Mineralisation Influence of Betamethasone on Lipopolysaccharide-Stimulated Dental Pulp Cells

Yuan Yuan WANG[1], Ning Xin ZHU[1], Yu Ming ZHAO[1], Li Hong GE[1], Man QIN[1]

Objective: *To evaluate the mineralisation response of lipopolysaccharide (LPS)-induced dental pulp cells (DPCs) to betamethasone and the potential benefit of betamethasone application on the recovery of injured dental pulp.*

Methods: *The proliferation influence of betamethasone on DPCs was analysed through the cell counting kit-8 assay. To assess the anti-inflammatory effects of betamethasone, the expression levels of inflammatory factors IL-6, IL-1ß and TNF-∂ were determined by real-time polymerase chain reaction (PCR). Mineralisation was investigated through the detection of the mineralisation-related biomarkers alkaline phosphatase (ALP), dentine sialophosphoprotein (DSPP) and osteocalcin (OCN) through the ALP activity assay, immunohistochemistry staining, Alizarin Red and tissue nonspecific alkaline phosphatase (TNAP) staining, the reverse transcriptase PCR technique and western blot.*

Results: *A low concentration of betamethasone (1 μ/mL) promoted the proliferation of DPCs. The real-time PCR results demonstrated that inflammatory cytokines were downregulated by betamethasone treatment. The mineralisation outcome in DPCs treated with betamethasone was better than in those treated without betamethasone.*

Conclusion: *Betamethasone promoted the proliferation of DPCs. Betamethasone enhanced mineralisation in LPS-stimulated DPCs.*

Key words: *LPS-stimulated dental pulp cells (DPCs), betamethasone, mineralisation*

Chin J Dent Res 2019;22(2):123–129; doi: 10.3290/j.cjdr.a42516

In cases of tooth injury, healthy pulp tissue is a key prognostic indicator for long-term tooth preservation[1]. Unfortunately, the recovery and conservation of injured pulp tissue is complex and not well defined. When dental pulp is injured by caries, trauma or other injuries, vital pulp therapy is applied to protect the injured pulp. Vital pulp tissue is responsible for the formation of secondary dentine in response to various stimuli[2]. Inflammation reduction and hard tissue formation that protects the pulp tissue from injurious agents help inflamed pulp tissue to heal[3]. Widely used vital pulp capping agents such as calcium hydroxide and mineral trioxide aggregate mainly serve to close the exposed pulp; however, the anti-inflammatory outcome is not ideal. After direct pulp capping and pulpotomy, the differentiation and proliferation of dental pulp cells (DPCs) are influenced by the activity of dental materials[4]. To date, there is no ideal dental pulp capping material for the repair of inflamed pulp.

The first reported case of the use of steroidal medicine in dental pulp capping was performed in 1958[5], and only a few cases related to steroidal medicines used in dental pulp treatment have been reported since. In recent years, steroids have been systemically applied to endodontic treatment[6-8]. Topically applied corticosteroids such as betamethasone have confirmed anti-inflammatory and vasoconstrictive properties[9]. Direct delivery of corticosteroids has been reported to reduce pulpal inflammation, and betamethasone has demonstrated better anti-inflammatory effects than hydrocortisone[10] Specifically, when betamethasone was applied topically to the dentine of rat molars, the vascular phase

1 Department of Pediatric Dentistry, Peking University School and Hospital of Stomatology, National Engineering Laboratory for Digital and Material Technology of Stomatology, Beijing Key Laboratory of Digital Stomatology, Beijing, P.R. China.

Corresponding author: Dr Man QIN, Department of Pediatric Dentistry, 22# Zhongguancun South Avenue, Haidian District, Beijing 100081, P.R. China; Tel: 8610 82195306; Fax: 8610 62173402; Email: qin-man@fox-mail.com

This study has been funded by a grant from the National Natural Science Youth Foundation of China (No. 81500837) to Yuan Yuan WANG.

of pulpal inflammation was shortened[8]. Furthermore, the combined administration of betamethasone and gentamicin (an antibiotic) has been reported to have beneficial antimicrobial and anti-inflammatory effects on soft tissue[11,12]. In a direct pulp-capping study in a rabbit model, the topical application of betamethasone and gentamicin cream significantly reduced histopathological changes in dental pulp compared with calcium hydroxide treatment[13]. However, the role of betamethasone on inflamed cells has not yet been defined.

With the above in mind, the purpose of this study was to evaluate the mineralisation response of lipopolysaccharide (LPS)-induced DPCs to betamethasone. The hypothesis of this study was that betamethasone may reduce the inflammation reaction and promote the mineralisation of the LPS-induced DPCs; thus, betamethasone could be applied as a dressing medication with a view to improving the success rate of vital pulp therapy.

Materials and methods

Isolation and culture of human dental pulp cells (hDPCs)

The study protocol was approved by the Ethics Committee of Peking University School and Hospital of Stomatology, Beijing, China (Approval No.: PKUSSIRB-201732003). All experiments were performed in accordance with relevant guidelines and regulations. The research was conducted in full accordance with the World Medical Association Declaration of Helsinki. Human impacted third molars were collected from adults in-clinic at The Peking University School of Stomatology and used to culture hDPCs. All patients provided written informed consent. After the tooth surfaces had been cleaned, the teeth were cut around the cementoenamel junction with sterilised dental fissure burs to expose the pulp chamber. The pulp tissue was gently separated from the crown and root and subsequently digested in a solution of 3 mg/mL collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) and 4 mg/ml dispase (Sigma-Aldrich) for 1 h at 37ºC. Single-cell suspensions were obtained by passing the cells through a 70-mm strainer (Falcon; BD Biosciences, San Jose, CA, USA). hDPCs derived from passage 3~4 were used for the experiments in this study.

Determination of betamethasone concentration

hDPCs (1×10^3/well) expended *ex vivo* were seeded into 96-well plates, cultured with betamethasone (0, 1, 10, and 100 μg/l) for 24 h in a 37ºC incubator. The Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) assay was used, and repeated five times for each sample to evaluate the number of viable cells, in accordance with the manufacturer's instructions. Untreated cells were used as the control group. Briefly, a volume of 10 μL 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt was added to each well before the culture plate was incubated at 37ºC for 4 h. Absorbance was measured at 450 nm in a microplate reader. The mean values of the optical density were calculated and analysed statistically for the cell number at each dilution of the samples (SPSS software, version 13.0; SPSS, Chicago, IL, USA). The dilution of betamethasone at which hDPCs had the highest cell viability was chosen for subsequent studies.

Anti-inflammatory effects of betamethasone

DPCs (1×10^5/well) were seeded onto 6-well plates, grown to 90% confluence and exposed to 1 μg/ml LPS for 1, 3 or 6 h with/without betamethasone (1 μmol/L). At the times indicated, RNA was extracted from the DPCs using TRIzol (Invitrogen, Carlsbad, CA). Then, real-time PCR for IL-6, IL-ß and TNF-α was carried out in a total volume of 20 μl, as described.

Osteogenic differentiation

DPCs (1×10^5/well) were seeded onto 6-well plates and grown for 5 days in α-minimum essential medium containing 50 μg/ml ascorbate. Mineralisation was induced by the supplementation of the medium with 10 mM β-glycerophosphate. The medium was replaced daily. 1 μg/ml *Escherchia coli* LPS (Sigma-Aldrich) was added to the osteogenic medium. Cells without mineralisation or LPS treatment were used as the control group. After a 7-day induction, the following experiments were performed:

Calcium deposit assessment: Alizarin Red staining
DPCs were rinsed with phosphate-buffered saline (PBS), fixed with 100% ethanol for 30 min, and air dried. Cells were then incubated with 0.5% Alizarin Red-S for 30 min. Cells were washed with 70% ethanol and air dried. The intensity of the Alizarin Red stain was quantified by NIH Image software.

Alkaline phosphatase (ALP) enzyme activity assay
Tissue nonspecific alkaline phosphatase (TNAP) enzyme activity was assayed using the colorimetric substrate nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich). Cells were fixed in

Table 1 Primers used for quantitative PCR.

Gene name	5'-squence-3'	Product size (bp)	GenBank number
GAPDH	Forward: GCTCTCTGCTCCTCCTGTTC Reverse: TTCCCGTTCTCAGCCTTGAC	273	NM_002046.6
ALP	Forward: GCGCAGGACAGGATTAAAGC Reverse: TCCACTGCCACAGTCAATCC	246	NM_014476.5
DSPP	Forward: CATGGGCCATTCCAGTTCCTC Reverse: TTCATGCACCAGGACACCAC	152	NM_014208.3
OCN	Forward: ATGAGAGCCCTCACACTCCT Reverse: TGGGGCTCCCAGCCATT	180	NM_199173.4
IL-6	Forward: CTCAATATTAGAGTCTCAACCCCCA Reverse: GAGAAGGCAACTGGACCGAA	163	NM_001318095.1
TNF-a	Forward: CACTGAAAGCATGATCCGGG Reverse: TGGGGAACTCTTCCCTCTGG	188	NM_000594.3
IL-1β	Forward: CCACCTCCAGGGACAGGATA Reverse: TGGGATCTACACTCTCCAGC	176	NM_000576.2

70% ethanol for 10 min at room temperature, air dried and incubated with substrate for 1 h at 37°C. Cells were then rinsed with distilled H_2O, air dried and visualised macroscopically for evidence of staining. For quantification, wells were scanned, and densitometry was measured using NIH ImageJ software.

Osteocalcin (OCN) and dentine sialophosphoprotein (DSPP) detection: immunocytochemical staining

The DPCs were subcultured onto 12-chamber slides (2×10^4/well, second passage). After osteogenesis induction, the cells were fixed in 4% paraformaldehyde and washed three times with PBS. Antibodies to OCN (1:200 dilution, Wuhan Boster, China) and DSPP (1:200 dilution, Wuhan Boster, China) were applied in the immunocytochemical staining study. The antibodies were diluted with tris-buffered saline (TBS) in the ratio 1:50. The DPCs were incubated with one of the primary antibodies overnight at 4ºC, followed by consecutive incubation with polymer helper and polyperoxidase-anti-goat/rabbit/mouse immunoglobulin G (Polink-2 Plus HRP System Kits; Zhongshan Golden Bridge Biotechnology, Beijing, China). All incubations were followed by at least three washes in TBS. The sections were developed with a 3.3-inch diaminobenzidine tetrahydrochloride (DAB) substrate kit (Zhongshan Golden Bridge Biotechnology), counterstained with hematoxylin and examined under a light microscope (Nikon Eclipse TS100). In all cases, negative control staining was conducted in parallel by incubating sections with TBS instead of the primary antibody.

ALP, OCN and DSPP detection: real-time polymerase chain reaction (PCR)

Total RNA was isolated from cells after 21 days using TRIzol and 2 μg of RNA was reverse transcribed with TaqMan Reverse Transcription Reagents (Applied Biosystems, Branchburg, NJ, USA), following the manufacturers' instructions. The resulting cDNA was then amplified by RT-PCR using AmpliTaq Gold DNA Polymerase (Applied Biosystems). RT-PCR amplifications were performed at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s using specific primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ALP, OCN and DSPP. The design of the primers was based on published mouse cDNA sequences (Table 1). The RT-PCR products were sub-cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA), and RNA expression was confirmed by sequencing.

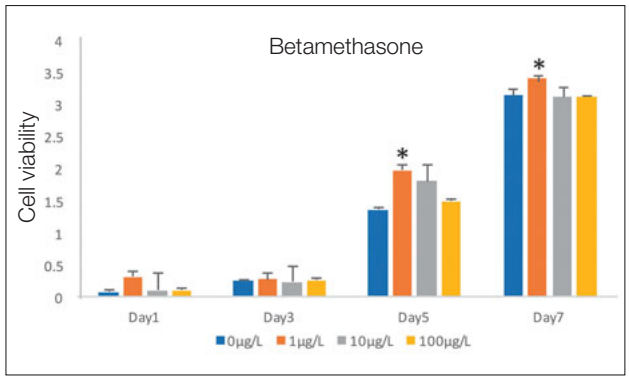

Fig 1 Influence of betamethasone on the proliferation of DPCs. The CCK-8 assay indicated that 1 μg/L betamethasone promoted the proliferation of DPCs to more than 0, 10 or 100 μg/L betamethasone after 1, 3, 5 and 7 days of cultivation ($P < 0.05$).

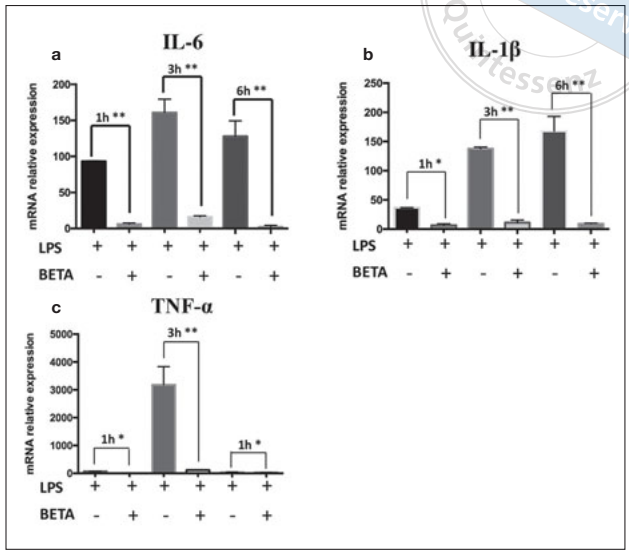

Fig 2 Influence of betamethasone on mRNA levels of inflammatory mediators in LPS-stimulated DPCs. The RT-PCR results showed that betamethasone significantly decreased the expression of IL-1β, IL-6 and TNF-α in DPCs that were stimulated by LPS for 1, 3 or 6 h (*$P < 0.05$, **$P < 0.01$).

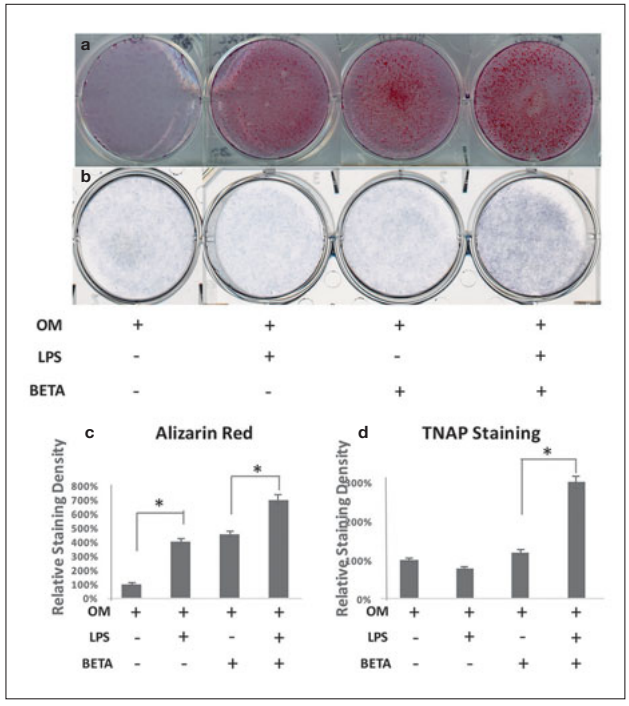

Fig 3 Influence of betamethasone on the formation of mineralised nodules. DPCs were cultured in induction medium for 7 days and then stained with Alizarin Red and TNAP. A representative photograph of Alizarin Red staining (a) and TNAP staining (b) are shown. The relative staining density was set at 100%, and the statistical results are shown in (c) and (d).

OCN and runt-related transcription factor 2 (RUNX2) detection: western blot

DPCs were seeded in 6-well plates (2×10^4/well, expanded *ex vivo*) and incubated until 80% confluence had been reached. DPCs were treated with LPS with or without betamethasone for 3 h, lysed in radioimmunoprecipitation assay buffer (RIPA buffer) containing protease inhibitors and quantified by using BCA Protein Assay (Pierce, Rockford, IL, USA). 40 μg of protein from each sample was separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene difluoride (PVDF) membranes (MilliporeSigma, Bedford, MA, USA) at 100 V for 60 min. The membranes were incubated in blocking buffer (5% non-fat dry milk in TBS containing 0.05% Tween-20, pH 7.4) for 1 h and incubated with the following antibodies in 1:500 dilutions overnight at 4°C: OCN (1:1000; Santa Cruz, CA, USA) and RUNX2 (1:1000; Santa Cruz, CA, USA).

Statistical analyses

Data are expressed as mean ± standard deviation (SD). Between-group differences were compared using one-way analysis of variance (ANOVA). The level of statistical significance was set at $P < 0.05$.

Results

Influence of betamethasone on the proliferation of DPCs

The growth of the DPCs was evaluated after exposure to various betamethasone concentrations (0, 1, 10 and 100 μg/L) after 0, 1, 3, 5 and 7 days. The CCK-8 assay results demonstrated that 1 μg/L betamethasone promoted the proliferation of DPCs to more than 0, 10 or 100 μg/L betamethasone after 1, 3, 5 and 7 days of cultivation ($P < 0.05$) (Fig 1). Considering this result, a concentration of 1 μg/L betamethasone was used for subsequent experiments.

Influence of betamethasone administration on mRNA levels of inflammatory mediators in LPS-stimulated DPCs

To investigate whether betamethasone reduces inflammatory cytokine expression in DPCs, we measured their expression in DPCs treated with LPS for 1, 3, or 6 h (Fig 2). The inflammatory genes were detected by quantitative PCR. The expression levels of IL-1β, IL-6 and TNF-α were stimulated by LPS, and betamethasone significantly decreased the expression of all three at each timepoint (*$P < 0.05$, **$P < 0.01$).

Betamethasone influence on expression of OCN and DSPP

The Alizarin Red staining results showed that significantly more mineralised nodes formed in the betamethasone-treated group than in the other groups (Fig 3a and c), while mineralised node formation was similar in the LPS and positive control groups (Fig 3b and d).

To evaluate the influence of betamethasone on osteogenesis in DPCs, osteogenic differentiation was investigated by immunostaining, Alizarin Red staining and alkaline phosphatase activity analyses. More positive OCN-stained (Fig 4a) and DSPP-stained (Fig 4b) cells presented in the betamethasone treated group than in the LPS and control groups.

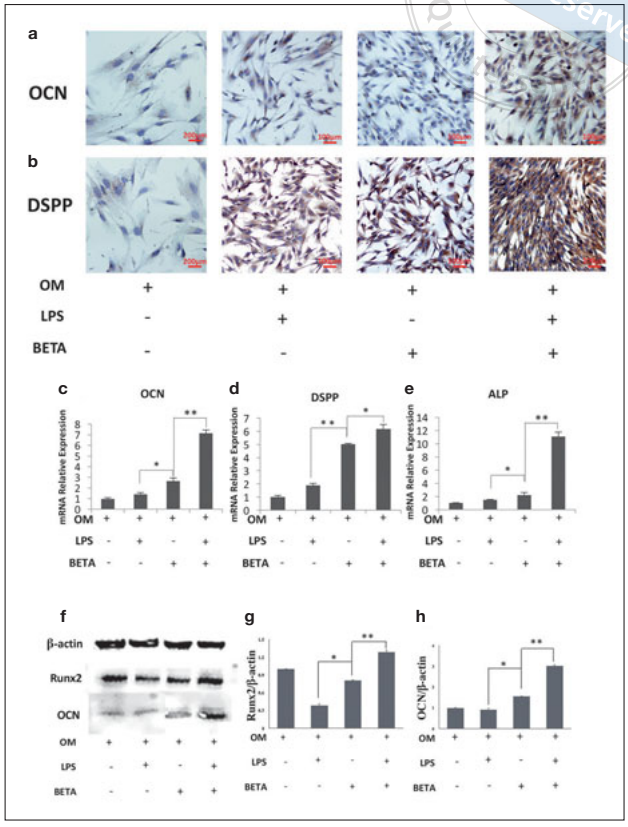

Fig 4 Influence of betamethasone on the mineralisation effects of LPS-induced DPCs. DPCs were cultured in induction medium for 7 days. Influence of betamethasone on OCN and DSPP expression, as assessed by immunostaining. More positive staining for OCN (a) and DSPP (b) presented in the betamethasone-treated group than in the LPS or control groups. OCN (c), DSPP (d) and ALP (e) messenger RNA levels were analysed by RT-PCR. The results indicate that betamethasone significantly promoted the expression of DSPP, OCN and ALP. Western blot results showed that betamethasone significantly promoted the expression of RUNX2 and OCN, which was further confirmed with real-time PCR results via densitometry using ImageJ software (f to h).

Messenger RNA level analysis of DSPP, OCN and ALP expression

Osteogenesis-related genes were detected by quantitative PCR. Total RNA was isolated from DPCs that were treated with betamethasone and LPS, which were then subjected to quantitative PCR after reverse transcription. The quantitative PCR results showed that no obvious difference was found in the expression of DSPP, OCN and ALP after being treated with LPS,while betamethasone significantly promoted the expression of DSPP, OCN and ALP (Fig 4c to e).

Western blot analysis of RUNX2 and OCN

Western blot results showed that betamethasone significantly promoted the expression of RUNX2 and OCN, which was further confirmed with real-time PCR results via densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA) (Fig 4f to h).

Discussion

LPS is the major component of the outer membrane in gram-negative bacteria and can induce the expression of inflammatory cytokines and cell apoptosis in DPCs[14-17]. Previous research has demonstrated that LPS can create an inflammatory environment in DPCs by upregulating the expression of proinflammatory cytokines IL-1β, IL-6 and TNF-α cytokines. Furthermore, LPS-induced inflammation is the main limiting factor in injured dental pulp tissue repair[18]. Lacey et al[19] reported that IL-1β and TNF-α proinflammatory cytokines inhibit mesenchymal stem cell differentiation into osteoblasts and suppress mineralisation processes. Additionally, Nomiyama et al[20] demonstrated that LPS inhibits the expression of ALP, DSPP and RUNX2 and the formation of mineralised nodules in odontoblasts. Sabbagh et al[21] verified that local inflammation has a negative influence on new bone formation. Both mineralisation and an anti-inflammatory effect are needed for the restoration of injured dental pulp tissue. Betamethasone, which exhibits both anti-inflammatory and osteoinductive properties, may be promising for the repair of injured dental pulp. Our results indicate that a low concentration of betamethasone promotes the proliferation of DPCs. This increased cell number should be beneficial to the healing process in dental pulp injuries.

The main function of DPCs in an injured environment is to form dentine, thus facilitating the biomineralisation process. Mineralised tissue-related markers include DSPP, OCN and ALP. These markers were quantified to determine if betamethasone exhibits osteoinduction potential. DSPP is a major non-collagenous dentine matrix protein expressed specifically by odontoblasts during dentinogenesis and is considered to be a dentine-specific protein[22]. OCN is the late-stage biomarker of osteogenesis, which is restricted to cells of mineralised tissues, including bone, dentine and cementum. ALP is an early-stage biomarker of osteodifferentiation that is highly expressed in mineralised forming cells.

Our results show that DSPP was significantly upregulated in betamethasone-treated DPCs, both in the immunostaining and RT-PCR studies. Notably, DSPP was found in high concentrations in mature dentine and was less expressed in odontoblasts. Mutations in the DSPP gene are associated with dentinogenesis imperfecta in humans[23]. Evidence shows that DSPP is necessary for dentine formation and important to mineralisation control[24,25].

The quantity and activity of ALP was significantly upregulated in betamethasone-treated DPCs. Moreover, the expression level of OCN in the betamethasone-treated group was higher than that in the other groups, indicating that betamethasone was capable of inducing osteogenic differentiation in DPCs.

In conclusion, betamethasone has the potential to promote the proliferation and growth of DPCs at an optimised concentration. Betamethasone can initiate biomineralisation and facilitate the formation of mineralisation nodes in DPCs. Betamethasone promotes the mineralisation process of DPCs by upregulating ALP, OCN and DSPP. Furthermore, betamethasone obviously downregulated IL-1β, IL-6 and TNF-α inflammatory cytokines, which were induced by LPS.

Conflicts of interest

The authors reported no conflicts of interest related to this study.

Author contribution

Drs Yuan Yuan WANG and Ning Xin ZHU carried out the biological studies, analysed the data and wrote the manuscript; Dr Ning Xin ZHU carried out the biological studies; Dr Yu Ming ZHAO contributed to the writing of the manuscript; Prof Li Hong GE contributed to the revision of the manuscript; Prof Man QIN was responsible for the overall project design and manuscript organisation, revision and finalisation.

(Received July 09, 2018; accepted Sep 18, 2018)

References

1. Goldberg M, Farges JC, Lacerda-Pinheiro S, et al. Inflammatory and immunological aspects of dental pulp repair. Pharmacol Res 2008;58: 137–147.
2. Al-Hiyasat AS, Barrieshi-Nusair KM, Al-Omari MA. The radiographic outcomes of direct pulp-capping procedures performed by dental students: a retrospective study. J Am Dent Assoc 2006;137: 1699–1705.
3. Tziafas D, Smith AJ, Lesot H. Designing new treatment strategies in vital pulp therapy. J Dent 2000;28:77–92.
4. Murray PE, Windsor LJ, Smyth TW, Hafez AA, Cox CF. Analysis of pulpal reactions to restorative procedures, materials, pulp capping, and future therapies. Crit Rev Oral Biol Med 2002;13:509–520.
5. Rapoport L, Abramson, II. Application of steroid hormones in pulp-capping and pulpotomy procedures; a preliminary report. Oral Surg Oral Med Oral Pathol 1958;11:545–548.
6. Chidiac JJ, Al-Asmar B, Rifai K, Jabbur SJ, Saadé NE. Inflammatory mediators released following application of irritants on the rat injured incisors. The effect of treatment with anti-inflammatory drugs. Cytokine 2009;46:194–200.
7. Mahmoud SH, Grawish Mel-A, Zaher AR, El-Embaby A, Karrouf GI, Sobh MA. Influence of selective immunosuppressive drugs on the healing of exposed dogs' dental pulp capped with mineral trioxide aggregate. J Endod 2010;36:95–99.

8. Fachin EV, Scarparo RK, Pezzi AP, Luisi SB, Sant'ana Filho M. Effect of betamethasone on the pulp after topical application to the dentin of rat teeth: vascular aspects of the inflammation. J Appl Oral Sci 2009;17:335–339.
9. Dyderski S, Grześkowiak E, Szałek E, Mrzygłód A. Pharmaceutical availability of betamethasone dipropionate and gentamicin sulfate from cream and ointment. Acta Pol Pharm 2002;59:99–103.
10. Fachin EV, Zaki AE. Histology and lysosomal cytochemistry of the postsurgically inflamed dental pulp after topical application of steroids. I. Histological study. J Endod 1991;17:457–460.
11. Aslan O, Teberik K, Yucel M, Gur N, Karakoc AE. Effect of topical netilmicin on the reduction of bacterial flora on the human conjunctiva. Eur J Ophthalmol 2008;18:512–516.
12. Friberg O, Svedjeholm R, Söderquist B, Granfeldt H, Vikerfors T, Källman J. Local gentamicin reduces sternal wound infections after cardiac surgery: a randomized controlled trial. Ann Thorac Surg 2005;79:153–161.
13. Aljandan B, AlHassan H, Saghah A, Rasheed M, Ali AA. The effectiveness of using different pulp-capping agents on the healing response of the pulp. Indian J Dent Res 2012;23:633–637.
14. Massey WL, Romberg DM, Hunter N, Hume WR. The association of carious dentin microflora with tissue changes in human pulpitis. Oral Microbiol Immunol 1993;8:30–35.
15. Rupf S, Kannengiesser S, Merte K, Pfister W, Sigusch B, Eschrich K. Comparison of profiles of key periodontal pathogens in periodontium and endodontium. Endod Dent Traumatol 2000;16:269–275.
16. Tokuda M, Sakuta T, Fushuku A, Torii M, Nagaoka S. Regulation of interleukin-6 expression in human dental pulp cell cultures stimulated with Prevotella intermedia lipopolysaccharide. J Endod 2001;27:273–277.
17. Yang H, Zhu YT, Cheng R, et al. Lipopolysaccharide-induced dental pulp cell apoptosis and the expression of Bax and Bcl-2 in vitro. Braz J Med Biol Res 2010;43:1027–1033.
18. Liu Z, Jiang T, Wang X, Wang Y. Fluocinolone acetonide partially restores the mineralization of LPS-stimulated dental pulp cells through inhibition of NF-κB pathway and activation of AP-1 pathway. Br J Pharmacol 2013;170:1262–1271.
19. Lacey DC, Simmons PJ, Graves SE, Hamilton JA. Proinflammatory cytokines inhibit osteogenic differentiation from stem cells: implications for bone repair during inflammation. Osteoarthritis Cartilage 2009;17:735–742.
20. Nomiyama K, Kitamura C, Tsujisawa T, et al. Effects of lipopolysaccharide on newly established rat dental pulp-derived cell line with odontoblastic properties. J Endod 2007;33:1187–1191.
21. Sabbagh Y, Jones AO, Tenenhouse HS. PHEXdb, a locus-specific database for mutations causing X-linked hypophosphatemia. Hum Mutat 2000;16:1–6.
22. Prasad M, Butler WT, Qin C. Dentin sialophosphoprotein in biomineralization. Connect Tissue Res 2010;51:404–417.
23. Xiao S, Yu C, Chou X, et al. Dentinogenesis imperfecta 1 with or without progressive hearing loss is associated with distinct mutations in DSPP. Nat Genet 2001;27:201–204.
24. Yamakoshi Y. Dentin Sialophophoprotein (DSPP) and Dentin. J Oral Biosci 2008;50:33–44.
25. Suzuki S, Haruyama N, Nishimura F, Kulkarni AB. Dentin sialophosphoprotein and dentin matrix protein-1: Two highly phosphorylated proteins in mineralized tissues. Arch Oral Biol 2012;57:1165–1175.