Abstract: The aim of this study was to investigate the effect of prolonged release of iloprost, a prostacyclin analog, on angiogenesis and dental pulp healing in a rat model of mechanical pulp exposure. The profile of iloprost release from poly (lactic-co-glycolic) acid (PLGA) microspheres was evaluated, and expression of vascular endothelial growth factor (VEGF) mRNA was determined. The molars of rats were subjected to mechanical pulp exposure and 5 different forms of treatment: Ca(OH)$_2$, PLGA (blank), iloprost, and iloprost/PLGA. Blood flow was determined at 0, 3, and 7 days using laser Doppler flowmetry. After 30 days, the tooth specimens were collected, and subjected to micro-CT and immunohistological analysis. The results showed that iloprost release from the microspheres was prolonged for 4 days, and that the treatment increased tooth blood flow for up to 7 days. At 30 days, an increase of mineralized tissue formation and dentin bridge formation was observed in the iloprost and iloprost/PLGA microsphere groups. VEGF expression was significantly increased in the iloprost/PLGA microsphere group relative to the other groups. In conclusion, this PLGA microsphere iloprost delivery system significantly increased dental pulp blood flow in a prolonged manner and increased tertiary dentin formation in this rat pulp injury model. Prolonged prostacyclin release could be a potentially useful approach for regeneration of dental pulp.

Keywords: angiogenesis; dental pulp; direct pulp capping; iloprost; PLGA.

Introduction

For several decades, tooth vital pulp therapy has been used to preserve teeth and avoid root canal treatment (1). The success of direct pulp capping can be defined as the formation of tertiary dentin and a dentin bridge covering the pulp exposure site (2). An important element of dentin
bridge formation is the induction of stem cell migration into the pulp chamber (3). Angiogenic factors, such as vascular endothelial growth factor (VEGF), play an important role in the early phase of vascularization and angiogenesis by recruiting stem cells from surrounding tissues to the site of injury (4).

Prostacyclin (prostaglandin I₂, PGI₂) is a potent chemoattractant and angiogenic molecule synthesized from arachidonic acid. Genetic deletion of PGI₂ synthase results in impaired bone formation in vivo (5), suggesting that PGI₂ is involved in hard tissue remodeling. Iloprost, a PGI₂ analog, has been used in animal models and in patients with syndromes involving vascular deficiency in order to promote revascularization and increase blood flow (6,7). Previous studies have shown that iloprost increases the expression of VEGF and proliferative growth factors in human dental pulp cells in vitro, as well as increasing dental pulp blood flow and tertiary dentin formation in vivo (8,9). A recent study has also demonstrated that iloprost can increase the expression of VEGF in periodontal ligament cells, which are another type of mesenchymal stem cell that could be employed to achieve dentin and hard tissue formation as well as dental revascularization (10). These previous findings suggest that iloprost could have potential application as a dental pulp capping material and/or scaffold in the root canal for regeneration of dentin.

Clearance of iloprost is relatively rapid, with a half-life of 30 min (11). To further explore the use of iloprost as a dental biomaterial for clinical use, development of an optimal delivery system is needed. Bioactive scaffolds have been widely used for tissue engineering. Microspheres can be modified by crosslinking to adjust their electrostatic and hydrophilic properties, allowing drugs to be incorporated into the scaffold (12). Poly (lactic-co-glycolic) acid (PLGA) microspheres are nanoparticles that have been widely employed for drug delivery and tissue engineering due to their biocompatibility and biodegradability (13). PLGA microspheres have the advantage of facilitating long-term drug release, as the particles swell and can release the drug over time (14). Use of PLGA as a delivery system for dental pulp tissue regeneration has been attempted in animals (5,15) and also for direct pulp capping (16). Therefore, a PLGA microsphere system could be used for prolonged-release drug delivery aimed at direct pulp capping. Consequently, the aim of the present study was to investigate the efficacy of PLGA-gelatin microspheres as a bioactive scaffold for prolonged release of iloprost to achieve dental pulp regeneration in a rat tooth pulp exposure model.

Materials and Methods

Microsphere preparation and evaluation of bovine serum albumin release

To determine the release of bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA), which is an approach commonly used for evaluating the release of a loaded substance from microspheres (17), 250 µg/mL BSA was loaded into PLGA microspheres that had been prepared as reported previously (18). Briefly, PLGA-coated gelatin microspheres containing iloprost (Ilomedin, Bayer, Germany) were created using a modified thermal gelation technique. Iloprost at a concentration of 1.2 × 10⁻⁶ M (0.4 µg/mL) was incorporated into 5 mg of PLGA microspheres. The characteristics of the microspheres, including their size, were studied at pH 7 using a polarizing optical microscope (DMRXP LEICA, Leica Biosystems, Wetzlar, Germany) at ×20 magnification with the UTHSCSA Image Tool, version 3.0 (UTHSCSA, San Antonio, TX, USA). Carbopol was used as a soluble carrier (Carbopol 21 Ultrez, Lubrizol, OH, USA) (19). The cumulative release of BSA in vitro after mixing with carbopol was determined for up to 7 days. The supernatant was assayed for the released BSA using a UV-Vis spectrophotometer at a wavelength of 215 nm. Optical images of the dry PLGA and swollen microspheres were also acquired.

Cell culture

Human dental pulp cells (HDPCs) were isolated from three extracted third molars extracted from healthy adults aged 18-50 years at the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Chulalongkorn University, under protocol number HREC-DCU 2012-024 (9). The explants were grown at 37°C in culture medium composed of Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, MD, USA) supplemented with 2 mM L-glutamine, antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin), 5 µg/mL amphotericin B, and 10% fetal bovine serum (FBS) at 5% CO₂. Confluent monolayers of HDPCs were used at subculture passages 3 through 5. The HDPCs (3 × 10⁵ cells/ well) were plated in 6-well plates, then treated with DMEM without FBS for 24 h prior to the experiment. Four experimental groups were prepared: 1) Blank (control), 2) PLGA, 3) iloprost (10⁻⁶ M), and 4) iloprost/PLGA (5 mg). To separate the microspheres (PLGA and iloprost/PLGA groups) from the HDPCs, 5 mg of microspheres, which contained iloprost was placed on a 0.45-µm pore size membrane (BD Falcon-cell culture insert, Franklin Lakes, NJ, USA) over the 6-well plate (20). All groups were incubated for 24 and 72 h. The
experiment was performed in triplicate.

**Proliferation assay**

The methyl thiazolyl tetrazolium (MTT) (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was used to determine the proliferation of the HDPCs. After 24 or 72 h of cell culture, each extraction medium was supplemented with 500 µL/well MTT solution (0.5 mg mL⁻¹). After 1 h of incubation at 37°C under 5% CO₂, each of the wells was filled with 1 mL of dimethylsulfoxide (DMSO) after removal of the MTT solution, and the optical density was measured. Cell proliferation was calculated as described previously (9).

**Real-time polymerase chain reaction analysis**

Total RNA was extracted with Trizol reagent (Roche Diagnostics, IN, USA). RNA samples (1 µg) were converted to cDNA using reverse transcriptase (Promega, Madison, WI, USA) with a LightCycler 480 SYBR Green I Master kit (Roche Diagnostics) for 90 min at 42°C and then incubated at 99°C for 2 min. Amplification was performed on a DNA thermal cycler (Biometra, Gottingen, Germany). Expression of VEGF mRNA was determined by quantitative polymerase chain reaction (qPCR) using the MiniOpticon System (Bio-Rad, Hercules, CA, USA) with a FastStart Essential DNA Green Master kit (Roche Diagnostics). The amplification profile was: 95°C/10 s, 60°C/10 s, and 72°C/20 s for 40 cycles. The reaction product was quantified using GAPDH expression as the reference. The VEGF primers were 5’-ATGAGGACACCGCTCTGACCA-3’ (forward) and 5’-AGGCTCCTGAATCTTCCAGGCA-3’ (reverse) (NM 001025366). The GAPDH primers were 5’-CACTGCCAACGTGTCAGTGGTG-3’ (forward) and 5’-GTAGCCCAGGATGCCCTTGAG-3’ (reverse) (NM 001289746.1). The experiment was performed in triplicate.

**Rat mechanical tooth exposure model**

The in vivo protocol was approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University (Protocol no. 1232004). Eight-week-old male Wistar rats (350-500 g body weight, n = 20) were used. During the mechanical pulp exposure procedure, the rats were placed under general and local anesthesia. Anesthesia was initiated by intraperitoneal injection of ketamine/xylazine (50 mg/kg/ 2.5 mg/kg) and maintained with isoflurane (1-5%/L O₂). Mechanical pulp exposure was performed using a no. 005 round steel bur on the mesial surface of the maxillary molars until near exposure, and the pulp was finally exposed using an explorer. The iloprost/PLGA microspheres (5 mg) were mixed with 50 µL of soluble carbopol carrier in an Eppendorf tube, loaded into a 1-cc syringe, and injected into the exposure site. The cavity was then restored with light-cured glass ionomer cement (Fuji II LC, GC, Tokyo, Japan). The control and experimental groups were (n = 5): 1) Ca(OH)₂ (Life, Kerr, Orange, CA, USA) as a control, 2) iloprost (10⁻⁶ M/0.2 mL) injected directly into the area of pulp exposure (iloprost), 3) PLGA microspheres without iloprost but with carbopol (PLGA), and 4) iloprost/PLGA microspheres with carbopol (iloprost/ PLGA). Postoperative analgesia was provided. During the 30 days of the experiment, the animals were observed every other day for any signs of swelling or discomfort. Before the endpoint, the presence of filling materials was confirmed before specimen collection.

**Laser Doppler flowmetry**

After pulp exposure and restoration, the pulpal blood flow in each animal was measured for 5 min using laser Doppler flowmetry (LDF) (Moor Instruments, Axminster, UK). Mean pulpal blood flow (PBF) was measured under general anesthesia using a VP3 needle probe (Moor Instruments) and recorded as Blood Perfusion Units (BPU) using Tissue Blood Flow Measurement software at 0, 3, and 7 days after restoration under general anesthesia. The measurement was performed on each tooth 3 times and the average BPU was calculated (9).

**Micro-CT analysis**

After 30 days, the rats were euthanatized and the maxilla specimens were collected as described previously (21). Block sections of the three maxillary molars and surrounding periodontal tissues were fixed in 4% paraformaldehyde buffered at pH 7.2-7.4 for 4 h, then placed in 70% ethanol for 24 h before micro-CT analysis using a µCT 35 instrument (Scanco Medical, Fabrikweg, Switzerland). Three-dimensional reconstruction of the treated teeth was performed using the 3D analytical software supplied (Scanco Medical). Bone volume/tissue volume (BV/TV) was calculated (22).

**Histological analysis**

Following micro-CT analysis, the samples were rinsed 3 times with PBS and demineralized in formic acid. The specimens were dehydrated in a graded ethanol series for paraffin embedding and preparation of sagittal sections (23). The sample blocks were oriented mesio-distally and sectioned using an automatic rotary tissue microtome (Leica RM 2255, Leica Biosystems) set at 3 µm thickness. The sections were subjected to either hematoxylin
and eosin (H&E) or Masson’s trichrome staining. The mineralized volume/tissue volume was measured using OsteoMeasure histomorphometry software (Osteometrics, Atlanta, GA, USA). In brief, the mineralized area was selected, and its size relative to the total tissue volume was calculated (24). Each slide was subjected to measurement in 5-8 different areas at ×10 magnification.

Immunohistochemical analysis
Paraffin sections were treated with methanol containing 0.4% hydrogen peroxide to inhibit endogenous peroxidases, rinsed in double-distilled water, and blocked with 5% BSA (w/v) at 4°C overnight. The sections were incubated with a primary antibody against VEGF (1:200) (Dako, Glostrup, Denmark) overnight at 4°C, then with a peroxidase-conjugated secondary antibody (Dako), and developed using a chromogen substrate, 3-3′ dianinobenzidine (Sigma, MO, USA). The slides were counterstained with hematoxylin. The intensity of VEGF expression was quantified using the ImageJ program (v1.33 downloaded from the NIH website [http://rsb.info.nih.gov/ij]) (25). Briefly, the images were obtained at ×4 magnification, the color channel was separated, and the intensity of the color was subtracted from the background.

Statistical analysis
The data are shown as mean ± SD. Shapiro-Wilk test for normal distribution and Levene’s test for homogeneity of variances were used in all experiments. Statistical analysis was performed using the two-independent Student’s t test for two-group comparisons. The data stratified by experimental group were compared using one-way ANOVA. LSD post-hoc test was performed to test the significance of changes resulting from different treatments in relation to the control group. Data that did not achieve normality were analyzed using non-parametric methods. For VEGF quantification and qPCR experiments, non-parametric tests were performed using the Kruskal-Wallis test followed by the Steel-Dwass test for comparison of changes in the treatment groups in relation to the control group. Differences at P < 0.05 were considered to be statistically significant. Each experiment was repeated three times.

Results
Prolonged release of iloprost from PLGA gelatin microspheres in vitro
The in vitro release of BSA from the microspheres in carbopol was prolonged for up to 7,000 min (4.86 days), whereas that in the BSA and PLGA (without carbopol) groups reached a peak at 1,440 and 4,680 min (1 and 3.25 days), respectively (Fig. 1A and B). Iloprost released from gelatin PLGA microspheres had no effect on HDPC morphology under microscopic examination (Fig. 1C). All of the iloprost groups showed a significant increase in cell proliferation and expression of VEGF mRNA under microscopic examination (Fig. 1C). The increase of cell proliferation at 24 and 72 h. Bars represent the average ± SD. (E) qPCR analysis of VEGF mRNA expression at 24 and 72 h. The data are shown as median, upper and lower quartiles (boxes), and minimum–maximum values. Asterisks indicate a statistically significant difference compared to the control.

Prolongation of increased dental pulp blood flow in vivo by iloprost/PLGA microspheres
After in vivo delivery of iloprost/PLGA microspheres into the exposed pulp of rat first molars, pulpal blood flow was measured by LDF. At 0 day, no difference was evident (Fig. 2A). However, at day 3, the directly added iloprost had increased pulpal blood flow in comparison with the other groups (Fig. 2B). In the iloprost/PLGA microsphere group, a significant increase in pulpal blood
flow was noted at day 7 compared to the control group. It was noted that the increase of pulpal blood flow was no longer observed in the directly added iloprost groups (Fig. 2C).

**Enhancement of dental pulp healing in vivo by iloprost/PLGA microspheres**

After 30 days, the animals were euthanatized and tooth specimens were obtained for micro-CT 3D reconstruction (Fig. 3A) and histological analysis. The iloprost/PLGA microsphere group showed an increase of calcified tissue in the dental pulp chamber relative to the control group. BV/TV was significantly increased in the iloprost and iloprost/PLGA microsphere groups relative to the Ca(OH)₂ group, as determined by micro-CT (Fig. 3B and 3C). However, there was no significant difference in BV/TV between the iloprost and iloprost/PLGA microsphere groups.

Histological analysis demonstrated the formation of dentin bridges and tertiary dentin along the dental pulp chamber walls in the iloprost and iloprost/PLGA groups. Figure 4A shows a schematic representation of the pulp exposure site. Histomorphometric analysis revealed that both the iloprost and iloprost/PLGA groups showed a significant increase of mineralized tissue compared with the control group (Fig. 4B). H&E staining showed increased tertiary dentin in the pulp chamber in the iloprost and iloprost/PLGA groups (Fig. 4C), and both groups also showed dentin bridge formation (Fig. 4C).

**Increased expression of VEGF in dental pulp tissue in vivo after treatment with iloprost/PLGA microspheres**

Masson’s trichrome staining was used to demonstrate connective tissue elements, principally those related to collagen synthesis. Masson’s trichrome-stained samples showed absence of collagen deposition in the control (PLGA) group. An increase of tertiary dentin was observed in the iloprost and iloprost/PLGA groups relative to the Ca(OH)₂ group (Fig. 5A). Immunohistochemical analysis demonstrated that the increase in VEGF protein expression in the dental pulp tissue was significantly higher in the iloprost/PLGA microsphere group than in the other groups (Fig. 5B and 5C).
Numerous systems for controlled drug delivery have been developed for regeneration of the dentin/pulp complex (26). The dosage of iloprost used in the present study was chosen on the basis of previous findings (9,27). BSA, which has been widely used to characterize the in vitro release of a substance from microspheres (17), was used as a substitute for iloprost in order to determine the release profile. Also, when mixing microspheres for a clinical delivery system, a soluble carrier is needed. Carbopol has been used clinically as a carrier to initiate drug release through a swelling and a gelling mechanism (28). In this study, when carbopol was used as a vehicle for the microspheres, the release of BSA into the carbopol was prolonged until day 4.

In addition to stimulating the formation of tertiary dentin in the pulp chamber, any regenerative procedure should provide an environment for restoring homeostasis in the dental pulp tissue (29). Teeth treated by direct pulp capping have a survival rate of more than 90% at 3 years. However, if an apical lesion or partial pulp necrosis was present, the overall 3-year success rate of direct pulp capping was considered to be below 75% (30). Therefore, the development of a more effective direct pulp capping biomolecule that is able to support the microenvironment in the restricted blood supply region is needed.

Angiogenesis is essential for the regeneration and healing of dental pulp. Local neovascularization around the site of injury during the early inflammatory phase is required for prevention of pulp tissue necrosis (29). In agreement with our previous findings (8,9), iloprost was able to increase HDPC proliferation and VEGF expression. The prolonged release of iloprost in vitro for up to 72 h might help to extend the healing of the dental pulp and further promote angiogenesis. In this animal tooth injury study, the increase in dental pulp blood flow in the iloprost/PLGA group was extended for a longer time than in the direct iloprost group, confirming the facilitating effect of prolonged iloprost release on dental pulp tissue.

During the first 7 days after pulp injury, angiogenesis enhances the healing process and resolves inflammation (31). Ossification and angiogenesis are closely related. VEGF not only functions during angiogenesis, but also supports hard tissue formation. In this connection, VEGF supports bone mineralization by regulating neovascularization and recruiting mesenchymal stem cells (32). Although it has been shown in vitro in this study that iloprost/PLGA enhances the expression of VEGF at 72 h, the pulpal blood flow in animals was not enhanced until 7 days after the delivery. Accordingly, this study further explored the presence of VEGF expression in the later phase of healing. At day 30, this study found that VEGF expression was maximally enhanced in the iloprost/PLGA group. A sustained increase of VEGF expression until the later phase of healing may further enhance stem cell proliferation and differentiation, and promote dentin formation.

Although VEGF expression in the PLGA/iloprost group was higher than in the other groups, histomorphometric analysis of hard tissue formation revealed no significant difference from the iloprost group. One possible explanation could be that the healing rate might...
have been different at an earlier time point, such as on day 15 (33). Further improvement of methodical sample rotation during tissue scanning and sectioning should be considered in future studies.

Although iloprost has been shown to be a promising therapeutic agent for promotion of dental pulp healing (8), the half-life of iloprost is considerably short (11). Moreover, commercially available prostacyclin analogs are generally available as aerosol or solution formulations, which would make them difficult to deliver into a tooth cavity. Therefore, there is a need to develop a delivery system that would be feasible in a clinical situation. The iloprost/PLGA microsphere delivery system is composed of powder (microspheres) and a liquid (carbopol). To initiate drug release after swelling, carbopol was chosen for this study based on its clarity in gel formulations and ease of manipulation, allowing delivery into the area of pulp exposure.

The cell-free approach using a material that can recruit local stem cells to the site of injury would be preferable to stem cell transplantation. Ca(OH)$_2$ has been considered the gold standard for direct pulp capping for several decades (34). It has been recently proposed that mineral trioxide aggregate might replace Ca(OH)$_2$ as a pulp capping agent due to its higher long-term success rate (35). A better understanding of stem cell signaling and the establishment of a controlled release system are needed (36,37). Since it has been proposed that vascularization of dental pulp is crucial for its regeneration (36), biomolecules that can enhance angiogenesis over a prolonged period are also required. PGI$_2$ analogs have been used clinically for treatment of several diseases characterized by ischemia (37). Recently, orally active prostacyclin agonist has been used in patients with pulmonary hypertension (38). Consequently, PGI$_2$ analogs and agonists are promising molecules for enhancing the effects of angiogenesis-based treatments and may be applicable for tooth revascularization and regeneration.

In conclusion, the present iloprost/PLGA microsphere delivery system significantly increases blood flow in dental pulp during the inflammatory phase after mechanical exposure. Iloprost/PLGA microspheres induce angiogenesis in a prolonged manner and increase tertiary dentin formation in this animal tooth injury model. PLGA microspheres might be applicable as a system for prolonged PGI$_2$ release, thus helping to improve the therapeutic effect and increasing the period of drug release for dental pulp regeneration.

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**References**

19. Paker-Leggs S, Neau SH (2009) Pellet characteristics and drug release when the form of propranolol is fixed as moles or mass in formulations for extruded and spheronzed carbopol-containing pellets. Int J Pharm 369, 96-104.


