differentiation markers (DSP, DMP-1, DSPP, and COL-I) at 7, 10, and 14 days were calculated. The data were normalized on a per million cells basis at the same time points. Statistical analysis was performed using software JMP Pro 13 (ver. 13.1.0). Multi-Way Analysis of Variance (Multi-Way ANOVA) with Wilks' lambda test is used for statistical analysis between the groups. Differences at $P \leq 0.05$ were considered statistically significant.

Results

Effect of MgCl2 on cell viability of HDPCs

For the 0.5 mM, 1 mM and 2 mM supplemental MgCl2 concentration groups, SDH activity presented 56.13%, 57.41%, and 46.52% at 16 hrs; 77.92%,79.93%, and 57.27% at day 3; 87.36%, 86.49%, and 70.23% at day 7; 91.14%, 91.73%, and 90.40% at day 10; 93.98%, 93.74% and 91.92% at day 14 compared to the negative control group (P<0.0001) (Figure 1). Wilks' Lambda interaction P-value for MgCl2 concentrations and SDH activity showed a statistically significant value (P<0.0001). However, for the 4 and 8 mM supplemental MgCl2 concentrations groups SDH activity showed 30.95%, and 34.20% at 16 hrs; 34.81%, and 34.20% at day 3; 28.57%, and 31.51% at day 7; 19. 66% and 21.02% at day 10; and 20.22%, 20.62% at day 14, showing a significant decrease in SDH activity comparative to the negative control (P<0.0001). Wilks' Lambda interaction P-value for MgCl2 concentrations and cellular activity showed a statistically significant value (P<0.0001).

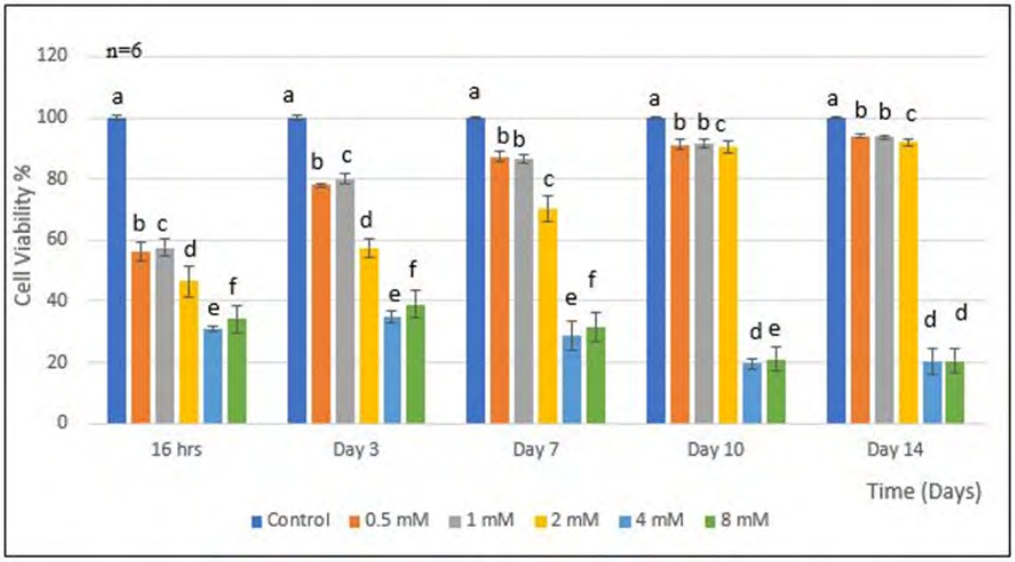

Figure 1. Histogram showing Cell Viability of Normal Human Dental Pulp Cells in Media with tested Supplemental Magnesium Chloride (MgCl2) Concentrations 0.5 mM, 1 mM, 2 mM, 4 mM, 8 mM and 0 mM as control group for time periods of 16 hr, 3,7,10, and 14 days.

The data is presented as means of six replicates with error bars indicating the standard deviation

Groups labeled with different letters differ statistically as compared to the control group and other study groups (P<0.0001)

Effect of MgCl2 on Dentin Sialoprotein (DSP) expression

At day 7, the 0.5 mM, 1 mM and 2 mM supplemental MgCl2 concentration groups presented significantly higher DSP expression of HDPCs comparable to the negative control (P<0.0001) (Figure 2). DSP expression in the 1 mM MgCl2 concentration group exhibited the highest expression in comparison to the negative control group and the other groups. Wilks' Lambda interaction P-value for MgCl2 concentrations and DSP expression at day 7 showed a statistically significant value (P<0.0001). At both days 10 and 14, a significantly higher increase in DSP expression was observed in 0.5 mM-4 mM supplemental MgCl2 concentration groups compared to the negative

control (P<0.0001) (Figure 2). Among these groups, 1 mM supplemented MgCl2 concentration group reached the highest value. Wilks' Lambda interaction P-value for MgCl2 concentrations and DSP expression showed a marked significant value (P<0.0001) at day 10 and day 14. However, the high (8 mM) supplemental MgCl2 concentration group showed significantly lower DSP expression compared to the negative control at days 7 and 14 (P<0.0001).

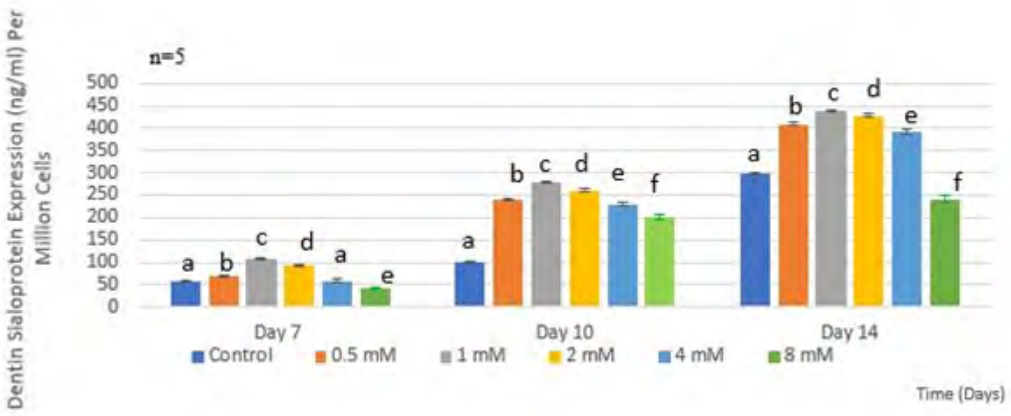

Figure 2. Histogram showing Dentin Sialoprotein (DSP) Expression of Normal Human Dental Pulp Cells in Media Supplemented with Magnesium Chloride (MgCl2) concentrations 0.5 mM, 1 mM, 2 mM, 4 mM, 8 mM and 0 mM as control group for time periods of 7,10, and 14 days.

Notes: Dentin sialoprotein expression in supernatants was normalized per million cells at each time interval. The control cells were treated with growth media without supplemental magnesium chloride.

The data is presented as means of five replicates with error bars indicating the standard deviation

Groups labeled with different letters differ statistically as compared to the control group and other study groups (P < 0.0001)

Effect of MgCl2 on Dentin Matrix Protein 1(DMP-1) expression

DMP-1 expression on HDPCs was analyzed at 7, 10, and 14 days. At day 7, a significantly higher level of DMP-1 expression was noted in the 0.5 mM, 1 mM, and 2 mM supplemental MgCl2 concentration groups compared to the negative control (P<0.0001) (Figure 3). In the 1 mM supplemental MgCl2 concentration group, the highest increase in DMP-1 expression was noted compared to the negative control group and the other tested groups. Wilks' Lambda interaction P-value for MgCl2 concentrations and DMP-1 expression at day 7 showed a statistically significant value (P<0.0001). At days 10 and 14, a significant time-dependent increase in DMP-1 expression level in the 0.5 mM-4 mM supplemental MgCl2 concentration groups was also observed (P<0.0001) (Figure 3). Similarly, the 1 mM supplemented MgCl2 concentration showed the highest level of DMP-1 expression compared to the negative control and other tested groups. On the other hand, cells grown in media containing higher 8 mM MgCl2 supplements showed a decreased DMP-1 expression compared to the negative control with a statistically significant difference (P<0.0001). Wilks' Lambda interaction P-value for supplemental MgCl2 concentrations and DMP-1 expression presented a statistically significant value (P<0.0001).

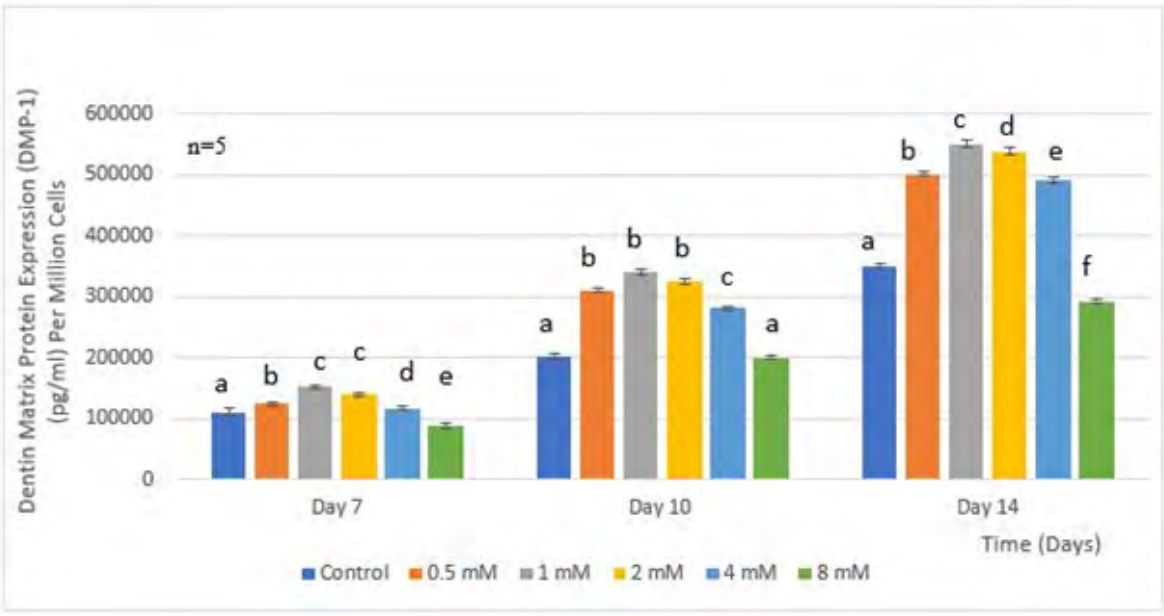

Figure 3. Histogram showing Dentin Matrix Protein-1 (DMP-1) Expression of Normal Human Dental Pulp cells in Media Supplemented with Magnesium Chloride (MgCl2) Concentrations 0.5 mM, 1 mM, 2 mM, 4 mM, 8 mM and 0 mM as control group for time periods of 7,10, and 14 days.

Notes: Dentin matrix protein-1 expression in supernatants was normalized per million cells at each time interval. The control cells were treated with growth media without supplemental magnesium chloride.

The data is presented as means of five replicates with error bars indicating the standard deviation

Groups labeled with different letters differ statistically as compared to the

control group and other study groups (P<0.0001)

Effect of MgCl2 on Dentin Sialophosphoprotein (DSPP) expression

At day 7, DSPP expression presented a statistically higher increase for the 0.5 mM-2 mM supplemental MgCl2 concentration groups compared to the negative control group and other tested groups (P<0.0001) (Figure 4). At days 10 and 14, a significant time-dependent increase in DSPP expression was observed in the same concentration groups. Wilks' Lambda interaction P-value for supplemental MgCl2 concentrations and DSPP expression demonstrated a statistically significant value (P<0.0001). Meanwhile, in comparison to the lower supplemental MgCl2 concentration groups 0.5 mM-2 mM, the cells grown in media containing higher MgCl2 supplement groups 4 mM and 8 mM showed significantly lower DSSP expression at days 7, 10, and 14 comparable to the negative control (P<0.0001) (Figure 4). Wilks' Lambda interaction P-value for supplemental MgCl2 concentrations and DSPP expression at these time points showed a statistically significant value (P<0.0001).

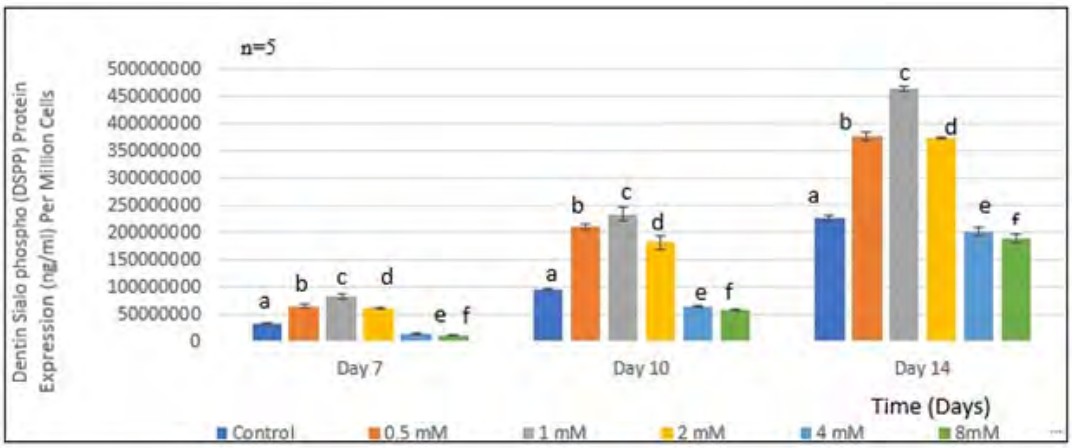

Figure 4. Histogram showing Dentin Sialophosphoprotein (DSPP) Expression of Normal Human Dental Pulp Cells in Media Supplemented with Magnesium Chloride (MgCl2) Concentrations 0.5 mM, 1 mM, 2 mM, 4 mM, 8 mM and 0 mM as control group for time periods of 7,10, and 14 days.

Notes: Dentin Sialophosphoprotein expression in supernatants was normalized per million cells at each time interval. The control cells were treated with growth media without supplemental magnesium chloride.

The data is presented as means of five replicates with error bars indicating the standard deviation

Groups labeled with different letters differ statistically as compared to the control group and other study groups (P<0.0001)

Effect of MgCl2 on Type I Collagen (COL-I) expression

The expression of COL-I at days 7, 10 and 14 showed significantly higher levels in the 0.5 mM-2 mM supplemental MgCl2 concentration groups compared to the negative control (P<0.0001) (Figure 5). Meanwhile, the highest increase in COL-I expression was observed in the 1 mM supplemental MgCl2 concentration group compared to the negative control group and all other concentration groups at days 10 and 14. Wilks' Lambda interaction P-value for supplemental MgCl2 concentrations and expression of COL-I at days 7, 10, and 14 showed a statistically significant value (P<0.0001). Meanwhile, cells grown in media containing higher MgCl2 supplements 4 mM and 8 mM showed significantly lower expression of COL-I compared to the negative control and other concentration groups (P<0.0001) (Figure 5). Wilks' Lambda interaction P-value for supplemental MgCl2 concentration and COL-I expression at the 14th daytime point presented the same statistically significant value (P<0.0001).

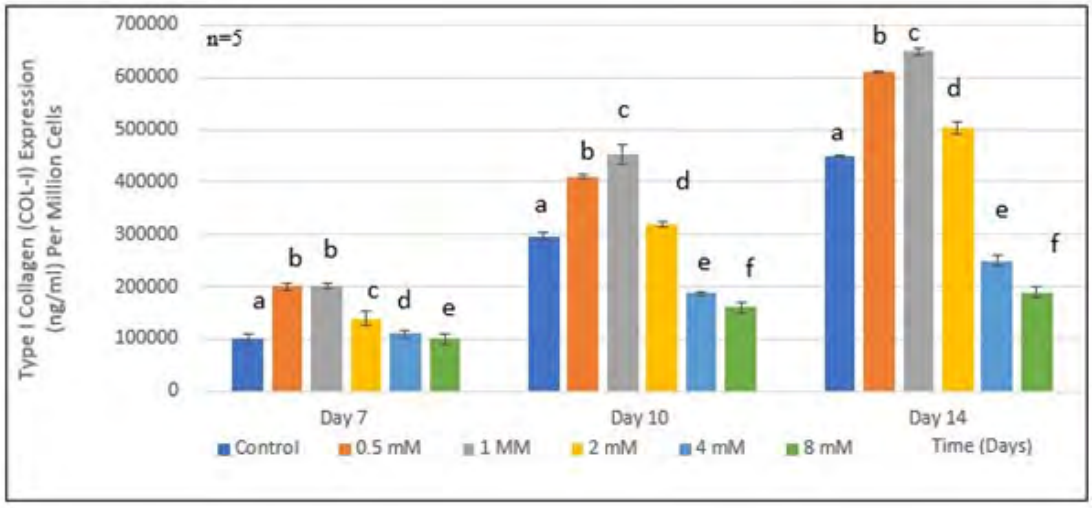

Figure 5. Histogram showing Type I collagen (COL-I) Expression of Normal Human Dental Pulp Cells in Media Supplemented with Magnesium Chloride (MgCl2) Concentrations 0.5 mM, 1 mM, 2 mM, 4 mM, 8 mM and 0 mM as control group for time periods of 7,10, and 14 days.

Notes: Type I collagen (COL-I) expression in supernatants was normalized per million cells at each time interval. The control cells were treated with growth media without supplemental magnesium chloride.

The data is presented as means of five replicates with error bars indicating the standard deviation

Groups labeled with different letters differ statistically as compared to the

control group and other study groups (P<0.0001)

Discussion

The advancement of regenerative endodontics is to restore vitality and functionality of permanent teeth with necrotic pulp by regenerative endodontic procedure. It could be presumed that if the bioactive materials could be enclosed and transferred to the target site using a particularly designed local delivery system, placed in near proximity to the injured pulp tissue, released in a controlled and sustained pattern, the dental pulp tissue could be engineered. Several studies already revealed the favorable behavior and the improved biological outcomes of Mg2+ ions on account of their documented benefits to bone regeneration. Magnesium doping of implant surfaces has been shown to result in an accelerated and enhanced osseointegration. Mg2+ and magnesium-hydroxyapatite (Mg-HA) coatings on titanium, improved alkaline phosphatase (ALP) activity in an (MC3T3-E1) osteoblast cell line. It has also been proposed that, with the application of local biodegradable/biocompatible carrier vehicles to transfer bioactive molecules to the dental pulp capping site, induction of odontoblast-like cell differentiation, formation of reparative dentin, and stimulation of fibrodentin may be predicted resulting in the reconstruction of normal tissue architecture at the dentin-pulp interface which might eventually result in better VPT outcome.

Thus, it is reasonable to suggest that the induced dentinogenesis could be triggered by interactions between MgCl2, extracellular matrix molecules, and HDPCs. HDPSCs represent an adult stem cell population that possess properties of high proliferative potential, the capacity of self-renewal, and multi-lineage differentiation. Data generated from this study would therefore be much more relevant towards clinical application of the material.

In the present study, cellular activity was assessed by MTT assay to measure mitochondrial succinic dehydrogenase (SDH) activity at several time points. The result showed that HDPCs treated with 0.5 mM-2 mM MgCl2 concentrations were initially severely cytotoxic to mitochondrial activity at 16 hr but exhibited slightly reduced cytotoxicity over time. The data also showed that low MgCl2 concentration supplements seemed to have a much less toxic effect on HDPCs compared with higher concentrations of MgCl2 (4 mM and 8 mM) at all time intervals. The results of the present study are consistent with Qu et al. [6], whom tested cell viability of human dental pulp stem cells (DPSCs) on 2D culture plates. Similarly, Zhao et al. [7] delineated that with the increase of Mg2+ ion concentration from 8 mM to 103 mM on human endothelial cells (HCAECs), viability decreased from 105% to almost 0. Low Mg2+ concentration had no significant adverse effects on HCAECs but improved cell proliferation and migration.

In case of reparative dentinogenesis, a cascade of events occurs involving cell division, chemotaxis, cell migration, cytodifferentiation, secretion of the extracellular matrix (ECM) and mineralization of ECM. Dentin matrix consists primarily of COL-1 and non-collagenous proteins (NCPs). The NCPs including DSP, DMP-1 and DSPP even though present in small quantities relative to collagen are of significant functional importance in the mineralization process. Thus, the expression of these critical dentin matrix proteins was assessed in the present study understanding their molecular and cellular bases for pulp regeneration and dental tissue repair.

DSP is one of the key extracellular matrix proteins, capable of stimulating cell attachment, spreading, migration and differentiation of dental mesenchymal cells. DSP may be used as an effective stimulator for dental pulp stem cell differentiation and dental tissue repair. Several studies advocated that DSP has a regulatory role in the initiation of dentin mineralization. Full-length and COOH-terminal DSP regulate bone/tooth related gene/protein expression and stimulate kinase phosphorylation.

Throughout the present study of DSP expression demonstrated the significantly stimulating effect by 0.5 mM-4 mM supplemental MgCl2 on HDPCs. At 7 days, optimal 1 mM MgCl2 concentration showed a significant increase in DSP expression. At days 10, and 14 the same optimal concentration groups had a significantly higher DSP expression. These data are in accordance with that of Kong [3] whom proposed that DPSCs cultured in 1 mM MgCl2 enriched medium, DSP protein amounts were markedly elevated, at all-time points of the culture. Therefore, these findings corroborate that both normal HDPCs and DPSCs cells could display resemblance in the DSP molecular mechanism under stimulation by MgCl2.

DMP-1 is expressed mainly in the ECM of bone and dentin. DMP-1 plays essential roles in osteogenesis and odontogenesis. The full-length DMP-1 and particularly the COOH-terminal fragment could accelerate the nucleation of hydroxyapatite crystals for in vitro mineralization. It was hypothesized that during maturation of the pre-odontoblast/odontoblast, nuclear DMP-1 would be phosphorylated by casein kinase II, leading to its exportation into the extracellular matrix where it promotes hydroxyapatite formation. In-vitro and in-vivo studies have shown that DMP-1 can induce pulp stem cells to differentiate into odontoblast-like cells as a morphogenetic protein targeting undifferentiated cells at the repair site. In the present study, DMP-1 expression of HDPCs was up-regulated during the entire experiment when the supplemented Mg2+ values ranged from 0.5 mM-4 mM, compared to the control group. These results are in accordance with the reports by Qu et al. with enhanced expression of DMP-1 by magnesium phosphate (MgP) in DPSCs [6] and by Kong et al. with markedly elevated expression of DMP-1 protein in DPSCs by 1 mM MgCl2 [3].

DSPP has a marked biological function in dentinogenesis, acts as a cell signaling molecule Earlier studies have reported DSPP as the sole odontoblast-specific gene, contains high levels of carbohydrate and sialic acid as well as aspartic acid and phosphoserine, proposing a function associated with dentin mineralization and mineral nucleation. Deletion of DSPP has altered the dental pulp stem cell fate, resulting in the appearance of chondrocyte-like cells. The conversion of predentin to dentin is impaired by a lack of DSPP, resulting in defects in dentin maturation. Recombinant DSPP stimulated phosphorylation of SMAD-1, induced osteogenic/dentinogenic differentiation suggesting that DSPP functions as a cell signaling molecule. DSPP expression at different time points throughout the present study clearly demonstrated the direct enhancing effect at day 7 with 0.5 mM-2 mM supplemental MgCl2 in HDPCs as compared to the negative control. At day 10, a higher increase in DSPP expression comparable to day 7 time point, with a markedly noticed increase in the 1 mM concentration group. At day 14, an enhanced increase in DSPP expression was observed. These results are in accordance with Qu et al. [6], Kong et al. [3] in DPSCs whom similarly showed an upregulatory effect by the same concentration (1 mM) at the same time points.

The primary function of the ECM is to provide a special physiological microenvironment for cells mediating signal transmission and furnish the template for mineral deposition in dentin and bone. Among families of proteins associated with the collagen matrix, type I collagen (COL-I) is a major organic component of dentin playing an important role in accelerating mineralized tissue formation by dental pulp cells. It has been postulated that collagen modulates several phenotypes of cells including osteoblastic differentiation. These effects are moderated by the interplay of collagen with integrin receptors located on the cell membrane. From these observations, it could be hypothesized that the physiological function of collagen is to promote the activity of dentin formation by promoting the odontogenic differentiation of HDPCs.

Studies demonstrated that COL-I scaffolds enhanced odontogenic differentiation and mineralization of stem cells from exfoliated deciduous teeth (SHED). COL-I hydrogel promoted proliferation of mouse primary calvarial cells (mPCs) and odontogenic differentiation of DPSCs. It has also been revealed that Mg2+ binds collagen to promote the proliferation and differentiation of different cell types through the expression of integrins and downstream signaling pathways. Mg2+ increased adhesion of human synovial MSCs to collagen, and this effect was inhibited by neutralizing antibodies for integrins a3 and b1. These findings were in accordance

with other studies that similarly reported high levels of COL-I integrins expression on substrata, such as hydroxyapatite (HAP), aluminum oxide (Al2O3), which may be due to extracellular changes in Mg2+ ions modifying integrin affinity to their respective ligands.

In the present study, data revealed that 0.5-2 mM MgCl2 concentrations had the highest effect on COL-I expression in HDPCs compared to the negative control at 7 days. A significantly higher increased expression of COL-I at days 10 and 14 were noted among the same groups. The results of the resent study are in accordance with those of Qu et al. [6] whom reported a upregulaory effect by 1% wt/wt Mg2+ ions in DPSCs. These results showed that the incorporation of Mg2+ions significantly enhanced the differentiation and biomineralization of both human DPSCs and HDPCs respectively.

In the present study, MgCl2 at its optimal concentration have shown a significant increase in dentin matrix protein expression (DSP, DMP-1, DSPP, COL-I) compared to the negative control, which exhibited even further upregulation with increasing time points. With higher concentrations (4-8 mM) of MgCl2, dentin matrix proteins were significantly downregulated, showing cytotoxic effect on normal HDPCs. These findings conform to the data of a previous study that introduced hydroxyapatite whiskers (HAp-Ws) with high levels of Mg2+ ions into a poly(d,l-lactide) PDLLA matrix showed an inhibition of osteogenic activity in MC3T3-E1 cells.

In our previous report, the stimulative effect of MgO on the expression of dentin matrix proteins was only limited to the 0.5 mM MgO concentration, showing a narrow window of effective concentration in comparison to a wider range of biological reactivity (0.5-2 mM) of MgCl2. MgO has conventionally been regarded as stable and relatively insoluble. It is speculated that MgO transformation within the culture medium might have occurred, with re-deposition of precipitated Mg (OH)2. Meanwhile, as the precipitation of Mg (OH)2 continues, it counteracts the dissociation of Mg2+ ions. This reaction is more likely to be the reason for the decreased amount of Mg2+ ions observed in MgO. While MgCl2 has been known to have significantly higher solubility (solubility ~5.5 g/L), believed to be responsible for the delivery of Mg2+ ions which in turn upregulated dentin matrix protein expression. It could also be postulated that the microenvironment where the cells lie could have been influenced by the ions arising from dissolution of MgCl2 and the associated pH changes affected cell behavior and extracellular matrix protein expression. Nevertheless, the mechanism underlying the presented data remains unclear and still needs to be addressed. The data from these two studies might indicate that MgCl2 could be a better choice with greater reactivity on dentin matrix expression in HDPCs than MgO.

Conclusion

The data presented in this study suggested the beneficial effect of 0.5 mM-2 mM MgCl2 on cell viability and expression of extracellular-matrix related proteins in HDPCs. This is the first report providing evidence on the effect of optimal MgCl2 concentrations needed to significantly enhance dentinogenic activities of HDPCs. Mg-containing biomaterials could be considered to develop pertinent regeneration therapies for the dentin-pulp complex

Disclosure Statement

No conflict of interest or competing financial interests.

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