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Magnesium Chloride Mediates Dentinogenesis in Normal Human Dental Pulp Cells via Activation of the P38 Mitogen-Activated Protein Kinase/Bmp-2/Smads Signaling Pathways: An in Vitro Study

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Abstract

Up-regulation of odontogenic differentiation and dentin formation in dental pulp are key factors in vital pulp therapy. In previous work, magnesium chloride ($MgCl_2$) has been contemplated for its potentiality of enhancing cell attachment, proliferation rate and expression of dentin matrix proteins of normal human dental pulp cells (HDPCs). However, the mechanism by which $MgCl_2$ stimulates p38 mitogen-activated protein kinase (p38MAPK)/bone morphogenic protein (BMP-2)/SMADS signaling pathways in dental repair remains rather obscure. This study was designed to study and compare the stimulatory effect of different concentrations of $MgCl_2$ on expression of BMP-2, SMADs 1/5/9, phosphorylated p38 (p-p38), and non-phosphorylated p38 MAPK in signal transduction pathways of HDPCs. HDPCs were cultured with 0.5 mm, 1 mm, 2 mm, 4m m, 8mm concentrations of supplemental $MgCl_2$, 0 mm as the negative control group. Statistical analysis using Multi-Way Analysis of Variance (MANOVA) with Wilks' lambda test. Results showed that 0.5, 1 mm, and 2mm supplemental $MgCl_2$ concentrations elicited the highest up regulatory effect on expression of BMP-2, phosphorylated SMADs 1/5/9, p-p38 compared to the negative control all time points ($P<0.0001$). However, 4 mm and 8 mm supplemental $MgCl_2$ concentrations downregulated BMP-2, phosphorylated SMADs 1/5/9, p-p38 expression at all-time intervals ($P<0.0001$). This is the first study to report that $MgCl_2$ at the optimal concentrations of 0.5 mm-2 mm might stimulate the differentiation of HDPCs via p38 mitogen-activated protein kinase (p38MAPK)/bone morphogenic protein (BMP-2)/SMADS signaling pathways.

Key words: Magnesium chloride • Bone morphogenic protein • SMADs • p38 mitogen-activated protein kinase • Regenerative endodontics

Introduction

Vital pulp therapy such as direct pulp capping, indirect pulp capping, and pulpotomy can be used to preserve the health status of the tooth [1]. Upregulation of odontogenic differentiation and dentin formation of HDPCs are the key factors in vital pulp therapy. During reparative dentin formation, the differentiation of dental pulp cells into odontoblasts depends on the regulation of multiple interacting signaling pathways and a variety of signaling molecules [2]. Among the molecules that participate in the regulation of dental pulp cell differentiation, include specific proteins and their signaling and regulatory molecules, such as bone morphogenetic proteins (BMPs) [3], SMADs [4], and mitogen-activated protein kinases (MAPKs) [5].

BMPs are multi-functional growth factors that belong to the transforming growth factor β (TGF-β) superfamily. Similar to their promotion of osteogenic differentiation. BMP-2 also mediates reciprocal interactions between epithelial and mesenchymal tissues, regulates tooth initiation and morphogenesis. Previous studies have elucidated that BMP-2 initiates signals from its receptor to the nucleus of targeted cells through receptor-regulated SMADs (R-SMADs) (SMAD1/2/3/5/8) [6]. On phosphorylation of the C-terminal SSXS motif, R-SMADs form the complex with the common partner SMADs 4 and are translocated into the nucleus. In addition to these positively acting SMADs, SMADs7 induced by BMP-2 blocks the phosphorylation of R-SMADs by acting as pseudo substrate for type I BMP receptor. BMP/SMADs signaling pathway might play a significant role in mediating odontoblastic differentiation and reparative dentin formation.

Members of the mitogen-activated protein kinase (MAPK) family, extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), and p38 kinase are the key elements, which transduce the signals generated by growth factors and stressing agents. Activation of p38 kinase in response to cytokines is associated with the expression of alkaline phosphatase (ALP) during osteoblastic cell differentiation. p38 α MAPK is involved in BMP-2 induced odontoblastic differentiation of HDPCs. Among signal transducers, it has been reported that phosphorylated sites for MAPKs existed in SMADs1/5/9 proteins. Previous reports demonstrated the cooperative interactions between SMADs, p38 pathways in vitro, associated with the rapid activation of SMADs [7]. Therefore, p38 MAPK might interact with BMP-2/SMADs signaling pathways through the phosphorylation process in HDPCs during reparative dentin formation.

Previous studies reported that $MgCl_2$ upregulated attachment efficiency, proliferation rate, alkaline phosphatase (ALP) activity, and enhanced expression of dentin matrix proteins including dentin sialoprotein (DSP), dentin matrix protein (DMP-1), dentin sialophosphoprotein (DSPP) and

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type I collagen (COL-I) in HDPCs in vitro [8,9]. However, the underlying molecular mechanisms of $MgCl_2$ for odontoblast differentiation remain obscure. The aims of the present study were to investigate the stimulatory effect of $MgCl_2$ on the expression of BMP-2, SMADs 1/5/9 and p38MAPK and on the odontoblast differentiation via SMADs 1/5/9 signaling pathways in HDPCs in vitro.

Materials and Methods

Magnesium chloride ($MgCl_2$) preparation

Magnesium chloride hexahydrate (Fisher Chemical, USA) was dissolved in deionized water and 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.

Human Dental Pulp Cells (HDPCs) culture

This study was approved by Boston University IRB committee under IRB approval H-33173. Human dental pulp explants were obtained from young and systemically heathy patients with an age range of 15-25 years requiring third molar or orthodontic premolar extractions. Patients were given the informed consent before the extraction procedure at the surgery department at Boston University School of Dental Medicine. HDPCs were isolated following a previously published protocol with modifications [10]. First, sagittal indentation was made using a high-speed handpiece and fissure bur with a coolant without exposing the pulp. 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 cm^2 culture flask (Thermo Fisher Scientific, USA). Culture medium consisted of 10% fetal bovine serum (FBS) (Life Technologies), 1X Penicillin antibiotic (100 U/mL), and 1X Streptomycin (100 ug/mL) (Life Technologies), Amphotericin B anti-fungal (0.25 ug/ml) in Eagle's Basal Medium (BME) (Life Technologies). The culture flasks were maintained at 37°C, in a standard CO_2 incubator, and saturated humidity and cultured up to the second passage. Growth media was changed every 72 hours. When nearly confluent cells were trypsinized with 0.05% Trypsin (Life Technologies). Re-suspended cells were then aspirated and collected in a sterile 15mL disposable tube placed in the TJ-6 Beckman Centrifuge at 1000 rpm for 5 minutes. After centrifugation, a pellet of cells formed. The cells were then counted using a hemocytometer. Characterization of dentinogenic phenotype of the cells was confirmed by expression of dentinogenic markers induced by vit D_3 stimulation. Human dental pulp cells were transferred to 24 well plates and grown to confluence supplemented with 0mm (control), 0.5 mm, 1 mm, 2 mm, 4 mm and 8 mm $MgCl_2$ concentrations respectively. For cellular differentiation studies, growth media were replaced with pre-inductive dentinogenic media with 10 nm Vitamin D_3 (172g/mol) (Sigma Aldrich, MA, USA) for two additional days before the predetermined time intervals of 4, 7, and 11 days. Dentinogenic media was composed of the following: 10% charcoal stripped fetal bovine serum (FBS) (Life Technologies, MA, USA), 100 IU/ml Penicillin (Gibco, Waltham, MA, USA), 100 µg/m streptomycin (Gibco, MA, USA), 10^{-8}M Menadione (Sigma Aldrich, Burlington, MA, USA), 10 mm β-Glycerophosphate (Sigma Aldrich, Burlington, MA, USA), 0.05 mg/mL L-ascorbic acid (Sigma Aldrich, MA, USA), 2mm L-glutamine (Gibco, Waltham, MA, USA). From measurements obtained from proliferation data in previously published study [9], the cell numbers for each time interval 7, 10 and 14 days were used to determine the values of extracellular protein level on a per million cell base formula.

Assessment of Bone Morphogenic Protein (BMP-2) expression in HDPCs

Cell culture supernatant samples at days 7, 10 and 14 were tested using bone morphogenic protein (BMP-2) Xpress BioTM ELISA Kit (Human), to assess expression levels of secreted BMP-2 protein. The kit is based on a sandwich enzyme-linked immune-sorbent assay technology. Anti-BMP-2 antibody and biotin conjugated antibody were used as detection antibodies. In a 96-well microplate, five replicate wells were labeled, the tested cell culture supernatants were collected from $MgCl_2$ concentrations: 0.5 mm, 1 mm, 2 mm, 4 mm, 8 mm and 0 mm as the control group. Supernatants with various conditions were centrifuged at 1000 xg (or 3000 rpm) for 20 minutes to remove cellular debris. Wash buffer was prepared by adding 30 mL of the wash buffer concentrate and 750 mL deionized water. Microtiter plate was washed two times with wash buffer before adding standards, sample, and controls (blank wells). The standard solutions were prepared in concentrations of 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.25 pg/mL, 15.625 pg/mL, 7.8 pg/mL and blank respectively. 0.3 mL of sample dilution buffer was added into each tube. Sample dilution buffer were aliquoted and added into the standard and the control (blank) wells. Microtiter plates with 100 µL of the diluted (2x) test samples, standards and blanks were incubated at 37°C for 90 minutes. The plate contents were then discarded and washed 2 times with wash buffer. 100 µL of the biotin conjugated detection antibody was added cautiously at the bottom of each well without touching the side wall to all standard, blank and test sample wells subsequently in the antibody pre-coated microtiter plate. The plate was then sealed with a cover and incubated for 60 minutes at 37°C. The incubation mixture was then discarded from each well. After, the plate was washed for three washes. Thereafter, 100 µL horseradish peroxidase streptavidin (HRP-Streptavidin) was added into each well and incubated for 30 minutes. Unbound conjugate was washed away five times with wash buffer. Before aspiration of the wash buffer, it was allowed to sit for 1-2 minutes, and then the plate was inverted and blotted onto a clean absorbent paper towel until no moisture was present. 90 µL TMB substrate was then added into each well, followed by incubation in the dark for 10-20 minutes. TMB was catalyzed by HRP-Streptavidin to produce a light blue color product. Finally, the HRP enzymatic reaction was brought to an end by adding 50 µL stop solution into each well in the same sequence as the TMB substrate solution, which in turn resulted in a yellow color immediately. Following the completion of the assay, the microplate reader (TECAN, Infinite 1000 Pro) was adjusted at a wavelength of 450 nm to identify the intensity of color (O.D) versus the respective BMP-2 concentration in the sample or the standard. BMP-2 concentration was calculated by a standard curve and normalized to BMP-2 expression measured per million of cells.

Assessment of SMADs 1/5/9 signaling down-stream effectors expression in HDPCs

Qualitative determination of target protein concentration of SMADs 1/5/9 in cultured cells was attained by using a colorimetric cell-based indirect ELISA Kit format CytoGlow™ (Assay Biotechnology, USA). Due to the qualitative nature of the cell-based kit, a monoclonal antibody specific for human glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was included to serve as an internal positive control in normalizing the target absorbance values. HDPCs were seeded at a density of 20,000 cells per well in 200 µL of culture medium in a 96-well plate (Thermo Fisher Scientific, MA, USA). Cells were treated with various concentrations of supplemental $MgCl_2$: 0.5 mm, 1 mm, 2 mm, 4 mm, 8 mm, 0 mm as negative control respectively. Each condition was repeated in five replicas. SMADs 1/5/9 protein expression profiles in cells were measured at 16hr, 7, 10 and 14 days according to the manufacturer's recommendations. The culture plates were incubated at 37°C and 5% CO_2. Cell culture medium was removed and rinsed with 200 µL of (1x) TBS twice. Growth medium for both the control and experimental groups was changed by a fresh medium every 3 days. Cells were then fixed in 100 µL of 4% formaldehyde for 20 min at room temperature. Fixing solution was removed and the plates then washed three times with 200 µL (1x) wash buffer for 5 min, each time with gentle shaking. 100 µL of quenching buffer was added, the plates were incubated for 20 min at room temperature. Thereafter, the plates were then washed with 1x wash buffer for 5 min. Then, 200 µL of blocking buffer was added for 1 hr at room temperature. The plates were then washed with 200 µL (1x) wash buffer. 50 µL of 1x primary antibody (Anti-SMADs 1/5/9) was added to the corresponding

wells containing different concentrations of $MgCl_2$. Anti-GAPDH antibody was added to the positive control wells. After incubation for 16 hrs at 4ºC, plates were washed three times with 200 µL (1x) wash buffer for 5 min at a time with gentle shaking on the shaker. Then, 50 µL of secondary antibody (HRP-Conjugated Anti-rabbit IgG) was added to the wells containing the Anti-SMADs 1/5/9 and to the negative control wells. HRP-Conjugated Anti-Mouse IgG antibody was then added to the positive control wells containing (Anti-GAPDH) antibody and incubated for 1.5 hr at room temperature with gentle shaking on the shaker. Plates were then repeatedly washed with 200 µL of (1x) wash buffer. Later on, 50µL of ready to use substrate was added to each well and incubated for 30 min at room temperature in the dark with shaking on the shaker. 50 µL of stop solution was then added and the ELISA plates were read using the microplate reader for HRP activity. After measuring absorbance at 450 nm, plates were washed twice with 200 µL of (1x wash) buffer, and then twice with 200 µL of 1x TBS. Plates were tapped on paper towels to remove excess liquid, then were left to air dry at room temperature. Then, crystal violet staining method was used to determine optical cell density number. 50 µL of crystal violet solution was added to each well and incubated for 30 min at room temperature with gentle shaking on the shaker. Followed by rinsing with 100 µL of distilled water, four to five times, till the wells became transparent. Plates were allowed to dry for 30 minutes. Thereafter, 100 µL of SDS solution was added into each well and incubated at room temperature for 1 hr. Absorbance was read with the microplate reader (TECAN, Infinite 1000 Pro) at 595 nm. The results were analyzed by normalizing the absorbance values to cell numbers. OD values at 450 obtained for the SMADs 1/5/9 target protein were normalized with the OD values at 450 obtained for GAPDH. For crystal violet staining normalization, the measured OD values at 450 were normalized with the OD values at 595 by dividing, OD 450/OD 595.

Assessment of expression and phosphorylation of p38 MAPK proteins in HDPCs

The effects of $MgCl_2$ on the expression of p38 MAPK (p38 MAPK) and phosphorylation of p38 MAPK (p-p38 MAPK) was determined using RayBio® cell-based Human/Mouse/Rat p38 MAPK ELISA kit. HDPCs were seeded at a density of 20,000 cells per well in 200 µL of culture medium in a 96-well plate. Cells were treated with various concentrations of supplemental $MgCl_2$: 0.5 mm, 1 mm, 2 mm, 4 mm, 8 mm, 0 mm as negative control. Both phosphorylated and non-phosphorylated p38 MAPK proteins in cells were measured at 16hrs, 7, 10 and 14 days. Each condition was repeated in five replicas. The culture plates were incubated at 37°C and 5% CO_2. Growth medium was changed by fresh medium every 3 days for both the control and experimental groups. Subsequently, cell culture medium was discarded and washed with 200 µL of wash buffe for three times, 5 min each with gentle shaking on a shaker. Wash buffer was discarded. After the final wash, plates were blotted on to a paper towel to remove any excess buffer. Cells were then fixed by incubating with 100 µL of fixing solution for 20 min at room temperature. Fixing solution was removed and the plates washed three times with 200 µL wash buffer (1x), 5 min each time with gentle shaking. 200 µL (1x) quenching buffer was added and the plates were then incubated for 20 min at room temperature. The plates were washed with wash buffer A (1x), four times for 5 min each on the shaker. Afterwards, 200 µL of (1x) blocking buffer was added, and the plates were incubated for 1 hr at room temperature. The plates were then washed three times for 5 min each on the shaker. 50 µL of 1x primary antibodies G-1 Mouse Anti-phosphorylated (p-p38) MAPK (Thr180/Tyr182) and H-1 Mouse Anti p38 MAPK were added into the corresponding wells and incubated for 2 hours at room temperature. Plates were then washed with wash buffer B (1x) 4 times. 50 µL of the conjugated secondary antibody (Anti-Mouse IgG) was added to the wells containing the primary antibody and to the negative control wells. Plates were then incubated for 1 hour at room temperature and washed four times with 1 wash buffer B (1x). Subsequently, 100 µL of TMB substrate was added into each well and incubated for 30 minutes in the dark at room temperature. 50 µL of stop solution was added into each well and plates were read immediately using the microplate reader (TECAN, Infinite 1000 Pro) at 450 nM. Negative control values were subtracted from OD 450 values which were obtained for the levels of target phosphorylated p38 MARK (p-p38) MAPK and non-phosphorylated p38 MAPK. The mean of the replicas of each of the p-p38 MARK and the non-phosphorylated p38 MARK was plotted for each of the time points against the various concentrations of $MgCl_2$. Crystal violet staining method was used to determine optical cell density number. 50 µL of crystal violet solution was pipetted into each well, placed for 30 min on the shaker at room temperature. Plates were then rinsed with 100 µL of distilled water, four to five times, till the wells became colorless. Then, plates were allowed to dry for 30 minutes. Thereafter, 100 µL of SDS solution was added into each well and incubated on the shaker at room temperature for 1 hr. Absorbance was with the microplate reader (TECAN, Infinite 1000 Pro) at 595 nm. The results were analyzed by normalizing the absorbance values to cell numbers. The measured OD values at 450 nm were normalized with the OD values at 595 by dividing, OD 450/OD 595.

Results

Effect of $MgCl_2$ on BMP-2 protein expression of HDPCs

At day 7, BMP-2 expression for 0.5 mm, 1 mm, 2mm supplemental $MgCl_2$ concentration groups showed a statistically significant difference in comparison to the negative control group. The 1 mm supplemental $MgCl_2$ concentration group presented the highest increase in BMP-2 expression of HDPCs comparable to the negative control group ($P<0.0001$) and other study groups (Figure 1) At day 10, significantly higher increase in BMP-2 expression was observed in the 0.5 mm, 1 mm and 2mm supplemental $MgCl_2$ concentration groups compared to the control ($P<0.0001$) (Figure 1). The 1 mm supplemental $MgCl_2$ concentration group presented the highest increase in BMP-2 expression. On the other hand, cells grown in media containing higher $MgCl_2$ supplements 4mM and 8mM showed statistically lower BMP-2 expression compared to the control group. At day 14, the 1mM supplemented $MgCl_2$ concentration still held the highest value compared to the negative control group and the other groups. ($P<0.0001$) (Figure 1). 0.5 mm and 2mm supplemented $MgCl_2$ concentrations also showed statistically higher levels of BMP-2 expression compared to control. However, significant lower BMP-2 expression was noted in cells grown in media containing higher $MgCl_2$ supplements 4mM and 8mM compared to the negative control group. The p-values of BMP-2 expression within groups for MANOVA of $MgCl_2$ concentrations showed statistically significant values ($P<0.0001$). Wilks' Lambda interaction P-value for $MgCl_2$ concentrations at different time intervals showed the same statistically significant value ($P<0.0001$).

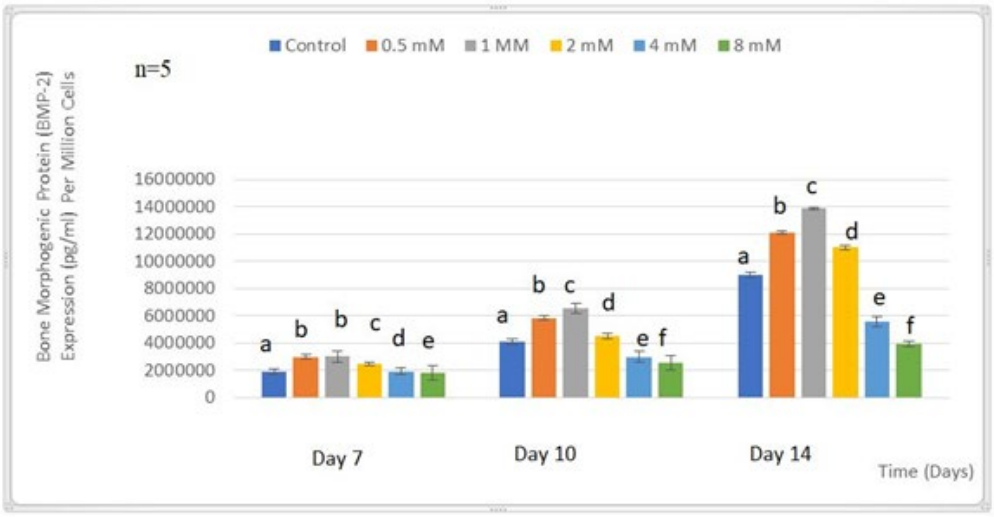

Figure 1. Histogram showing of Bone Morphogenic Protein (BMP-2) Expression of Normal Human Dental Pulp Cells in Supplemental Magnesium Chloride ($MgCl_2$) of Different Concentrations

Notes: Normal human dental pulp cells were cultured in media containing supplemental magnesium chloride ($MgCl_2$) concentrations: 0.5 mm, 1mm, 2 mm, 4mm, 8 mm and 0 mm as negative control group at time periods of 7, 10 and 14 days. Bone Morphogenic Protein (BMP-2) 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 $MgCl_2$ on SMADs 1/5/9 expression of HDPCs

SMADs 1/5/9 expression at 16 hrs, showed a statistically significant increase in 0.5 mm, 1 mm, 2mm supplemental $MgCl_2$ concentration groups of HDPCs in comparison with the negative control group. The 1mm supplemented $MgCl_2$ concentration group presented the highest value in SMADs 1/5/9 expression comparable to the negative control and the other $MgCl_2$ concentration groups (P<0.0001) (Figure 2). At day 7, SMADs 1/5/9 expression presented a markedly significant increase in 0.5 mm, 1 mm supplemental $MgCl_2$ concentration groups of HDPCs comparable to the negative control. The 1 mm showed the highest increase in value. (P<0.0001) (Figure 2). Meanwhile, there was no significant difference in SMADs 1/5/9 expression between the 2mm supplemental $MgCl_2$ and the negative control group. For the 4 mm and 8 mm $MgCl_2$ concentration groups SMADs 1/5/9 expression was significantly lower than that of the negative control group (P<0.0001). At both day 10 and day 14, the significant higher increase in SMADs 1/5/9 expression was observed in the 0.5 mm and 1 mm supplemental $MgCl_2$ concentration groups. The 1 mm supplemented $MgCl_2$ concentration reached the highest value compared to other concentration groups and the negative control group (P<0.0001) (Figure 2). On the other hand, cells grown in media containing high $MgCl_2$ supplements 4 mm and 8 mm presented statistically lower SMADs 1/5/9 expression compared to the negative control. The p-values of SMADs 1/5/9 expression within groups for MANOVA of $MgCl_2$ concentrations showed statistically significant values (P<0.0001). Wilks' Lambda interaction P-value for $MgCl_2$ concentrations at different time intervals showed the same statistically significant value (P<0.0001).

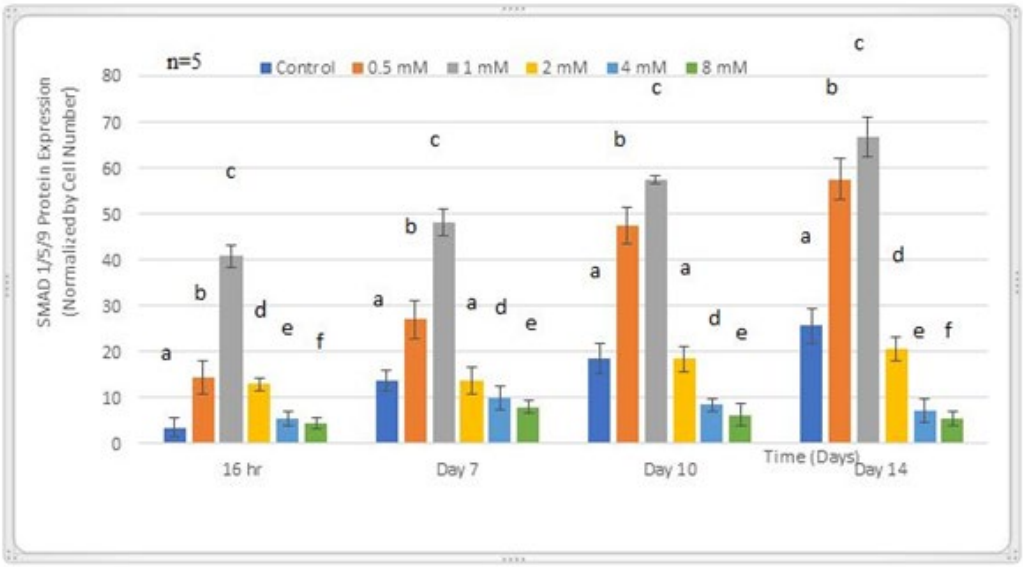

Figure 2. Histogram showing SMADS (1/5/9) Expression of Normal Human Dental Pulp Cells in Supplemental Magnesium Chloride ($MgCl_2$) of Different Concentrations

Notes: Normal human dental pulp cells were cultured in media containing supplemental magnesium chloride ($MgCl_2$) concentrations: 0.5 mm, 1 mm, 2 mm, 4 mm, 8 mm and 0 mm as negative control group at time periods of 16 hr, 7, 10 and 14 days. SMADs 1/5/9 expression was normalized per cell number 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 $MgCl_2$ on levels of phosphorylated p38 MAPK and non-phosphorylated p38 MAPK of HDPCs

At both time points, 16 hr and day 7, a significant increase in levels of the phosphorylated-p38 MAPK (p-p38 MAPK) and non-phosphorylated p38 MAPK (p38 MAPK) was seen in the 0.5 mm and 1 mm supplemental $MgCl_2$concentration groups of HDPCs (P<0.0001) (Figures 3A and 3B). Also, a significantly higher levels of p-p38 MAPK than p38 MAPK in both 0.5 mm and 1 mm groups at these two time-intervals (P<0.001). On the other hand, 4 mm and 8 mm $MgCl_2$ supplements revealed statistically lower p-p38 MAPK, and non-phosphorylated p38MAPK levels compared to the negative control. Wilks' Lambda interaction P-value for $MgCl_2$ concentrations at 16 hrs and day 7 for p-p38 MAPK presented a statistically significant value (P<0.0001). At day 10, a significant increase in levels of the phosphorylated-p38 MAPK (p-p38 MAPK) and non-phosphorylated p38 MAPK (p38 MAPK) was seen in the 0.5 mm, 1 mm and 2mm supplemental $MgCl_2$ concentration groups of HDPCs (P<0.0001) (Figure 3C). Also, a significant higher level of p-p38 MAPK was observed in the 0.5 mm, 1 mm, and 2mm supplemental $MgCl_2$ concentration groups comparable to the non-phosphorylated p38 MAPK, (P<0.0001) (Figure 3C). At day 14, the significantly higher levels of p-p38 MAPK comparable to the non-phosphorylated p38 MAPK were shown in all concentration groups while a significant increase in levels of the phosphorylated-p38 MAPK (p-p38 MAPK) and non-phosphorylated p38 MAPK (p38 MAPK) was seen in the 0.5 mm and 1 mm supplemental $MgCl_2$ concentration groups of HDPCs (Figure 3D) (P<0.0001). Wilks' Lambda interaction P-value for $MgCl_2$ concentrations at day 14 for p-p38 MAPK presented a statistically significant value (P<0.0001). In viewing the ratio of phosphorylated p38 MAPK to non-phosphorylated p38 MAPK (p-p38 MAPK: p38 MAPK). HDPCs treated with the 0.5 mm, 1 mm, and 2mm supplemental $MgCl_2$ concentration groups showed increased ratios by 1.07, 1.19, 1.13 (P<0.0001) at 16 hours, 1.24 1.38, and 1.41 at 7 days (P<0.0001), 1.44,1.82, and 1.91 at 10 days (P<0.0001) and 1.66,1.95 and 1.99 at 14 days (P<0.0001). Wilks' Lambda interaction P-value for $MgCl_2$ concentrations at different time intervals for ratio of phosphorylated p38 MAPK presented a statistically significant value (P<0.0001).

Notes: Normal human dental pulp cells were cultured in media containing supplemental magnesium chloride ($MgCl_2$) concentrations: 0.5 mm, 1 mm, 2 mm, 4 mm, 8 mm and 0 mm as negative control group at 16 hr, 7 days, 10 days, and 14 days. Phosphorylated p38 MAPK and non-phosphorylated p38 MAPK were normalized per cell number at each time interval. The control cells were treated with growth media without supplemental magnesium ($MgCl_2$) chloride. The data is presented as means of six replicates with error bars indicating the standard deviation. Groups labeled with different letters differ statistically for the phosphorylated p-38 MAPK and non-phosphorylated p38 MAPK, as compared to the control group and other study groups (P<0.0001).

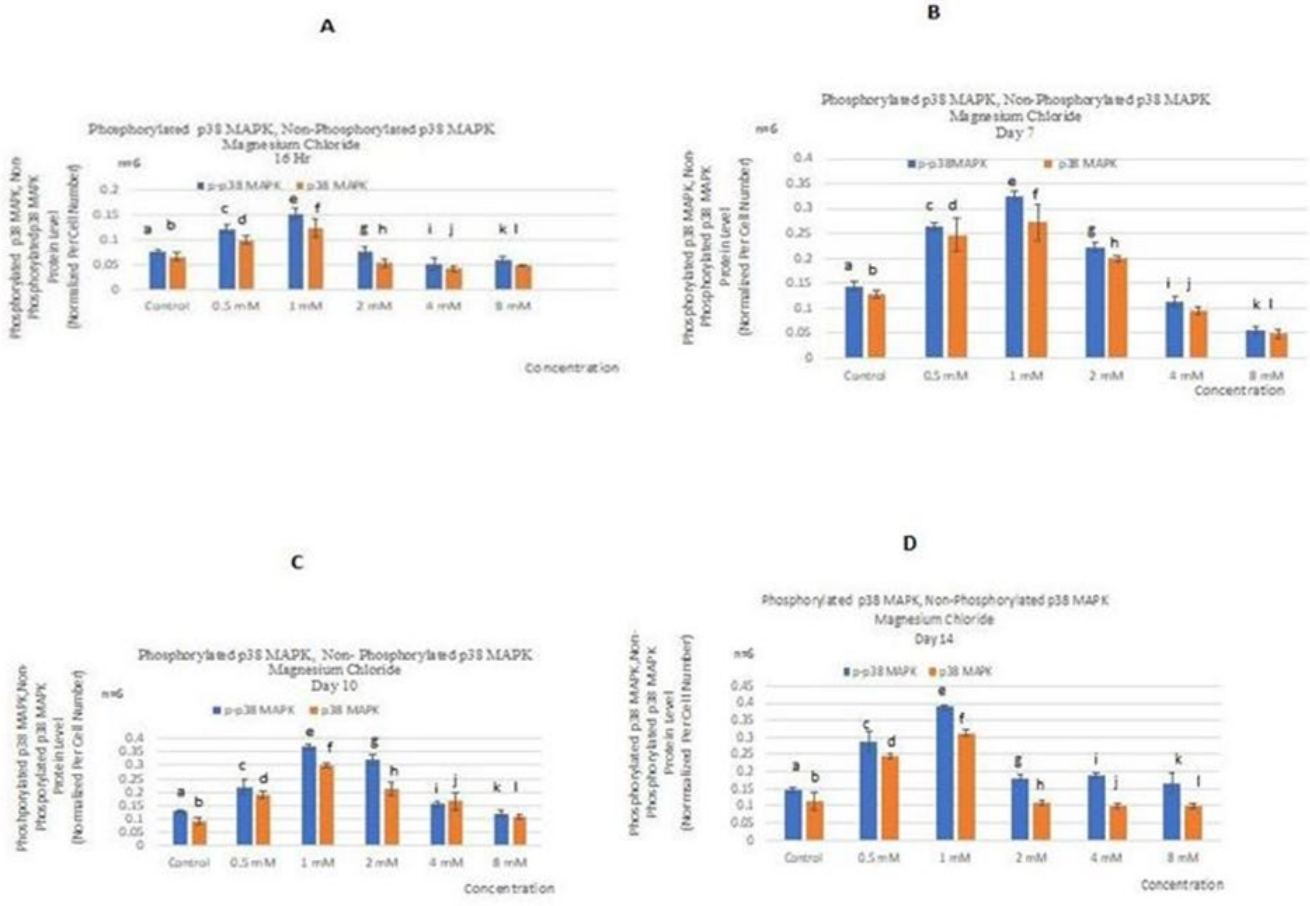

Figure 3. Histogram showing Phosphorylated p38 MAPK and Non-Phosphorylated p38 MAPK Levels of Normal Human Dental Pulp Cells in Supplemental Magnesium Chloride ($MgCl_2$) of Different Concentrations A) 16 Hrs B) Day 7 C) Day 10 D) Day 14

Discussion

The vitality of the dental pulp has always been the goal for a successful long-term restorative dental treatment. In clinically relevant insults due to trauma or infection, the pulp has some ability to form reparative dentin. The differentiation of dental pulp cells plays a key role in pulp/dentin regeneration. A variety of factors participate in the regulation of dental pulp cell differentiation, including specific proteins and cytokines such as bone morphogenetic protein (BMP), SMADs 1/5/9 complex and p38 MAPK. BMP-2 is an important mediator of extracellular matrix biosynthesis and is involved in regulation of cell growth, differentiation and function. Previous studies reported that BMP signaling is initiated by receptor binding, propagated by phosphorylation of the SMADs 1/5/9 complex and finally transduced into the nucleus resulting in regulation of target protein transcription [11]. SMADs 1/5/9 is involved in BMP-2-induced odontoblastic differentiation in dental pulp cells. Loss of BMP signaling resulted in reduced crown and root dentin formation, but restoring SMADs signaling activity rescued dentin formation, suggesting that BMP-SMADs signaling regulates dentinogenesis [12]. Several studies demonstrated the effect of Mg^{2+} on BMP-2 signaling. Ding et al. [11] investigated the effect of Mg^{2+} modification on the osteogenic bioactivity of rhBMP-2 with calcium phosphate cement (CPC) and modified calcium phosphate cement (MCPC) as substrate models. When compared with CPC/rhBMP-2, C2C12 myoblasts or bone marrow mesenchymal stem cells (BMSCs) cultured on 5 MCPC/rhBMP-2 substrates exhibited dramatically enhanced in vitro osteogenic differentiation and phosphorylation of SMADs 1/5/9. Cheng et al. [12] also stated that Mg^{2+} scaffolds promoted bone regeneration via activation of BMP-2 signaling by releasing extracellular Mg^{2+}. ERK signaling inhibition by U0126 resulted in significantly decreased type II BMP receptor, type I BMP receptor, and phosphorylated SMADs 1/5/9 amounts, indicating that BMP2/SMADs is a downstream signaling of ERK. These above studies are in accordance with the experimental data in relation to the effect of Mg^{2+} supplement on odontogenic differentiation of HDPCs and BMP-2 expression in the present study. In the present study, a significant increase in BMP-2 expression was noted in 0.5-2mm supplemental $MgCl_2$ concentration groups of HDPCs comparable to the negative control at all-time points of 7,10 and 14 days. As for the 1 mm supplemented $MgCl_2$ concentration group, BMP-2 expression reached the highest level of expression compared to the negative control at all-time points.

A number of studies have used primary dental pulp cells, existing pulp cell lines, and a few odontoblastic cells lines to elucidate SMADs signaling pathway activation. Woo et al. [13] reported that the combination of MTA and platelet rich fibrin extract (PRFe) induced the activation of BMP/SMADs signaling and enhanced the expression of BMP-2/4 and phosphorylation of SMADs 1/5/9 in HDPCs in vitro showing a synergistic effect on the stimulation of odontoblastic differentiation of HDPCs via the modulation of the BMP/SMADs signaling pathway. In a study by Zhang et al. [14] the protein levels of SMADs 4 and type I BMP receptor were significantly upregulated by Mg^{2+} ions. The upregulation of BMP-2 activated the BMP-2/SMADs signaling pathway in bone marrow mesenchymal stem cells (BMSCs) played the key pathway for osteogenic differentiation. Taken together, these findings suggested that $MgCl_2$ enhanced odontogenic differentiation in dental pulp cells by activating ERK/BMP2/SMADs signaling. The data of the present study demonstrated that at 7 days optimal 0.5 mm and 1 mm $MgCl_2$ concentration groups showed an increase in the SMADs 1/5/9 expression. Moreover, at days 10, and 14 the same concentration groups had a significantly higher increase in SMADs 1/5/9 expression. These results are also in agreement with Kong et al. [15] whom substantiated that dental pulp cells cultured with 1 mm $MgCl_2$ concentration, SMADs 1/5/9 protein amounts were markedly elevated showing the same trend regarding the concentration range and the time points. Moreover, Zhang et al. [14] corroborated that $MgCl_2$ induced more expression and more robust phosphorylation of SMADs 1/5/9 protein of C2C12 myoblasts and BMSCs cells in vitro. The upregulation of BMP-2 activated BMP-2/SMADs signaling, subsequently phosphorylation of SMADs 1/5/9 is the key pathway for differentiation of both cell types. Taken together, these findings suggested that Mg^{2+}promoted odontogenic and osteogenic differentiation by activating BMP2/SMADs signaling. Conclusively, the present study revealed that Mg^{2+} handled at the optimal concentrations promoted the activation of BMP/SMADs signaling pathway in odontogenic differentiation.

Among signal transducers that have been reported to participate in TGF-β signaling, MAPKs are a group of well-described serine/threonine kinases including extracellular-signal regulated kinases (ERKs), Jun amino terminal kinase (JNK) and p38 mitogen activated protein kinase implicated in the transmission of extracellular signals to intracellular target [10]. Several studies indicated that MAPKs are involved in the regulation of odontoblastic differentiation. Zhao et al. [16] reported that MTA induced the phosphorylated and unphosphorylated levels of p38 and p-JNK MAPK. MTA significantly increased the expression of ALP, DSPP, COLI, OCN and BSP mRNAs. MTA-induced odontoblastic differentiation of human DPSCs via p38 MAPK pathways. Qin et al. [17] reported BMP-2 upregulated phosphorylation and unphosphorylated p38 α of HDPCs. Moreover, suppression of p38 MAPK depressed odontoblastic differentiation in HDPCs. Studies by Wang et al [18] and Wu et al. [19] noted that the levels of the p38 protein was activated in response to BMP-2 and BMP-4 in dental pulp cells, while phosphorylated-p38 was significantly decreased with SP600125 inhibition. Similarly, the above forementioned studies convincingly supported that MAPKs play essential roles in regulating osteogenic/odontogenic activity. This is the first report that reveals that treatment with $MgCl_2$ triggered the activation of the p38MAPK cascade in HDPCs. At day 7 time point, treatment with 0.5 mm, 1 mm and 2mm supplemental $MgCl_2$ concentration groups induced the expression of p38 MAPK and activation of the phosphorylated-38 (p-p38) in HDPCs. At both 10 and 14 days, the same concentration groups showed a significantly higher increase in expression and phosphorylation of p38 MAPK. The enhanced phosphorylation of p38 MAPK at different time points throughout the experiment with the 0.5 mm, 1 mm and 2mm supplemental $MgCl_2$ concentration groups yielded enhanced signaling effect in odontogenic differentiation. Meantime, contrastingly phosphorylation rate of p38 MAPK was down regulated with higher 4 and 8 mm supplemental $MgCl_2$ concentrations at different time points. However, the molecular mechanism by which $MgCl_2$ induced p38 activation driving odontoblastic differentiation needs further investigation.

Conclusion

The data presented in this study suggested the beneficial effect of 0.5 mm, 1 mm, and 2mm supplemental $MgCl_2$ on promoting odontoblastic differentiation, through the activation of BMP-2/SMADs/p38 signaling pathways. This is the first study to reveal the optimal $MgCl_2$ concentrations required to significantly promote dentinogenic activities of HDPCs via phosphorylated p38 MAPK signaling pathway. $MgCl_2$ containing biomaterials could be considered to provide a novel therapeutic strategy for the dentin-pulp complex.

Conflict of Interests

The authors declare no conflict of interest.

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