รายงานวิจัยฉบับสมบูรณ์

โครงการ **ผลของพรอสตาไซคลินอนาล็อคที่มีต่อการส่งเสริมการสร้างหลอดเลือดของเนื้อเยื่อในฟันผ่านทางวาสคูลาร์เอนโดทีเลียลโกรทแฟกเตอร์ ไฟโบรบลาสท์โกรทแฟกเตอร์ทูและเพลทเลทดีไรฟ์โกรธแฟกเตอร์**

นางชลิดา ลิ้มจีระจรัส (นาคเลขา) (ทพญ.ดร.)

เดือน ปี ที่เสร็จโครงการ

26 พฤศภาคม 2558

สัญญาเลขที่ MRG 056

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ผู้วิจัย นางชลิดา ลิ้มจีระจรัส (นาคเลขา) (ทพญ.ดร.)
สังกัด จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

เอกสารแนบหมายเลข 2

รูปแบบ Abstract (บทคัดย่อ)

Project Code : MRG 056
(รหัสโครงการ)

Project Title : ผลของพรอสตาไซคลินอนาล็อคที่มีต่อการส่งเสริมการสร้างหลอดเลือดของเนื้อเยื่อในฟันผ่านทางวาสคูลาร์เอนโดทีเลียลโกรทแฟกเตอร์ ไฟโบรบลาสท์โกรทแฟกเตอร์ทูและเพลทเลทดีไรฟ์โกรธแฟกเตอร์

Investigator : นางชลิดา ลิ้มจีระจรัส (นาคเลขา) (ทพญ.ดร.)
สังกัด จุฬาลงกรณ์มหาวิทยาลัย
E-mail Address : kandychula@yahoo.com, nchalidachula@gmail.com

Project Period : 2 ปี
(ระยะเวลาโครงการ)

บทคัดย่อ

ไอโลพรอสเป็นสารอนุพันธุ์ของพรอสตาไซคลินซึ่งมีฤทธิ์ในการเสริมสร้างการสร้างหลอดเลือดใหม่ การนำมาใช้ในทางวิศวกรรมกระดูกและฟันยังมีจำกัด การศึกษานี้ทำขึ้นเพื่อศึกษาถึงผลของไอโลพรอสที่มีต่อเนื้อเยื่อฟัน จากผลการศึกษาพบว่ามีการเพิ่มของยีนส์ที่เกี่ยวข้องกับการสร้างหลอดเลือดและการเพิ่มจำนวนเซลล์ ได้แก่ยีนส์ VEGF, FGF-2 and PDGF ตามลำดับ ในการศึกษาในสัตว์ทดลองพบว่าไอโลพรอสสามารถเพิ่มการไหลเวียนโลหิตในฟันของหนูทดลองและเพิ่มการสร้างเนื้อฟันทดแทน เมื่อให้เป็นระยะเวลาสามสิบวันได้อย่างมีนัยสำคัญ จากผลการศึกษาที่กล่าวมาสามารถสรุปได้ว่าไอโลพรอสสามารถเพิ่มการสร้อางหลอดเลือดใหม่ในเซลล์เนื้อเยื่อในฟันมนุษย์และเพิ่มการหายของเนื้อเยื่อฟันในสัตว์ทดลองได้ ปัจจุบันโครงการที่กำลังดำเนินการอยู่การพัฒนาการใช้ยาในลักษณะตัวพา เพื่อให้สามารถปล่อยยาออกมาอย่างช้าๆและมีประสิทธิภาพมากยิ่งขึ้น

Abstract

Iloprost is a stable long acting prostacyclin analogue. The usage of iloprost in dental pulp regeneration is limited. In this study, the investigation of the effect of iloprost on the proliferation and vascularization on Human Dental Pulp cells (HDPCs) was

performed. The significant increase of VEGF, FGF-2 and PDGF was marked in HDPCs treated with iloprost in dose dependent manner. The delivery of iloprost in animal tooth injury model showed that the increase of vascularization and reparative dentin was found at day 30. In conclusion, iloprost increased vascularization and healing in dental tissues. Iloprost could be used as adjuvant in drug delivery therapy. Our current ongoing project is on improving the drug delivery system for prolonging and sustainable release of the iloprost for using in bone and tooth regeneration.

Keywords : iloprost, prostacyclin, tooth, dental pulp, animal

Rationale

Dental pulp vitality is extremely important for the tooth viability, since it provides nutrition and acts as biosensor to detect pathogenic stimuli. In clinical situations, exposure of dental pulp as a result of dental caries or injuries could lead to the inflammation of the pulp and end up in the necrosis of the pulp. Prostacyclin (PGI_2), a strong physiologic vasodilating agent, has a biogenic property by enhancing angiogenesis and cellular proliferation, and probably bone remodeling[1]. PGI_2 and VEGF are strongly related in many cell types and promoted vascular proliferation, yet the studies of the relationships between these two molecules are limited. In this study, we aim to investigate the role of PGI_2 analogue; iloprost, to dental pulp tissue in the promoting vascularization and ultimately promoting dentin repair in both laboratory and animal aspects.

Objectives

The objectives of this study are to;

1. To investigate the interaction and the possible roles of prostacyclin analogue in promoting the angiogenesis of human dental pulp cells, particularly via the up-regulation of proliferating factors; VEGF,FGF-2 and PDGF.

2. To study the effect of prostacyclin analogue on promoting dental pulp healing in animal by establishing the application of prostacyclin delivered to the tooth injured model and further study the possibility of applying prostacyclin as an adjuvant for drug delivery treatment in clinical situation.

Methodology

Strategy 1: The investigation of the interaction of prostacyclin analogue in promoting the proliferation and angiogenesis of human dental pulp cells.

Experimental Design

Human dental pulp cell (HDPC) will be treated with exogenous prostacyclin (iloprost) in variation of dosages[2]. The level of cytokine, such as VEGF, FGF2 and PDGF and proteins release shall be determined by RT-PCR and ELISA, respectively.

Materials and Methods

Tooth

Tissue specimens will be acquired from extracted tooth from the patients at department of oral surgery, faculty of Dentistry, Chulalongkorn university. Patients' consent will be taken after patients were fully informed about information, procedure and material. Patients are allowed to ask questions and given plenty of time to consider and sign before giving consent.

Cell cultures

Human dental pulp cells (HDPC) will be prepared as previously described[3]. In short, the dental pulp tissues specimens are obtained from extraction of third molars with the informed consent of three patients. The explants are cultured in Dulbecco's modified minimum essential medium (DMEM), supplemented with 10% fetal bovine serum in plastic culture dishes with medium changes every 3 days for 10 to 15 days until confluent cell monolayer were formed. After 3 subcultures, homogeneous, slim, spindle-shaped cells growing in characteristic swirls will be obtained. The HDPC will be used as confluent monolayer at subculture passages 3 through 5. All experiments shall be performed in triplicate with cells obtained from three different donors.

Treatment of iloprost

HDPC suspensions (3×10^5 cells/well) are seeded in the 6- well plates culture dishes in DMEM supplemented with 10%FBS. After incubation for 1 day at 37°C in a 5% CO_2 atmosphere, the cell monolayers will be treated with DMEM without FBS, and then cultured for 24 hrs. The monolayers are subsequently treated by the addition of iloprost

in DMEM without FBS at various concentrations (10^{-6}, 10^{-7},10^{-8} and 10^{-9} M). After further incubation for 24 hrs, the supernatant and cells are collected and used in the experiments. Total RNA will be isolated for PCR assay of VEGF and FGF2 and other cytokines expression.

Treatment of selective IP receptor antagonist

It is known that PGI_2 analogues, such as iloprost, exert their function through the IP receptor, leading to increased levels of intracellular cAMP[4] . To examine whether, indeed, the effect of PGI_2 analogues was mediated through the IP receptor, pDCs will be pre-treated with an IP receptor antagonist , CAY10449,1hour prior to the treatment of iloprost [5]. The concentrations of inhibitors were chosen based on the IC_{50} values of each inhibitor in other cell types[6]. The effects of iloprost on the cytokine expression in pDCs will be then analysed.

PCR expression of cytokines

Total RNA is extracted, using TRIzol reagent. The cDNA will be generated from 5 mg of RNA using reverse transcriptase. The polymerase chain reaction (PCR) reactions are performed by qPCR.

Enzyme-linked immunosorbent assay (ELISA)

Cells will be cultured and treated as indicated previously. The culture media is collected and assayed to quantify concentrations of VEGF, cAMP by ELISA kit according to the manufacture's protocol. Each sample will run in duplicate and the concentration of VEGF and cAMP is determined by comparison of the optical densities of the sample wells with that of the standard curve. Data is represented by graph as a fold-induction compared to the control.

Measurement of intracellular cyclic AMP

Intracellular cyclic AMP (cAMP) was measured by cAMP immunoassay kit (CA-201 Sigma-Aldrich). Briefly, HPDCs ($1.5x10^5$/mL) were stimulated with different concentrations of iloprost for 30 min at 37 C, and then supernatant will be collected and cAMP levels were measured according to the manufacturer’s instruction. This assay uses a polyclonal antibody to cAMP to competitively bind cAMP or cAMP which has been covalently linked to an alkaline phosphatase molecule. The assay is performed in a 96 well plate coated with anti-rabbit IgG antibody. The colored end product, produced

by the additon of substrate to the wells, is read at 405 nm on a multiwell plate readerThe cAMP levels are expressed as an index representing the ratio between values obtained in stimulated cells and cells incubated in the control medium.

Strategy2; The study of the effect of prostacyclin analogue on promoting dental pulp healing in animal by establishing the application of prostacyclin delivered to the tooth injured model.

- **Design and plan**

  Iloprost delivery system

Iloprost comes in solution of 20ug/mL (vial) with the molecular weight at 352.465 g/mol. Iloprost is diluted with PBS to the desired concentration at 10^{-6}, 10^{-7} and 10^{-8} M. The delivery of iloprost to the tooth cavity is carried by pre-mixing the solution with the PLGA/gelatin microsphere carrier (Fig.3). The fabrication of the microsphere has been previously reported[7]. PLGA/Gelatin microsphere has biocompatibility and biodegradability and was well accepted in commercial industry.

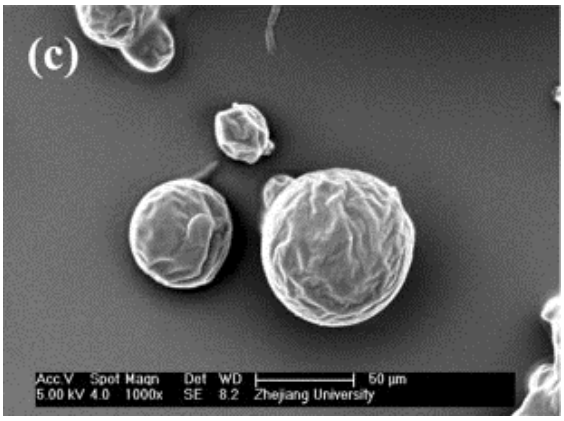
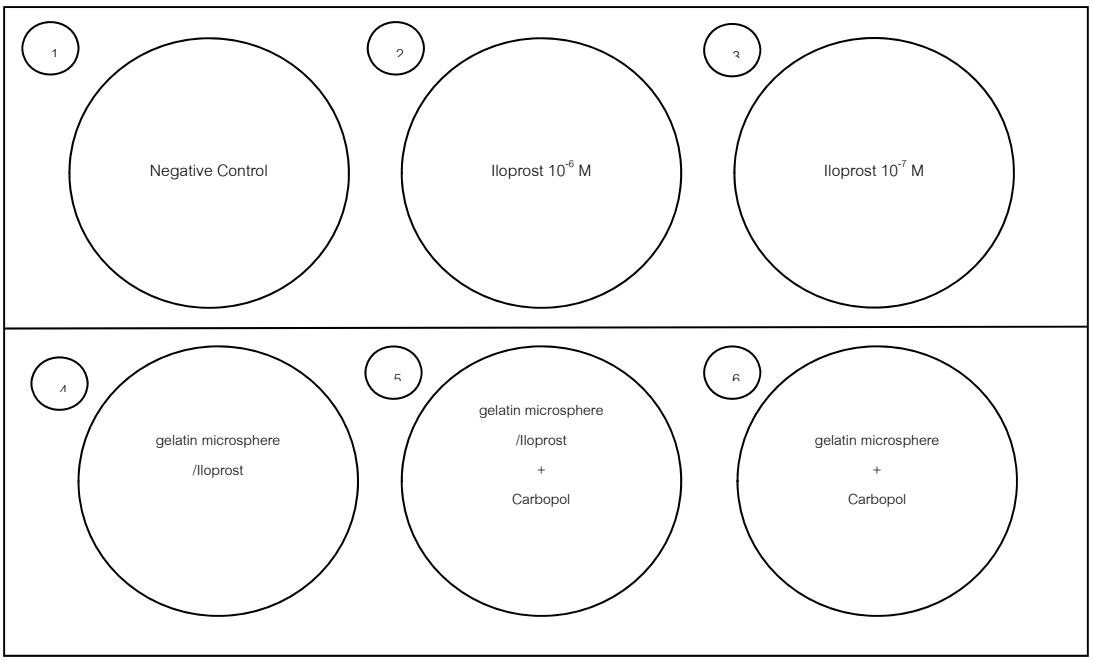


Fig.3 PLGA/gelatin microsphere; from *Journal of biomedical materials research. Part B, Applied biomaterials* **2009**, *91*, 228-38.

Treatment of iloprost

HDPC were seeded in the 6- well plates culture dishes at a density of 3 x 10^{5} cells/well in DMEM supplemented with 10%FBS. Then, incubated at 37°C in a 5% CO_2 atmosphere for 24 hrs. The cell monolayers were treated with DMEM without FBS, and then cultured for 24 hrs. Each well plate was treated in difference condition (fig.2 and

fig.3). All wells were incubated for 24 hrs. The supernatant were collected for ELISA. The cells were collected for PCR.



Control :

Plates without insert

Experimental :

Plates with insert

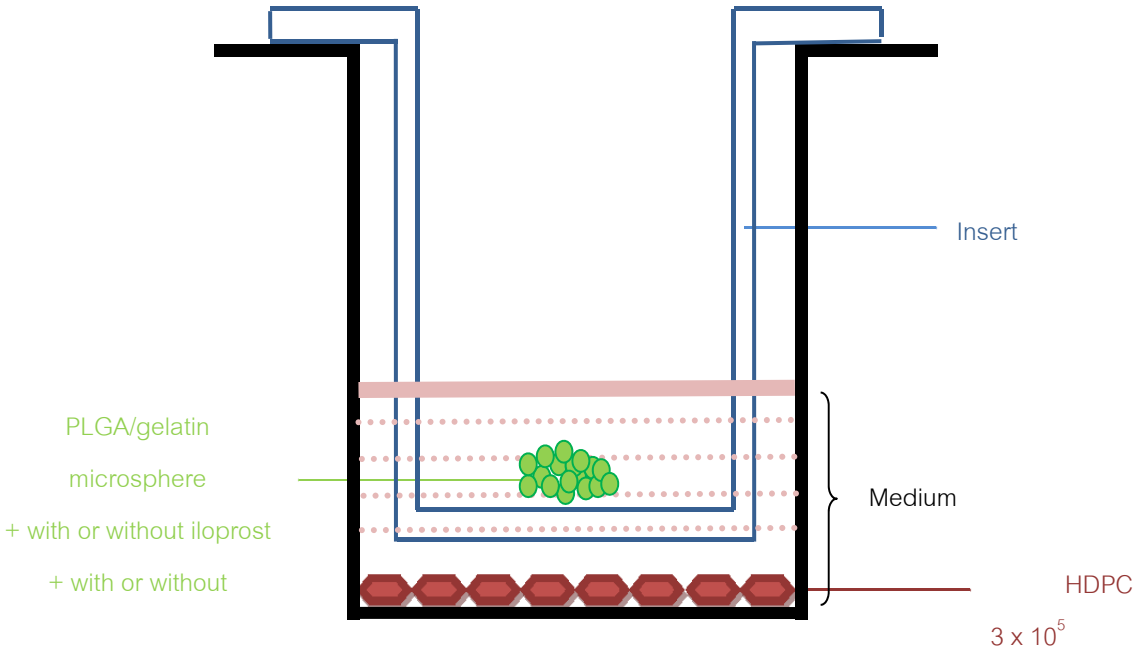


Fig.3: illustrate of plate with insert

Animal

Eight-week- old, male Wistar rats (n=20) will be used in this study. During an operative procedure, the rats are given general and local anesthesia. Intentional mechanical injury will be performed by drilling on the mesial surface of upper molars until the near exposure, followed by a sharp instrument to make a mechanical exposure. Each of the animal will be used as both control and study groups.

Control groups are right upper molars (Q1) that have mechanical exposure with no medication and restored with glass ionomer cement (GIC); GI lining Fuji II.

Left upper molars that have mechanical exposure (study groups) will be divided into separated 4 subgroups; medication with iloprost in various concentrations from 10^{-6}, 10^{-7} and 10^{-8} M and $CaOH_2$(n=5 each group). Solution of iloprost is mixed with gelatin beads as carriers and were carried to the cavity by dycal carrier instrument[8, 9]. After the application of iloprost, the cavities will be filled with GIC.

Measuring level of blood flow

Laser Doppler Flowmetry (LDF) can measure the rate of blood flow in specific area per time, by placing the laser probe at the position designated area. After the operation, the rate of blood flow of each sample will be immediately measured for 5 minutes by LDF. “Mean pulpal blood flow” will be calculated immediately before the mechanical exposure, right after the iloprost application and then at next 24 and 72 hours by comparing between the control and study groups.

Tooth’s preparation

After 30 days, the rats will be euthanized for collecting the tooth specimens. The decalcifying method is modified from the proposing method by Decup et al (2000) [8]. In brief, the maxilla specimens will be preserved in formaldehyde for 3 days. Then they will be dissected for removing soft tissues following with the procedure of decalcifying and embedding in paraffin blocks. The specimen blocks will be cut with microtome machine at 5 micron thickness. The section will be stained for H&E staining and inspected under microscopic examination.

Histological examination

Specimen were placed in 10% formaline solution overnight for fixation, decalcified by 20% formic acid for 2 days before embedding in paraffin. Three-micrometer-thick

sections were cut with an automatic rotary microtome, then stained with hematoxylin and eosin stain.

Immunohistochemistry

The sections from biopsies of the tooth specimen will be used for VEGF, FGF-2 staining. In brief, sections will be fixed and incubated for primary antibodies overnight at 4 c. After rinsing, sections were incubated for secondary antibodies and then rinsed and exposed to chromogen solution. Counterstaining and final rinsing will be performed before mounting. Examination will be conducted under microscope. Series of all samples and controls will be run at least twice.

Statistics

Data are presented as means ± standard deviation. Data between groups were compared using a 2-tailed *t* test or ANOVA, as appropriate, followed by Tukey's post hoc test (or other appropriate tests). Differences at $p<0.05$ will be considered to be statistically significant.

Results

Currently, the revascularization concept has become widely accepted as a regenerative endodontic procedure [10]. Dentin matrix protein could enhance angiogenesis by enhancing endothelial cell proliferation, new vessel formation as well as promoting the expression of the proangiogenic factor and their receptors [11]. The proper use of scaffolds, cells, and growth factors could enhance the clinical outcome of dental stem cell therapy. Thus, the *in vivo* mechanism(s) of iloprost-induced neovascularization should be further examined. It would be interesting to investigate its effects on promoting angiogenesis when used in a well-designed delivery system as a direct pulp-capping agent in *vivo*.

In conclusion, the current study suggests that the prostacyclin analogue iloprost is part of a signaling network between the vascularization and the proliferation of the dental pulp via the up-regulation of *VEGF*, *FGF-2*, and *PDGF*. Our *in vivo* study of pulpal blood flow at early time points of dental pulp healing suggested that neovascularization was promoted by iloprost. Thus, we propose that iloprost might be used as an adjuvant for promoting the regeneration of dentin/pulp tissues, especially

when an increase in blood flow in the dental pulp is required; such as the treatment of a necrotic pulp or in an avulsed immature tooth.

To examine the effect of iloprost *in vivo*, iloprost was directly applied to the pulpal exposure area in rat first molars. Because our *in vitro* results indicated that most of the significant increases in osteogenic markers were found using 10^{-6} M iloprost, this concentration was used to evaluate the pulpal response *in vivo*. We found that iloprost dramatically enhanced tertiary dentin formation by mechanically injured rat dental pulp. Although the mechanisms of tertiary dentin formation in humans are not yet completely known [12], it is believed that the dental pulp healing process begins with new blood vessel formation, followed by the proliferation, migration, and subsequent differentiation of stem/progenitor cells into osteo/odontoblast-like cells [13, 14]. The success of a pulp capping procedure depends on many factors under which it is performed and the prognosis depends upon the patient's age, type of injury, and site and size of the pulp exposure. The odontogenic/osteogenic differentiation of dental pulp cells can be promoted by exogenous molecules and biomaterials. MTA and calcium-enriched mixtures, together with other medications or biomolecules, have been shown to possess osteogenic activity and promoted hard tissue formation in dental pulp through several mechanisms. These mechanisms included the up-regulation of DSPP, BMPs, and TGF-β, but further investigation is still needed [15-17].Glass ionomer, which provides a good bacterial seal and biocompatibility also enhanced the mineralization of dental pulp tissue [18]. Although many biomaterials have been recently introduced, the appropriate delivery systems should be further established.

A limitation of the present study is that we did not investigate the controlled delivery of iloprost. When directly applied to the exposure area, iloprost may be cleared relatively quickly. Although iloprost is a long-acting PGI_2 analogue, it has a short half-life of approximately 20-30 minutes in lung tissues [19]. The proper control of iloprost levels is important because although iloprost is considered safe to be administered systemically, including to pediatric patients, when given at high doses some adverse effects have been observed, such as hypotension and nausea [20, 21]. Thus, the development of a controlled delivery system is needed to maintain appropriate iloprost levels at the defect site. The controlled and sustained delivery of iloprost/PGI_2 should mimic its temporal profile during natural healing *in vivo* [22]. The retention of iloprost in the dental pulp should be further investigated.

In conclusion, iloprost could be a potential agent for promoting pulp healing and inducing tertiary dentin formation in traumatized dental pulp tissues. Thus, we propose

that iloprost could potentially be used as an adjuvant in direct pulp capping. Furthermore, investigation into developing a delivery system is needed.

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เอกสารแนบหมายเลข 3

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. **Nakalekha Limjeerajarus C.**, Osathanon T., Manokawinchoke J. and Pavasant P., Iloprost Upregulates VEGF Expression in Human Dental Pulp Cells In Vitro and Enhances Pulpal Blood Flow In Vivo, *Journal of Endodontics* Volume 40, Issue 7, Pages 925–930, July 2014 (impact factor 3.122)

2. **Nakalekha Limjeerajarus C.**, Chanarattanubol T., Trongkij P., Rujiwanichkul M. and Pavasant P., Iloprost enhances the regeneration of reparative dentin: *In Vitro* and *In Vivo* Study, *J of Endodontics.* Volume 40, Issue 11, November 2014, Pages 1784–1790, (impact factor 3.122)