

The influence of addition of osteogenic supplements to mineral trioxide aggregate on the gene expression level of odontoblastic markers following pulp capping in dogs

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ABSTRACT

This study investigates the effect of addition of dexamethasone, vitamin D₃, or chitosan to mineral trioxide aggregate (MTA) on the gene expression level of dentin sialophosphoprotein (DSPP) and matrix extracellular phosphoglycoprotein (MEPE) after pulp capping in dogs. Pulp exposure was performed in sixty dogs' teeth. The teeth were classified into 3 equal groups according to the evaluation period. Group 1: 7 days, group 2: 21 days and group 3: 60 days. Each group was further subdivided according to the pulp capping material used, into subgroup A: MTA + dexamethasone, subgroup B: MTA + dexamethasone + vitamin D₃, subgroup C: MTA + chitosan and subgroup D: MTA. According to the group, the pulps of the capped teeth were removed for analysis of the relative mRNA expression level of DSPP and MEPE using PCR. Statistical analysis of all data was performed. In subgroup A, significant expression was observed of DSPP ($P \leq 0.05$) in group 2 up to 18.8 relative fold change while in subgroup B a significant upregulated gene expression of DSPP ($P \leq 0.05$) up to 29.4 relative fold change was seen. Significant upregulated DSPP expression ($P \leq 0.05$) was recorded in groups 1 and 2 up to 6.9 and 3.6 relative fold change, respectively in subgroup C. In conclusion, dexamethasone, with or without vitamin D₃ and chitosan, are synergistic odontogenic inducers with MTA for differentiation of dental pulp cells in dogs. The upregulation of DSPP is a good marker for this differentiation.

Key words: chitosan, dexamethasone, dentin sialophosphoprotein, matrix extracellular phosphoglycoprotein, mineral trioxide aggregate, pulp capping

Introduction

Direct pulp capping is considered a well-established method of treatment where the exposed pulp tissue is covered by a suitable capping material. This material protects

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the pulp from additional injury, and allows healing and repair. The goal of treating the exposed pulp with an effective pulp capping material is to promote the dentinogenic potential of pulp cells (OKAMOTO et al., 2009; ZARRABI et al., 2010). Mineral trioxide aggregate (MTA) is widely used for pulp capping, root-end filling, different perforation repairs and many other endodontic procedures (TAWFIK et al., 2013; NAGY et al., 2014). Mineral trioxide aggregate is the most commonly recommended repair material used for capping exposed dental pulp. It is a bioactive material, with a high sealing ability, relative antibacterial properties, and excellent biocompatibility (NAGY et al., 2014). Unfortunately, delayed setting time and poor handling characteristics, including the physical and mechanical properties, are the main drawbacks of MTA, on which numerous investigations have been conducted (PARIROKH and TORABINEJAD, 2010). Therefore, there have been many attempts to improve the handling characteristics of MTA by addition of various additives (KOGAN et al., 2006; PARIROKH et al., 2011), or to develop new capping materials as an alternative to MTA that can improve pulp tissue repair (KIBA et al., 2010; WANG et al., 2014). The traditional method to assess the hard tissue forming capability of pulp capping materials was by histologic investigations (KIBA et al., 2010). However, it is now possible to evaluate it by the gene expression level of odontoblastic differentiation markers, such as dentin sialophosphoprotein, dentin matrix protein, matrix extracellular phosphoglycoprotein, bone sialoprotein or osteonectin (LIU et al., 2005; MIN et al., 2009; WANG et al., 2010; KARANXHA et al., 2013). Mineral trioxide aggregate is a pulp capping material that has a positive affect on a series of genetic changes in the pulp cells. Several studies have investigated the genetic expression of mineralizing genes after the application of MTA to dental pulp cells (KIM et al., 2010; PARANIPE et al., 2010). Most of these studies used quantitative real-time polymerase chain reaction to quantify the gene expression level of odontoblastic markers. This test provides more accurate and indicative results than those given by the previously used qualitative polymerase chain reaction, which only shows the presence or absence of gene expression. Several studies have been conducted to evaluate the effect of addition of various additives to MTA in order to improve the quality of the dentinal bridge that follows pulp capping procedures, thus improving its clinical outcomes (PARIROKH and TORABINEJAD, 2010; PARIROKH et al., 2011). These studies were mainly *in vitro* and did not cover all the physical properties and clinical applications of the material. The aim of this study was to investigate the effect of the addition of dexamethasone, vitamin D₃, or chitosan to MTA on the gene expression level of dentin sialophosphoprotein (DSPP) and Matrix extracellular phosphoglycoprotein (MEPE), for monitoring the mineralization and odontoblastic differentiation of dental pulp stem cells.

Materials and methods

Experimental animals. The experimental protocol was approved by the Ethical Committee of the Faculty of Dentistry, Ain Shams University and the Animal Use and Care Committee of the Faculty of Veterinary Medicine, Cairo University, Egypt. This study was performed on sixty intact incisors, canines and premolars, related to three dogs with intact dentitions, aged 2-3 years and weighing 20-25 kilograms. These teeth were divided into 3 equal groups (one dog each) according to the evaluation period, that is: group 1 (7 days), group 2 (21 days) and group 3 (60 days). The three dogs were clearly labeled by ear tags for identification of the experimental period.

General anesthesia and pulp exposure. Each animal was put under general anesthesia using Atropine sulphate (Sopharma, Bulgaria) at a dose of 0.1 mg/Kg given subcutaneously, then Xylazine HCl (Alfasan International B.V., Werden, Holland) at a dose of 1 mg/Kg given intravenously. General anesthesia was induced using Ketamine HCl (Alfasan International B.V., Werden, Holland) at a dose of 5 mg/Kg given intravenously, then maintained by Thiopental sodium (Egyptian Inter Pharmaceutical Industries Co, Tenth of Ramadan City, Egypt) at a dose of 25 mg/Kg, 2.5%, solution given intravenously (EL ASHRY et al., 2013).

On the day of the operative procedure, all the teeth were scaled and polished with a rubber cup. Quadrants of the teeth were isolated using sterile cotton rolls. Pulp exposure procedures were performed in 5 teeth of each quadrant of the experimental dogs. Class V cavities were prepared on the buccal surfaces of teeth (1.5-2 mm depth) at ultra-high speed, with a copious water spray. The preparations were cut 0.5-1 mm above the free gingiva. Pulp exposure was performed in the middle of the cavity floor using a sterile round tungsten carbide bur (0.8 mm in diameter; Komet) at high speed and water cooling. After exposure, the cavities were washed with sterile saline and dried with cotton pellets. Hemorrhage was controlled by light pressure.

Pulp capping procedures. The experimental teeth (n = 60) were divided into 3 equal groups according to the evaluation period (20 teeth each). The exposed teeth in each group were then assigned to four equal subgroups (A, B, C and D), representing three experimental and one control subgroup (5 teeth each), according to the capping material used.

In subgroup A (upper right quadrant) the exposed teeth were capped by a combination of MTA (ProRoot MTA; Dentsply, Tulsa Dental, Tulsa, OK), and 0.4% w/v dexamethasone (Amriya Pharmaceutical Industries, Alexandria, Egypt).

In subgroup B (upper left quadrant), the teeth were capped by a combination of MTA and dexamethasone with 7% w/v 1,25 dihydroxy vitamin D₃ {1,25(OH)₂D₃} (Medical Union Pharmaceuticals Ismailia, Egypt).

In subgroup C (lower right quadrant), the teeth were capped by a combination of MTA and chitosan (Al-Debeiky Pharmaceuticals and Chemicals, El-Obour City, Egypt), where the MTA was prepared with a modified form of its original liquid containing 10% wt chitosan.

In subgroup D (lower left quadrant), the teeth were capped by MTA only as control.

Following pulp capping procedures, all the cavities were restored with self-curing glass ionomer cement (GC International Corp, Tokyo, Japan) to provide the suitable conditions for pulpal repair.

Intra-muscular Cefotaxime sodium at a dose of 10 mg kg⁻¹ and Diclofenac sodium at a dose of 1.1 mg kg⁻¹ once/day for 5 days after surgery were given to control the post-operative infection and pain (ABU-SEIDA, 2012).

Pulp extirpation and real time polymerase chain. At the end of the evaluation period of each dog, the animals were placed under general anaesthesia by the previously described technique, and the pulp chamber was aseptically reached from the occlusal surface using a high-speed hand piece. New burs were used for each tooth to reduce the risk of contamination. The powder formed during access opening was removed by a 3 way syringe. Pulp was carefully extirpated using a suitable barbed broach (Dentsply, Tulsa Dental, Tulsa, OK) to avoid shredding of pulp tissue. A new broach was used for every tooth. The pulp tissue of each tooth was placed carefully in a coded Eppendorf tube containing RNA later (QIAGEN, Hilden, Germany) solution (in a volume of 10 mL/mg) to avoid breakdown of the RNA. Quantitative RT-PCR was performed to quantify the gene expression level of odontoblastic markers.

The RNA was converted to cDNA, and 2.0 µL of the resulting cDNA product was used per 20 µL reaction in a real-time PCR Roche LightCycler system (Roche Diagnostics GmbH, Mannheim, Germany). Polymerase chain reactions were carried out with the DNA Master SYBR Green I kit (Roche Diagnostic Co., Indianapolis, IN, USA), with a total volume of 20 µL. These genes included glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Dentin sialophosphoprotein (DSPP) and Matrix extracellular phosphoglycoprotein (MEPE).

The mRNA expression of GAPDH, DSPP and MEPE was determined after extraction of total RNA using RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, followed by reverse transcriptase polymerase chain reaction (RT-PCR) with dog-specific primers. The samples were subjected to 40 cycles of amplification at 95 °C for 15 seconds, followed by 64 °C for 20 seconds, and 72 °C for 25 seconds, using the dog-specific primers. The relative level of gene expression was normalized against the GAPDH messenger RNA signal and the control was set as 1.0. Relative gene expression was calculated using the relative quantification method (delta

delta Ct), comparing messenger RNA expressions between the experimental and control samples.

Statistical analysis. Data were presented as mean and standard deviation (SD) values. Data showed non-parametric distribution, so the Kruskal-Wallis test was used to compare the groups. The Mann-Whitney U test was used for pair-wise comparison when the Kruskal-Wallis test was significant. Friedman's test was used to compare the subgroups. The Wilcoxon signed-rank test was used for pair-wise comparison when Friedman's test was significant. The significance level was set at $P \leq 0.05$. Statistical analysis was performed with IBM SPSS Statistics Version 20 for Windows (SPSS Inc, NY USA).

Results

The relative mRNA expression level of DSPP. The relative mRNA expression of DSPP in all groups and subgroups is shown in Table 1 and Fig. 1. The relative gene expression level of DSPP differed according to the treatment period and the type of additive added to MTA. After the addition of dexamethasone to MTA (Subgroup A), the relative gene expression increased up to an 18.8 fold change in relation to the control in group 2 (21 days) and showed the statistically significantly highest mean expression ($P \leq 0.05$) when compared with the samples of group 1 (7 days) and group 3 (60 days). There was no statistically significant difference ($P > 0.05$) between 7 day and 60 day samples; both showed the lowest mean expression, with down regulation of the target gene in the 60 day samples.

Table 1. The mean relative fold change in relation to the control and standard deviation (SD) values of DSPP gene expressions of all additives among different groups.

Subgroups	Group 1 (7 days)	Group 2 (21 days)	Group 3 (60 days)	P-value
Subgroup A MTA + Dexamethasone	1.1 (0.3) ^b	18.8 (13.2) ^a	0.1 (0.1) ^b	0.030*
Subgroup B MTA + Dexamethasone + Vitamin D3	0.6 (0.6) ^b	29.4 (28.7) ^a	0.1 (0.1) ^b	<0.001*
Subgroup C MTA + Chitosan	6.9 (4.2) ^a	3.6 (5.9) ^a	0.2 (0.2) ^b	0.045*

* Significant at $P \leq 0.05$. Different superscripts in the same row are statistically significantly different. Control was set as 1.0.

When dexamethasone and vitamin D were added to MTA (Subgroup B), the expression of DSPP increased in group 2 (21 days) up to a 29.4 fold change in relation to the control, showing the statistically significantly highest mean expression ($P \leq 0.05$)

in comparison with the other treatment periods. There was no statistically significant difference ($P > 0.05$) between the 7 day and 60 day samples; both showed the lowest mean expression characterized by down regulation of the target gene in relation to the control.

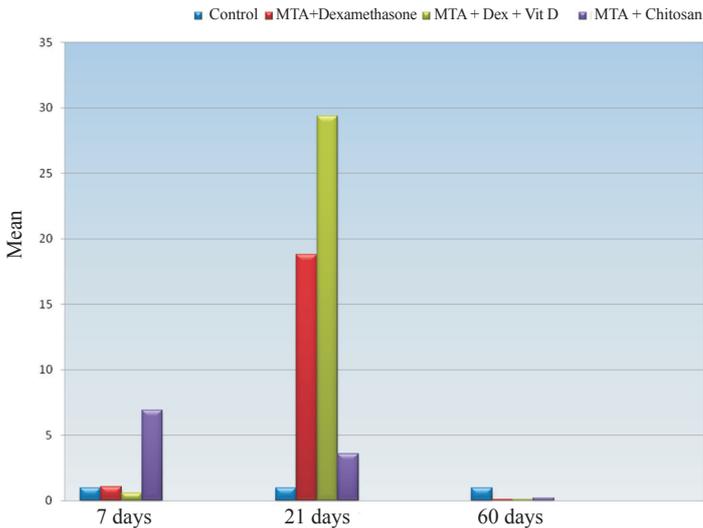


Fig. 1. Bar chart representing the gene expression of DSPP at different treatment periods. The relative gene expression level was normalized against the GAPDH messenger RNA signal, and the control was set as 1.0.

Addition of chitosan to MTA (Subgroup C) upregulated the expression of DSPP in groups 1 and 2, up to a 6.9 and 3.6 fold change, respectively, in relation to the control. These groups showed the statistically significant highest mean expression ($P \leq 0.05$) when compared with group 3, which showed the statistically significantly lowest mean expression ($P \leq 0.05$), and was characterized by down regulation of DSPP in relation to the control. In contrast, no statistically significant difference ($P > 0.05$) in relative gene expression was observed between groups 1 and 2.

Interestingly, our results revealed that the relative gene expression of DSPP was down expressed in group 3 (60 days) samples of all additives.

The relative mRNA expression level of MEPE. There was no significant difference in the relative mRNA expression of MEPE between all the additives mixed with MTA at any time point (Table 2 and Fig. 2). In subgroup A, down regulation of mRNA expression of MEPE occurred in samples of groups 1 and 2 (7 and 21 days) in relation to that of the control (subgroup D). However, the relative gene expression in the samples of group 3 (60

days) showed only a 1.4 fold change in relation to the control. However, no statistically significant differences ($P > .05$) in the relative gene expression of MEPE were observed in the three groups of this subgroup.

Table 2. The mean relative fold change in relation to control and standard deviation (SD) values of MEPE gene expressions of all additives among different groups

Subgroups	Group 1 7 days	Group 2 21 days	Group 3 60 days	P-value
Subgroup A MTA + Dexamethasone	0.1 (0.04)	0.4 (0.1)	1.4 (2.5)	0.330
Subgroup B MTA + Dexamethasone + Vitamin D3	0.03 (0.03)	2.1 (0.1)	0.5 (0.9)	0.061
Subgroup C MTA + Chitosan	2.0 (4.1)	0.4 (0.3)	0.6 (1.1)	0.477

Significant at $P \leq 0.05$. Control was set as 1.0.

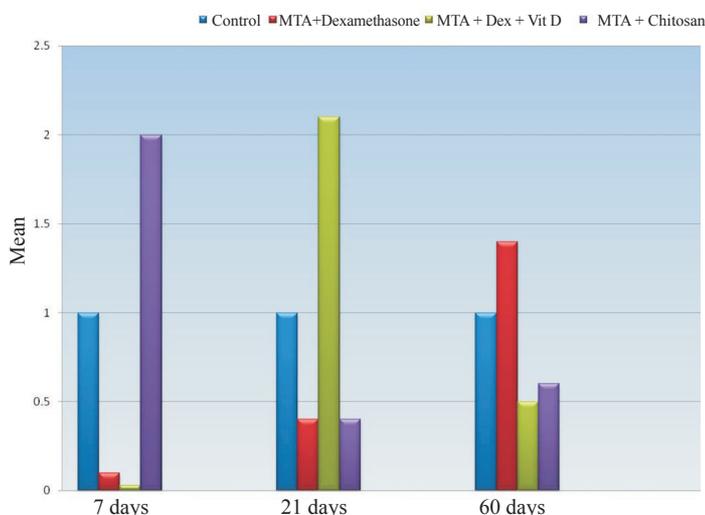


Fig. 2. Bar chart representing the gene expression of MEPE at different treatment periods. The relative gene expression level was normalized against the GAPDH messenger RNA signal, and the control was set as 1.0.

Subgroup B showed down regulation of the mRNA expression of MEPE in samples of groups 1 and 2 (7 and 60 days) in relation to that of the control, while the relative gene expression of the samples of group 2 (21 days) showed a 2.1 fold change in relation to the control. However, no statistically significant differences ($P > .05$) in the relative gene expression of MEPE were observed between the three groups of this subgroup.

In subgroup C, down regulation of the relative mRNA expression of MEPE in the samples of groups 2 and 3 (21 and 60 days) in relation to that of the control was reported, whereas a fold change of 2 occurred in the samples of group 1 (7 days) in relation to that of the control. In contrast, there was no statistically significant difference ($P > .05$) in the relative mRNA expression between all the treatment periods of the samples treated with chitosan.

Discussion

The present study discusses whether the addition of some osteogenic supplements, such as dexamethasone, vitamin D, or chitosan, to MTA would affect the process of odontoblastic differentiation. Pulpal injury might send signals to stimulate the stem cell migration, proliferation, and differentiation into odontoblasts. However, the factors regulating the differentiation of dental pulp cells into odontoblasts remain unclear. A number of studies have indicated the important role of MTA in dentinal bridge formation following exposure of vital dental pulp (HASSANIEN et al., 2015). The level of expression of the mineralizing gene markers, after the application of pulp capping agents or cements to dental pulp cells, has been the aim of many studies (YASUDA et al., 2008; CHANG et al., 2014). Mineral trioxide aggregate can stimulate dentin bridge formation in a greater frequency compared with calcium hydroxide, with no difference between gray MTA and white MTA regarding their effect on dental pulp during pulp capping (FARACO et al., 2001).

Dentin sialophosphoprotein (DSPP) is mainly expressed in odontoblasts and is considered a marker of the differentiation of dental pulp cells into odontoblasts (PARIROKH et al., 2005). The phosphophoryn covalently cross-linked to type I collagen stimulates the mineralization of the collagen in calcium phosphate solutions and has a major role in the calcification of dentin (YE et al., 2006). The DSPP gene product, phosphophoryn, is a hyperphosphorylated protein which comprises nearly 50% of the noncollagenous protein in dentin (SAITO et al., 2000). Therefore DSPP is considered a positive regulator of hard-tissue mineralization acting on dentin, and its stimulation might lead to enhanced mineralization. When MTA was applied to human dental pulp cells it increased the messenger RNA expression level of DSPP (GUZMAN-MORALES et al., 2009). In our study, the level of expression of DSPP is time dependent, and this correlates with the findings of previous studies (OKAMOTO et al., 2009; HASSANIEN et al., 2015).

Dexamethasone is a glucocorticoid that can induce the osteogenic differentiation of human bone marrow stromal cells *in vitro* (SRISAWASDI et al., 2007), and can stimulate the differentiation of hard tissue forming cells when used in pulp injury (KHANNA-JAIN et al., 2010). Our results revealed that the addition of dexamethasone to MTA significantly stimulated the expression of DSPP in the 21 day samples, compared with the 7 day or 60 day

samples. Our findings are in agreement with others who emphasized the enhancing effect of dexamethasone in inducing pulp cell differentiation into odontoblast-like cells, and its important role in dental pulp tissue healing (MATSUNAGA et al., 2006). The positive role of dexamethasone in dental pulp cell differentiation might be attributed to its capability to promote fibronectin synthesis, that may help in the migration and proliferation of dental pulp cells to the site of injury (KHANNA-JAIN et al., 2010). Moreover, GUZMÁN-MORALES et al. (2009) reported that dexamethasone promotes osteoblastic differentiation *in vitro* partly by inhibiting gelatinase activity, and by suppressing inflammatory cytokines which results in increased cell attachment and cell cycle exit.

Vitamin D₃ metabolites have been shown to induce odontoogenic differentiation in human dental pulp and human dental follicle cells. It has been reported that the addition of 1 α ,25(OH)₂D₃ metabolite to dexamethasone and beta-glycerophosphate had a synergistic effect on the alkaline phosphatase activity of human dental pulp cells (MATSUNAGA et al., 2006). This supports the data of our results that showed over expression of the relative mRNA level of DSPP up to a 29.4 fold change when vitamin D₃ was added to dexamethasone in the 21 day evaluation period, compared with the 7 day or 60 day samples. KHANNA-JAIN et al. (2010) attributed the odontogenic effect of vitamin D₃ on human dental pulp to the increased expression of vitamin D receptor (VDR), upregulated vitamin D₃ regulating genes, especially CYP24 expression, and the decrease or inhibition of cell proliferation.

Chitosan is a biocompatible, biodegradable natural biopolymer originated from chitin, and it is considered to be a copolymer of glucosamine and N-acetylglucosamine. There are many studies regarding the use of chitosan in dentistry. Chitosan has been shown to promote osteogenesis *in vivo* through indirect mechanisms (RAKKIETTIWONG et al., 2011).

Our results showed that the addition of chitosan to MTA significantly increased the expression of the relative mRNA level of DSPP in the 7 day and 21 day samples, compared with that of the 60 day samples. The reason behind the odontogenic effect of chitosan when added to MTA in the 7 day and 21 day periods might be its ability to enhance the mechanical properties of MTA, which is considered to be an important factor for biomaterials used in tooth repair or pulp capping treatment. However, further investigations should be done to examine the exact effect of chitosan on the mechanical properties of MTA. According to our results, it is interesting to know that the expression of DSPP was down-regulated in relation to the control in the 60 day samples of all additives. However, this might be attributed to the decrease in the rate of odontoblastic differentiation after 2 months of pulp capping by these modified capping agents.

Another gene marker of odontogenesis is called matrix extracellular phosphoglycoprotein (MEPE). Matrix extracellular phosphoglycoprotein is an

extracellular matrix protein that is mainly expressed in mineralizing tissues, including the dental pulp. Several studies have been conducted to investigate the effect of MEPE on the process of mineralization (LIU et al., 2005; WEI et al., 2012). The results of these studies provide conflicting data about the exact role of MEPE in regulating mineralization, and the underlying mechanisms. It appeared to play an important positive role in the process of mineralization and dental pulp repair (MATSUNAGA et al., 2006). On the other hand, other studies revealed that MEPE has a negative effect on mineralization (LIU et al., 2005; WANG et al., 2010). According to our results, the addition of chitosan, or dexamethasone (with or without Vitamin D) to MTA did not significantly enhance the level of mRNA expression of MEPE during pulp capping in any of the evaluation periods (7, 21, or 60 days). The relative fold change of MEPE differed throughout the capping periods of each additive, but with no significant differences between them. However, MEPE was down-regulated in relation to the control (MTA only) in most of the samples, a finding that might correlate with the results of a previous study (LIU et al., 2005). On the other hand, a few samples showed slight upregulation of MEPE in relation to the control (60 day samples that were treated with dexamethasone, 21 day samples that were treated with dexamethasone and vitamin D, and 7 day samples treated with chitosan), but there were no significant differences in the relative mRNA expression of MEPE between the samples of any of the additives at any time point. However, the mechanism that controls the upregulation or down-regulation of DSPP and MEPE at different time points using these additives requires further investigations.

In conclusion, dexamethasone, with or without vitamin D₃, and chitosan are synergistic odontogenic inducers with MTA for differentiation of dental pulp cells in dogs. The upregulation of DSPP is a good marker for this differentiation.

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SAŽETAK

Istraživan je utjecaj dodatka deksametazona, vitamina D₃ ili hitozana agregatu mineralnog trioksida na razinu ekspresije gena za dentin-sijalofosfoprotein (DSPP) i gena za ekstracelularni matriks fosfoglikoprotein nakon zatvaranja pulpe u pasa. Pulpa je bila otvorena na 60 zuba. Ti su zubi bili razvrstani u tri jednake skupine ovisno o trajanju promatranja. Prva skupina bila je promatrana sedam dana, druga skupina 21 dan, a treća skupina 60 dana. Svaka skupina bila je podijeljena u podskupine ovisno o materijalu rabljenom za zatvaranje pulpe. Podskupini A bio je primijenjen agregat mineralnog trioksida + deksametazon, podskupini B bio je primijenjen agregat mineralnog trioksida + deksametazon + vitamin D₃, podskupini C agregat mineralnog trioksida + hitozan, a podskupini D samo agregat mineralnog trioksida. Sukladno skupinama, pulpe zatvorenih zuba bile su uzete za analizu relativne ekspresije mRNA za dentin-sijalofosfoprotein i za ekstracelularni matriks fosfoglikoprotein PCR-om. Svi podatci bili su statistički obrađeni. Značajna ekspresija gena za dentin-sijalofosfoprotein ($P \leq 0,05$) bila je dokazana u podskupini A druge skupine u relativnoj vrijednosti 18,8, dok je u podskupini B značajna ekspresija gena za dentin-sijalofosfoprotein ($P \leq 0,05$) bila 29,4 puta veća. Značajno povećana ekspresija DSPP ($P \leq 0,05$) u prvoj i drugoj skupini sve do 6,9 odnosno 3,6 puta ustanovljena je u podskupini C. Može se zaključiti da deksametazon (s vitaminom D₃ ili bez njega) i hitozan djeluju sinergistički s agregatom mineralnog trioksida kao odontogenični pokretači za diferencijaciju stanica zubne pulpe u pasa. Povećana razina dentin-sijalofosfoproteina dobar je pokazatelj te diferencijacije.

Ključne riječi: hitozan, deksametazon, dentin-sijalofosfoprotein, agregat mineralnog trioksida, zatvaranje pulpe
