Protein expression and inflammatory response after delivery of mRNA encoding *platelet-derived growth factor-BB* in sucrose citrate buffer into rat gingiva

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A Thesis Submitted in Partial Fulfillment of the Requirements

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การแสดงออกของโปรตีนและการตอบสนองของระบบภูมิคุ้มกัน เมื่อนำส่งเมสเซนเจอร์อาร์เอ็นเอที่เข้ารหัสเพลทเลทดีไรฟ์ โกรทแฟกเตอร์-บีบี โดยใช้ซูโครสซิเตรทบัฟเฟอร์ในเหงือกของหนูแรท

น.ส.พรรณ พงษ์เสฐียร

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พรรณ พงษ์เสฐียร : การแสดงออกของโปรตีนและการตอบสนองของระบบภูมิคุ้มกันเมื่อนำส่งเมสเซนเจอร์อาร์เอ็นเอที่เข้ารหัสเพลทเลทดีไรฟ์ โกรทแฟกเตอร์-บีบี โดยใช้ซูโครสซิเตรทบัฟเฟอร์ในเหงือกของหนูแรท. (Protein expression and inflammatory response after delivery of mRNA encoding *platelet-derived growth factor-BB* in sucrose citrate buffer into rat gingiva) อ.ที่ปรึกษาหลัก : อ. ทญ. ดร.วิชญา วิศิษฐ์รัศมีวงศ์, อ.ที่ปรึกษาร่วม : ศ. ทญ. ดร.รังสินี มหานนท์

ปัจจุบัน เอ็มอาร์เอ็นเอหรือเมสเซนเจอร์ อาร์เอ็นเอได้รับการพัฒนาให้สามารถใช้กระตุ้นเซลล์เป้าหมายให้ผลิตโปรตีนที่ต้องการ เช่น โกรทแฟคเตอร์ ขึ้นเองภายในเซลล์เพื่อทดแทนการใช้โปรตีนลูกผสมได้ ดังนั้น การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของการนำส่งเอ็มอาร์เอ็นเอดัดแปลงที่เข้ารหัสเพลตเลตดีไรฟ์โกรทแฟคเตอร์-บีบีซึ่งเป็นโกรทแฟคเตอร์ที่นิยมศึกษาเพื่อการฟื้นฟูเนื้อเยื่อปริทันต์ใหม่ ด้วยการฉีดเข้าที่เหงือกของหนูแรท ต่อระดับโปรตีนเพลตเลตดีไรฟ์โกรทแฟคเตอร์-บีบีและการกระตุ้นการอักเสบของเนื้อเยื่อ โดยเอ็มอาร์เอ็นเอดัดแปลงที่เข้ารหัสเพลตเลตดีไรฟ์โกรทแฟคเตอร์-บีบี ในซูโครสซิเตรทบัฟเฟอร์ จะถูกฉีดเข้าที่เหงือกด้านเพดานของหนูแรท จากนั้นจึงทำการเก็บเนื้อเยื่อเหงือกบริเวณดังกล่าวที่ระยะเวลา 1 2 3 5 และ 7 วันภายหลังการฉีด เพื่อตรวจวัดปริมาณโปรตีนที่สนใจด้วยวิธีอีไลซา ผลการศึกษาพบว่า การนำส่งเอ็มอาร์เอ็นเอข้างต้นสามารถเพิ่มการแสดงออกของโปรตีนเพลตเลตดีไรฟ์โกรทแฟคเตอร์-บีบี ได้ 40-100 เท่าเมื่อเปรียบเทียบกับกลุ่มควบคุม โดยพบระดับโปรตีนสูงสุดที่ 24 ชั่วโมงแรกหลังการนำส่ง และลดลงจนใกล้เคียงกับระดับเดิมในวันที่ 3 ไม่พบการเพิ่มขึ้นของสารสื่ออักเสบอินเตอร์ลิวคิน-6 และทูเมอร์ เนคโครซิส แฟคเตอร์-อัลฟาในเนื้อเยื่อภายหลังการนำส่ง จึงสรุปได้ว่า การนำส่งเอ็มอาร์เอ็นเอดัดแปลงที่เข้ารหัสเพลตเลตดีไรฟ์โกรทแฟคเตอร์-บีบี ในซูโครสซิเตรทบัฟเฟอร์ ด้วยวิธีการฉีดเข้าที่เหงือกของหนูแรท สามารถกระตุ้นการผลิตเพลตเลตดีไรฟ์โกรทแฟคเตอร์-บีบี ได้ในปริมาณสูง และมีผลกระตุ้นการอักเสบที่เนื้อเยื่อเหงือกของหนูแรทน้อยมากหรือไม่มีเลย ดังนั้น เทคโนโลยีเอ็มอาร์เอ็นเอนี้อาจนำไปใช้ในการฟื้นฟูเนื้อเยื่อปริทันต์ใหม่ได้

สาขาวิชา	ปริทันตศาสตร์	ลายมือชื่อนิสิต
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KEYWORD: periodontal regeneration, mRNA, platelet-derived growth factor, rat gingiva

Phan Bhongsatiern : Protein expression and inflammatory response after delivery of mRNA encoding *platelet-derived growth factor-BB* in sucrose citrate buffer into rat gingiva. Advisor: Dr. WICHAYA WISITRASAMEEWONG, D.D.S., M.Sc., D.M.Sc. Co-advisor: Prof. RANGSINI MAHANONDA, D.D.S., M.Sc., Ph.D.

Messenger RNA (mRNA) has emerged as a novel therapeutic modality in medical fields, including regenerative medicine. The concept of mRNA-based therapy is to use synthesized mRNA encoding therapeutic protein delivered to target tissue. Thus, mRNA encoding growth factor could be a promising alternative to recombinant protein. Platelet-derived growth factor (PDGF) is one of most extensively studied growth factors for periodontal regeneration. To date, mRNA therapy has never been explored in the field of periodontal regeneration. The aim of the study is to examine the effect of nucleoside-modified mRNA encoding PDGF-BB on the level of PDGF-BB protein and inflammatory response at local tissue upon intragingival injection. Sprague-Dawley rats were injected with pseudouridine-modified mRNA encoding PDGF-BB in sucrose citrate buffer at palatal gingiva. Gingiva was collected at day 1, 2, 3, 5 and 7 for analysis of PDGF-BB, VEGF-A protein and pro-inflammatory cytokines IL-6 and TNF-a, using ELISA. The results showed that intragingival injection of pseudouridine-modified mRNA encoding PDGF-BB significantly promoted transient PDGF-BB protein expression up to 40 to 100-fold as compared to control. PDGF-BB level peaked at 24-hour post-injection and declined to baseline within 3 days. Neither IL-6 nor TNF-a in gingiva were affected. The findings from this study demonstrated the potential of mRNA for periodontal regeneration.

Field of Study: Periodontics Student's Signature

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Academic 2019 Advisor's Signature

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Phan Bhongsatiern

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CHAPTER I

INTRODUCTION

Background and rationale

Periodontitis is one of the most common chronic diseases, affecting up to 743 million adults worldwide(Kassebaum et al., 2014). In Thailand, more than 25% of middle-aged and 35% in elderly population are diagnosed with periodontitis(สำนักทันตสาธารณสุข กรมอนามัย กระทรวงสาธารณสุข, 2017). The breakdown of tooth-supporting structures, in particular alveolar bone, is the hallmark of the disease. This eventually leads to early tooth loss in severe cases, which significantly impinges on patients' quality of life.

Regeneration of damaged tooth-supporting structures is indeed required to restore full function of the periodontium and become the ultimate goal of periodontal treatment. Several regenerative approaches have been investigated yet the results of current treatments are mostly unpredictable. Despite the fact that guided tissue regeneration (GTR) and/or bone grafting procedures have showed to be effective for treating some types of periodontal defects, they have failed to attain complete periodontal regeneration(Bartold, McCulloch, Narayanan, & Pitaru, 2000). Undoubtedly, new therapeutic approaches to promote regeneration such as the use of growth factors, stem cells or gene-based therapy have been investigated. Nevertheless, the clinical results are limited and inconsistent. Transplantation of mesenchymal stem cells, for

example, exhibits disadvantages in term of cost, time-consuming, and potential immunological responses. Moreover, the survival and differentiation of the target cells are difficult to manipulate(Wada et al., 2011). Recombinant growth factor delivery has gained more interest and various commercial products are currently available. However, the rapid degradation *in situ* and the cost of treatment probably hinder its use(Mitchell, Briquez, Hubbell, & Cochran, 2016). Recently, a gene-therapy has been introduced to enhance the ability to regenerate periodontal tissues(Ramseier, Abramson, Jin, & Giannobile, 2006).

The gene therapy could be achieved by viral vector or plasmid DNA (pDNA). This approach has been studied in the field of periodontology since early 2000s. For example, adenovirus encoding PDGF-A transduced gingival fibroblast and enhanced cell proliferation and migration *in vitro*(Q. P. Chen & Giannobile, 2002). In animal model, plasmid DNA encoding BMP-4 with a scaffold delivery system was found to enhance bone formation in rat cranial defect(Huang, Simmons, Kaigler, Rice, & Mooney, 2005). Injection of plasmid DNA (pDNA) encoding Osteoprotegerin (OPG) was found to reduce progressive alveolar bone resorption in experimental-induced periodontitis in rat(Tang et al., 2015). However, integration of DNA into host genome may pose the risk of insertional mutagenesis and often associated with low levels of protein expression. Therefore, mRNA-based approach has recently emerged as a novel alternative in the non-viral gene therapy. In contrast to DNA, mRNA does not integrate into host genome,

transiently expresses protein of interest and subsequently be degraded through metabolic pathway(Sahin, Kariko, & Tureci, 2014; Weissman, 2015).

Nevertheless, delivery of mRNA encoding growth factor remains challenging in the aspect of its immunogenicity and limited stability(Sahin et al., 2014; Weissman, 2015). Several methods have been applied to overcome these limitations, including base modification and delivery system optimization. Base or nucleoside modification, such as replacing uridine with pseudouridine, evidently offers an advantage of diminishing immunogenicity when deliver into target cells(Kariko et al., 2008). In term of stability, studies have shown that the stability of mRNA can be improved when the proper vehicles, for example, lipids, polymers and peptides are employed(Martin & Rice, 2007; Mintzer, Merkel, Kissel, & Simanek, 2009; Pack, Hoffman, Pun, & Stayton, 2005). Recently, citrate-buffer has also been proven to be a potential carrier for mRNA(Sultana et al., 2017). This knowledge has advanced the used of mRNA for a therapeutic purpose. mRNA has now been employed for immunotherapy, inhibition of pathogenic mRNA translation, genetic reprogramming and protein replacement/growth factor therapy. A pioneered work on mRNA-based therapy in the field of tissue regeneration by Zangi *et al.* has demonstrated that a single dose of mRNA encoding vascular endothelial growth factor-A (VEGF-A) could successfully restore the ischemic heart in a mouse model(Zangi et al., 2013). This study underscored the potential of a

synthetic mRNA as a tool for tissue regeneration. Currently, mRNA encoding VEGF-A are under clinical trials, aiming to develop regenerative therapies for the treatment of cardiometabolic diseases. Nevertheless, the use of nucleoside modified mRNA has never been investigated in periodontal tissue regeneration.

Given the potential of mRNA-based therapy in tissue regeneration, we hypothesize that mRNA encoding specific growth factors could be a promising alternative for periodontal therapy. As an initial step in our research study, we propose to develop nucleoside-modified mRNA encoding platelet-derived growth factor-BB (PDGF-BB) which its transfection and expression into target cells has been successfully demonstrated *in vitro.* Therefore, in this study, we aim to examine the effect upon delivery of nucleoside-modified mRNA encoding platelet-derived growth factor-BB (PDGF-BB) *in vivo*. We will investigate protein expression in rat gingival tissue at different time points and the inflammation at a site of mRNA delivery.

Objectives

To examine the PDGF-BB protein expression, as well as VEGF-A, IL-6 and TNF-α production in gingival tissue upon local delivery of pseudouridine-modified mRNA encoding platelet-derived growth factor-BB (PDGF-BB) in sucrose citrate buffer *in vivo*

Hypothesis

Delivery of pseudouridine-modified mRNA encoding PDGF-BB in sucrose citrate buffer into rat gingiva would result in PDGF-BB protein expression with minimal immune or inflammatory stimulation.

Field of research

In vivo study of pseudouridine-modified mRNA encoding PDGF-BB in sucrose citrate buffer as a next generation therapeutics for periodontal regeneration

Limitation of research

This *in vivo* study is the pilot study. Thus, safety, efficacy and effectiveness of the mRNA should be evaluated, and larger sample size should be employed in future study.

Application and expectation of research

This preliminary study provides a set of data, which will help in developing further pre-clinical and clinical studies for novel therapeutic agents to regenerate periodontal defects. We expect to have this mRNA encoding PDGF-BB as a next generation therapeutic platform, which when applied to periodontal defect will lead to complete periodontal regeneration in patients with periodontitis and peri-implantitis.

Keywords

Periodontal regeneration, mRNA, Platelet-derived growth factor, Rat gingiva

CHAPTER II

REVIEW LITERATURE

Periodontal disease and regeneration

The Global Burden of Disease Study in 2010 ranked severe periodontitis as the sixth-most prevalent disease in the world, affecting 11.2% worldwide(Kassebaum et al., 2014). In Thailand, severe periodontitis has been recognized as the major oral health problem and cause of teeth loss among adults and elderly people, afflicting up to 18% of the population(สำนักทันตสาธารณสุข กรมอนามัย กระทรวงสาธารณสุข, 2017). Periodontitis is a chronic inflammatory disease resulting from host response against bacteria and its components in dental plaque. The microbial dysbiosis in dental plaque around tooth leads to the destruction of tooth-supporting structures including gingiva, cementum, periodontal ligament (PDL) and alveolar bone.

The ultimate goal of periodontal treatment is to regenerate the lost periodontal tissues and restore periodontal health. However, non-surgical therapy and the majority of surgical periodontal procedures usually result in long junctional epithelium or connective tissue attachment which lead to a reduced periodontium with compromised function and esthetics.

Although regenerative surgical procedures, such as guided tissue regeneration (GTR) or bone grafting, have the potential to restore the damaged periodontium, their outcomes are not predictable and their efficacy and effectiveness are still in questioned

due to several limitations(Bartold et al., 2000). GTR could provide predictable outcome in specific types of periodontal defects, such as two to three-walled bony defects, some furcation defect with proper level of proximal bone[18]. Current evidence suggested that the complete regeneration might not be achievable(Avila-Ortiz, De Buitrago, & Reddy, 2015; Kao, Nares, & Reynolds, 2015). Moreover, the treatment cost is relatively expensive. It can cost up to 500 USD per procedure. Therefore, the new therapeutic approaches aiming for periodontal regeneration such as the use of stem cells, growth factors and gene therapy have been investigated, albeit produced mixed clinical outcomes.

Cell-based approaches, for example; cell-sheet tissue engineering or stem cell application, have been introduced to the field of periodontology. Despite the promising outcome in regenerating periodontium, the techniques utilized in cell-based approaches are complicated, costly and time-consuming. In addition, the source of mesenchymal stem cells (MSC) is limited and *in vitro* cell expansion may pose the risk of cell transformation(Kao, Murakami, & Beirne, 2009), as it is difficult to control the survival and differentiation of target cells(Wada et al., 2011).

Instead of cells, the use of growth factors has gained more attention recently as a tool to stimulate multi-potent cells within the periodontium to proliferate and differentiate into desired tissues(Kao et al., 2009). Growth factors function as signaling molecules, which regulate and cue the cells engaged in periodontal regeneration and

to create the proper microenvironment for cell differentiation(Bartold et al., 2000). Various recombinant growth factors have been studied and found to be promising. Platelet-derived growth factors (PDGF) and insulin-like growth factor-I (IGF-I), for example, have been shown to enhance periodontal regeneration both *in vitro* and *in vivo*(Li et al., 2017; Lynch et al., 1991; Rutherford, Ryan, Kennedy, Tucker, & Charette, 1993). Bone morphogenetic proteins (BMP), which render a good result in inducing bone formation, are considered as another propitious alternative(Choi et al., 2002; Jepsen & Terheyden, 2002; Vandana, Singh, Prakash, Bhushan, & Mahajan, 2016), Fibroblast growth factors (FGF), especially FGF-2, has potent angiogenic, osteogenic activities and found to effectively stimulate mesenchymal stem cell proliferation and differentiation(Kao et al., 2009; Li et al., 2017; Murakami, 2016).

In spite of their attractive properties for tissue regeneration, several studies showed inconclusive clinical efficacy of recombinant growth factor application in periodontal regeneration. A rapid clearance of growth factor *in vivo* is the primary issue. Topical administration of recombinant growth factor resulted in transient release of growth factor *in vivo*, typically less that 24-hour(Nevins et al., 2005). In order to maintain the therapeutic level, growth factor has to be incorporated into a scaffold or synthetic matrix(Anusaksathien & Giannobile, 2002), otherwise supra-physiologic dose or several administrations are required. Such high dose of growth factors may cause

undesirable side effects and increase the cost of therapy. Thus, the new periodontal regeneration platform that is safe and cost effective is still in search.

Platelet-derived growth factor (PDGF)

Two most extensively studied growth factors for periodontal regeneration are platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF-2). Recent meta-analysis by Li *et al.* indicated that the use of 0.3 mg/ml recombinant human PDGF-BB (rhPDGF-BB) positively increased defect bone fill, bone height and clinical attachment gain in periodontal defects(Li et al., 2017). Importantly, rhPDGF-BB was the first growth factor to be evaluated in pre-clinical periodontal regenerative studies and is one of the few growth factors approved by Food and Drug Administration (FDA) for clinical use. Thus, PDGF-BB is selected as the candidate growth factor used in the present study.

Platelet-derived growth factor (PDGF) is a natural protein, abundantly found in bone matrix. Regarding evidence from *in vitro* studies, PDGF attracts osteoblasts to the injured or inflamed site, thus promoting new bone formation(Sanchez-Fernandez, Gallois, Riedl, Jurdic, & Hoflack, 2008). PDGF has also been found to cue proliferation and enhance osteogenic differentiation of mesenchymal stem cells (MSCs)(Pountos et al., 2010). Moreover, recent data demonstrated that rhPDGF-BB can induce osteoblasts to produce vascular endothelial growth factor (VEGF), one of the most potent mediators promoting angiogenesis(Bouletreau et al., 2002; Cooke et al., 2006).

The isomeric forms of PDGF, namely PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD have been identified. All forms of PDGF molecules bind to two distinct type of receptors. PDGF alpha-receptors could bind to all isoforms with high affinities, while beta-receptors bind PDGF-BB with high affinity and PDGF-AB with a lower affinity and do not bind PDGF-AA(Hart et al., 1988). In regenerated periodontal tissues, expression of beta-receptors of PDGF, but not PDGF alpha-receptors, could be detected. This suggests that the PDGF beta-receptors are likely to play a role in periodontal regeneration to a greater extent as compared to PDGF alpha-receptors. Accordingly, the PDGF-BB may contribute more in promoting periodontal tissue regeneration(Parkar, Kuru, Giouzeli, & Olsen, 2001).

Pre-clinical study utilizing rhPDGF-BB with collagen carrier matrix in periodontal defects of non-human primates showed a significant alveolar bone fill with higher new cementum and periodontal ligament formation as compared to the use of collagen carrier matrix alone(Rutherford et al., 1993). In canine model, treating class III furcation defect with rhPDGF-BB and ePTFE membrane was found to significantly promote new bone formation, compared to the control sites(Park et al., 1995). In another study, natural bone matrix or biphasic calcium phosphate soaked with rhPDGF-BB could induce new bone formation in chronic-type lateral ridge defects in dogs(Schwarz, Ferrari, Podolsky, Mihatovic, & Becker, 2010; Schwarz, Sager, Ferrari, Mihatovic, & Becker, 2009). In multi-centered, human studies, randomized clinical trial comparing

the efficacy of rhPDGF-BB with β-tricalcium phosphate (β-TCP) and β-TCP alone in regenerating human intra-osseous periodontal defects resulted in a significant new bone formation at 6 month post-treatment with rhPDGF-BB(Jayakumar et al., 2011). The positive outcome of rhPDGF-BB is supported by another multi-center, randomized clinical controlled trial, which showed a significantly more attachment gain and bone fill when used rhPDGF-BB with β-TCP compared to β-TCP alone(Nevins et al., 2005).

Although rhPDGF-BB offers a great therapeutic potential, the major drawback of recombinant growth factors is its short half-life. The mean half-life of rhPDGF-BB at the periodontal defect was 4.2 hours. Only 7% of growth factors remained after 48 hours post application(Lynch et al., 1991). The half-life of recombinant growth factors at the periodontal defect is significantly reduced due to the proteolytic breakdown and rapid dilution by gingival crevicular fluid and saliva(Anusaksathien & Giannobile, 2002). Owing to the low bioavailability *in vivo*, recombinant growth factors require the supra-physiologic dose administration to exert its biologic effect, which in turn add up the cost of treatment. In addition, the uncontrolled high dose growth factors within the defect site might lead to serious side effects such as increasing the risk of cancer. Recombinant growth factors could possibly trigger immune response through minor histocompatibility antigens (MHC) pathway, which hampers the repeat administration(Magadum, Kaur, & Zangi, 2019). Hence, alternative approaches to deliver PDGF-BB for periodontal regeneration are in search. Gene therapy approach,

for example, may provide greater bioavailability of desired growth factor within the damage tissue leading to greater tissue regeneration.

mRNA-based therapy

Gene therapy is the delivery of nucleic acids, either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) into patient's cells for therapeutic purpose. It was first conceptualized in 1972 by Friedmann and Roblin(Friedmann & Roblin, 1972), yet not widely accepted until the first approved gene therapy trial was launched in 1990(Blaese et al., 1995). Considering the higher stability of DNA compared to mRNA(Lodish et al., 2000), DNA-based gene therapy has gained more interest and been widely studied in the medical field. Nevertheless, the major challenge of DNA is that nuclear membrane breakdown is required to activate its function. The delivered DNA has to integrate into host genome, thus, may pose the risk of insertional mutagenesis. Moreover, monitoring and controlling their expressions is relatively more difficult(Weissman, 2015). Therefore, mRNA-based therapy has been developed. The successful direct mRNA transfer was first demonstrated in the study by Wolff and coworkers in 1990. It was reported that injection of *in vitro* transcribed messenger RNA (IVT mRNA) into murine muscle resulted in the encoded proteins synthesis, corresponding to their codons(Wolff et al., 1990). Since then, the use of mRNA has emerged as an alternative to DNA in the gene therapy.

mRNA-based therapy is the use of synthesized mRNA encoding a potentially therapeutic protein delivered into targeted tissue. mRNA-based therapy has several advantages over DNA-based gene therapy. First, mRNA-based therapy does not require host genome integration. Second, mRNA is directly uptaken into cytosol for translation, thus facilitating more rapid protein production. Lastly, mRNA provides only transient translation, and therefore, the protein produced is controllable.

In contrast to recombinant proteins, the construction of mRNA is more simple and cheaper. mRNA technology could be applied to produce any protein of interest with a similar platform, while the construction of each type of recombinant proteins requires different process and optimization technique. Another advantage is that mRNA is synthesized without animal or cell component contamination, hence, the risk of stimulating host immune response is lower, as compared to cell therapy or recombinant proteins(Weissman, 2015). Owing to the aforementioned benefits, mRNA has recently emerged as a potential tool for immunotherapy, inhibition of pathogenic mRNA translation, genetic reprogramming and protein replacement therapy.

The use of mRNA encoding growth factor for protein replacement therapy remains challenging in the aspect of its immunogenicity and limited stability(Weissman, 2015). However, these limitations could be overcome by the modification of bases in mRNA sequences. Base modification evidently offers an advantage of diminishing immunogenicity. Previous observations showed that nucleoside modification with

pseudouridine or N1-methypseodouridine effectively reduced innate immune responses and simultaneously enhanced protein expression(Andries et al., 2015; Kariko et al., 2008). The reduction of type I interferon is thought to occur as a result of nucleosides rendering mRNA undetectable by TLRs 3, 7, 8(Andries et al., 2015; Kariko, Buckstein, Ni, & Weissman, 2005). Removal of contaminated double-strand (dsRNA) by high performance liquid chromatography could also reduce immune activation and enhance protein translation(Kariko, Muramatsu, Ludwig, & Weissman, 2011).

In the field of tissue engineering, Zangi *et al.* utilized mRNA-based therapy as a novel alternative to stem cell, recombinant growth factors or DNA-based gene therapy. The study showed that a single dose of modified mRNA encoding vascular endothelial growth factor-A (VEGF-A) could successfully restore the ischemic heart in a mouse model. Mice receiving the mRNA treatment showed an increase in capillary density, reduction of infarcted area and longer survival time, compared to mice receiving plasmid DNA vector or control. The result from this study emphasizes the potential benefit of a synthetic mRNA as a tool for tissue regeneration(Zangi et al., 2013). This mRNA encoding VEGF-A is now under human clinical trials, aiming to develop regeneration therapies for the treatment of cardiometabolic diseases. Nevertheless, mRNA-therapy has never been explored in the field of periodontal regeneration.

Delivery of mRNA therapeutics

Efficient delivery of mRNA to target cells is crucial for its success in protein expression *in vivo*. mRNA is sensitive to degradation by enzyme nuclease in extracellular space and, therefore, must be fabricated to resist the degradation in order to be translated more efficiently. Encapsulation of RNA by lipids(Mintzer et al., 2009), polymers(Pack et al., 2005) and peptides(Martin & Rice, 2007) has been shown to enhance mRNA stability and also promote cellular uptake and endosomal escape.

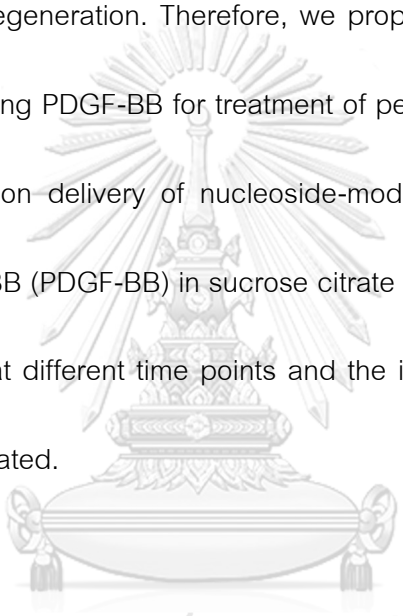
The most studied RNA delivery system is the use of positively charged or cationic lipids, which has consistently shown promising results(Kormann et al., 2011; Midoux & Pichon, 2015; Mintzer et al., 2009; Pardi et al., 2015; Thess et al., 2015; Whitehead, Langer, & Anderson, 2009). Cationic lipids are used to bind the negatively charged RNA, forming lipoplexes, which could facilitate endosomal escape and protect mRNA from nucleases, thus enhancing the efficacy of transfection and translation(Guan & Rosenecker, 2017; Kaczmarek, Kowalski, & Anderson, 2017; Schroeder, Levins, Cortez, Langer, & Anderson, 2010). Due to high efficacy across diverse cell lines and the reproducibility of formulation with cationic lipid liposomes, many commercial products such as Lipofectamine, have been used for mRNA delivery *in vivo*(Kariko, Muramatsu, Keller, & Weissman, 2012; Kormann et al., 2011; Thess et al., 2015; Zhao, Li, Zhang, Gong, & Sun, 2016). In addition to cationic lipids, combining cholesterol and PEG-lipid into lipoplex to form cationic lipid nanoparticles (LNPs) could

also significantly enhance efficacy of RNA delivery(D. Chen et al., 2012). However, their complicated formulating process and high cost limit the widespread application of lipid nanoparticles. Although the cationic lipids offer an efficient vehicle for mRNA delivery, toxicity regarding their use has been reported(Guan & Rosenecker, 2017; Sultana et al., 2017). For instance, injection of cationic lipid liposomes intravenously can cause liver damage(Landesman-Milo & Peer, 2014) and trigger interferon-γ induced inflammatory response in mice(Ma et al., 2005).

Recently, study in small animals reported that mRNA can be expressed in the heart without the need for lipid carriers(Carlsson et al., 2018; Sultana et al., 2017). Sultana and coworkers suggested the use of sucrose-citrate buffer in delivery of modified mRNA into cardiomyocytes in mice model. The result from that study showed that sucrose-citrate buffer yielded highest gene expression of transfected mRNA compared to encapsulated nanoparticles (both *in vivo* JetPEI and *in vivo* fectamine), calcium phosphate and saline. Moreover, citrate-buffer, as a non-lipid-based carrier, does not provoke innate immunity and local pro-inflammatory effects, and therefore, potentially prolong mRNA protein expression(Sultana et al., 2017). The study of Carlsson and coworkers also showed an improvement of cardiac function using citrate-saline buffer as a vehicle for vascular endothelial growth factor – A (VEGF-A) mRNA in swine model of myocardial infarction(Carlsson et al., 2018). Another study by Magadam and coworkers, delivery of modified mRNA encoding the N180Q mutant human

follistatin-like 1 (N180Q hFSTL1) in sucrose citrate buffer could trigger cardiomyocyte proliferation, decrease scar size and improve heart function in mouse myocardial infarction (MI) model(Magadum et al., 2018).

With growing knowledge of mRNA application, this mRNA technology could be a promising alternative approach to deliver growth factors, such as PDGF-BB, to enhance periodontal regeneration. Therefore, we propose to develop pseudouridine-modified mRNA encoding PDGF-BB for treatment of periodontitis. In this study, we will examine the effect upon delivery of nucleoside-modified mRNA encoding platelet-derived growth factor-BB (PDGF-BB) in sucrose citrate buffer into rat gingiva. The level of protein expression at different time points and the inflammation at a site of mRNA delivery will be investigated.



CHAPTER III

MATERIALS AND METHODS

Construction of modified mRNA encoding PDGF-BB

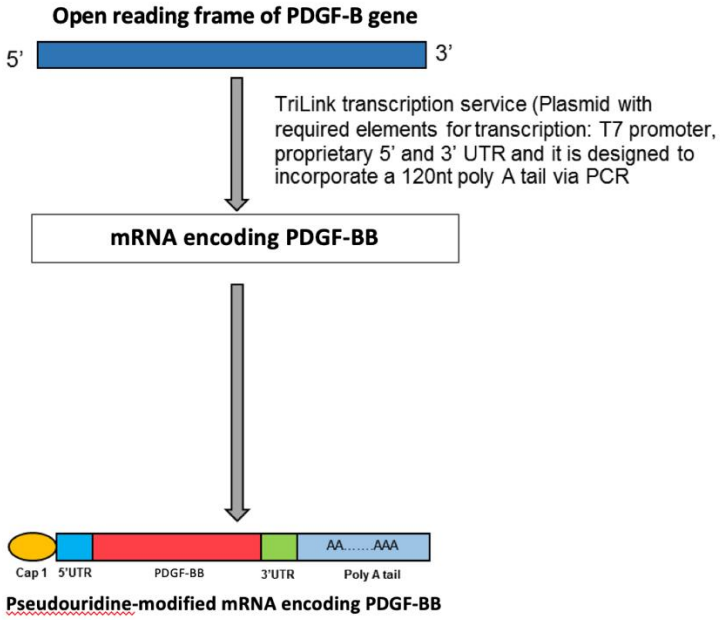


Figure 1 The design of pseudouridine-modified mRNA encoding PDGF-BB flanked by 5' and 3' untranslated regions (UTRs) and a poly A tail with cap1 structure.

Open reading frame of human PDGF-BB gene was provided to Biotechnology company (TriLink Biotechnologies Co., Ltd., San Diego, CA, USA) for mRNA transcription service. A specially designed cDNA with the required elements for transcription: proprietary 5'and 3' UTR, cap1 and its designed to incorporate a 120nt poly-A tail via polymerase chain reaction (PCR) was constructed. Since pseudouridine-modified mRNA has been reported to achieve higher protein expression compared to

unmodified mRNA or other formulation (Kariko et al., 2008). The modified mRNA encoding PDGF-BB was synthesized, utilizing pseudouridine modification (Fig.1).

Animals

All rat experimental protocols in this study were reviewed and approved by Institute of Animal Care and Use Committee of Faculty of Tropical Medicine, Mahidol University (FTM-IACUC) and the Ethics committee of the Faculty of Dentistry, Chulalongkorn University. Wild-type Sprague-Dawley male rats, aged 6-week old were purchased from Nomura Siam International Co., Ltd. (Bangkok, Thailand) and adopted in individually ventilated cages with 12-hour light/dark cycle for a week before beginning of the experiment. Rats were randomly divided into six groups of three mice each (Zangi et al., 2013). One group, served as a control, was injected with sucrose citrate buffer only and then sacrificed 1-day post-injection. The rest were injected with pseudouridine-modified mRNA encoding PDGF-BB in sucrose citrate buffer and then sacrificed at five different timepoints post-injection (day 1, 2, 3, 5 and 7).

Delivery of mRNA encoding PDGF-BB *in vivo* using rat intragingival injection model

Rats were anaesthetized with sodium pentobarbital (50 mg/kg body weight). The 30 μg of pseudouridine-modified mRNA encoding PDGF-BB (Ψ-modified mRNA) was prepared in sucrose citrate buffer. All rats received intragingival injection of the solution of Ψ-modified mRNA encoding PDGF-BB in sucrose citrate buffer at the palatal

gingiva, using needle gauge 31 under the loupe magnification. The injection was performed at 6 sites with the volume of 3 μl solution containing 5 μg Ψ-modified mRNA per site (Fig.2). Thus, the total Ψ-modified mRNA of 30 μg in a total volume of 18 μl solution was given to each rat.

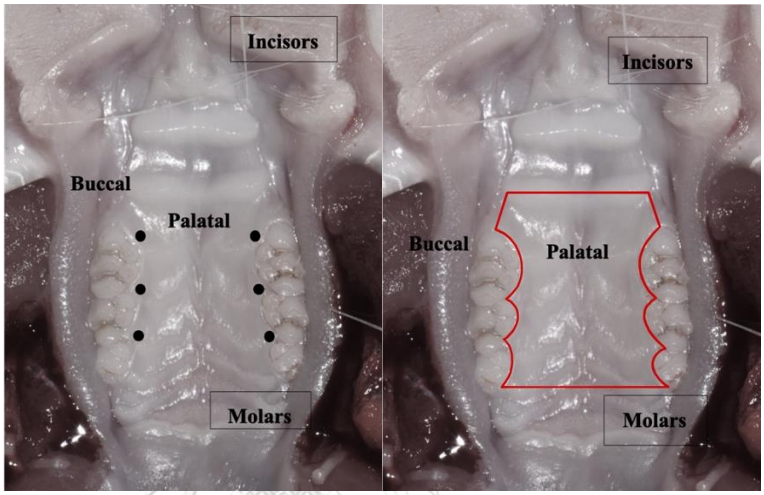


Figure 2 Diagram of rat maxillary teeth: Intragingival injection 6 sites per rat

(A) Black dot (•) indicated site for intragingival injection.

(B) Red line outlines the part of palatal gingiva that was collected.

Preparation of gingival tissue homogenates

Rats were sacrificed at 1, 2, 3, 5 and 7-day post-injection, regarding the experimental groups previously assigned. Palatal gingival tissues were collected as shown in Fig.2. The collected gingival tissues were weighed and homogenized with a micro tissue homogenizer in RIPA Lysis and Extraction Buffer (Pierce™ RIPA Buffer; Thermo scientific, Co., Ltd, Rockford, IL, USA) supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO, USA). The homogenates were centrifugated at 16,000

rpm for 15 minutes at 4°C, and the supernatant was collected for further protein analysis by enzyme-linked immunosorbent assay (ELISA).

Measurement of protein expression *in vivo*

Total protein in tissue homogenates was measured using BCA protein assay kit (Pierce™ BCA Protein Assay; Thermo scientific, Co., Ltd, Rockford, IL, USA). The productions of human PDGF-BB, rat VEGF-A and rat pro-inflammatory cytokines including TNF-α and IL-6 were determined using commercially available ELISA kits (Quantikine® ELISA; R&D Systems, Inc., Minneapolis, MN, USA). ELISAs were performed according to the manufacturer's protocols. The minimal levels of rat TNF-α and IL-6 detected by ELISA kit were 5 and 21 pg/ml, respectively. The results were presented as the amount of interested growth factors or pro-inflammatory cytokines per total protein.

Statistical Analysis

The statistical software SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Normal distribution of data was tested by Shapiro-Wilk normality test. Non-parametric Kruskal-Wallis and Mann-Whitney U test were performed for within group and between group comparisons, respectively. A *P*-value<0.05 was considered statistically significant.

CHAPTER IV

RESULTS

One rat in test group at 7-day time point was lost within 24 hours post-anesthetization and delivery of mRNA. Thus, the total number of rat gingival samples analyzed was 17.

Analysis of PDGF-BB expression

The delivery of Ψ-modified mRNA encoding PDGF-BB in sucrose citrate buffer attained efficient PDGF-BB protein production *in vivo*. Direct single intragingival injection of this modified mRNA yielded a robust PDGF-BB protein expression, 40- to 100-fold increase compared to control. The amount of PDGF-BB production in gingiva peaked at 24-hour post-injection, then rapidly decreased on the second day and returned to baseline at day-3 (Fig.3). The mean concentration of PDGF-BB protein at 24-hour was 6886.32 ± 1213.81 pg/mg protein. At 48-hour, the mean concentration dropped by 98% to 191.34 ± 68.10 pg/mg protein. On the third day, the elevated level of PDGF-BB protein decreased to the level comparable to that of control, which were 68.21 ± 9.50 pg/mg protein and 55.08 ± 22.87 pg/mg protein in test and control, respectively (Supplementary Appendix; Table 1-2). A statistically significant difference was observed between test and control group only at day-1 ($p<0.05$).

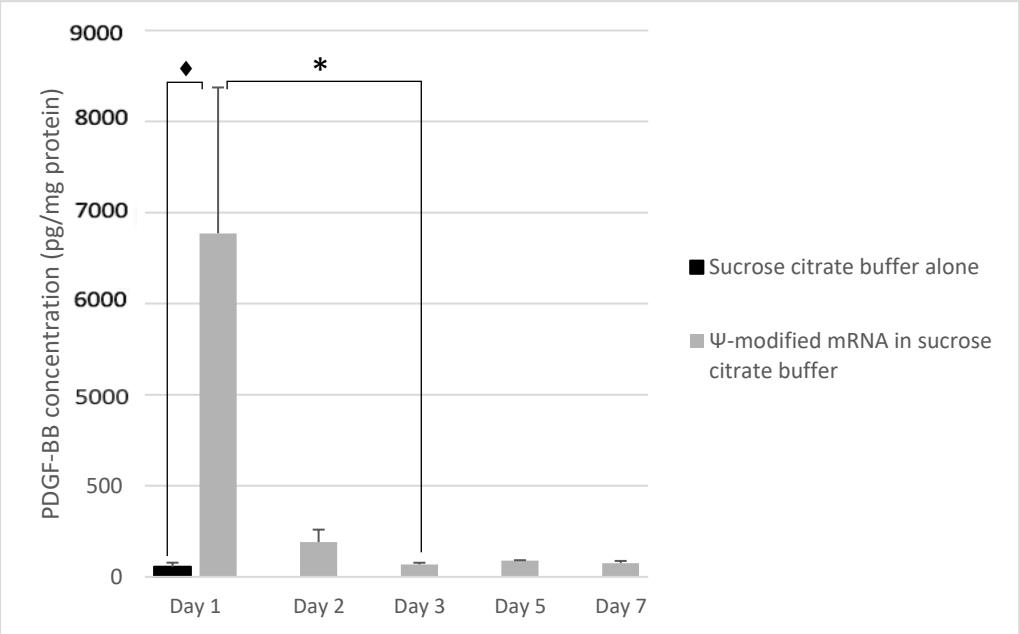


Figure 3 Kinetics of PDGF-BB protein expression in rat gingival tissue after injection with pseudouridine-modified mRNA encoding PDGF-BB

(N = 3 for Day 1, 2, 3 and 5; N = 2 for Day 7, Data are presented as mean ± SD)

* *indicates statistically significance, p<0.05; Kruskal-Wallis with pairwise comparison;*

♦ *indicates statistically significance, p<0.05; Mann-Whitney U test*

Analysis of VEGF-A expression

Previous study demonstrated that PDGF-BB could promote angiogenesis through stimulating vascular endothelial growth factor-A (VEGF-A) production (Affleck et al., 2002). Therefore, the level of VEGF-A protein was assessed. However, the levels of VEGF-A protein in experimental and control group were not statistically different at all time points (Fig.4, Supplementary Appendix; Table 3-4).

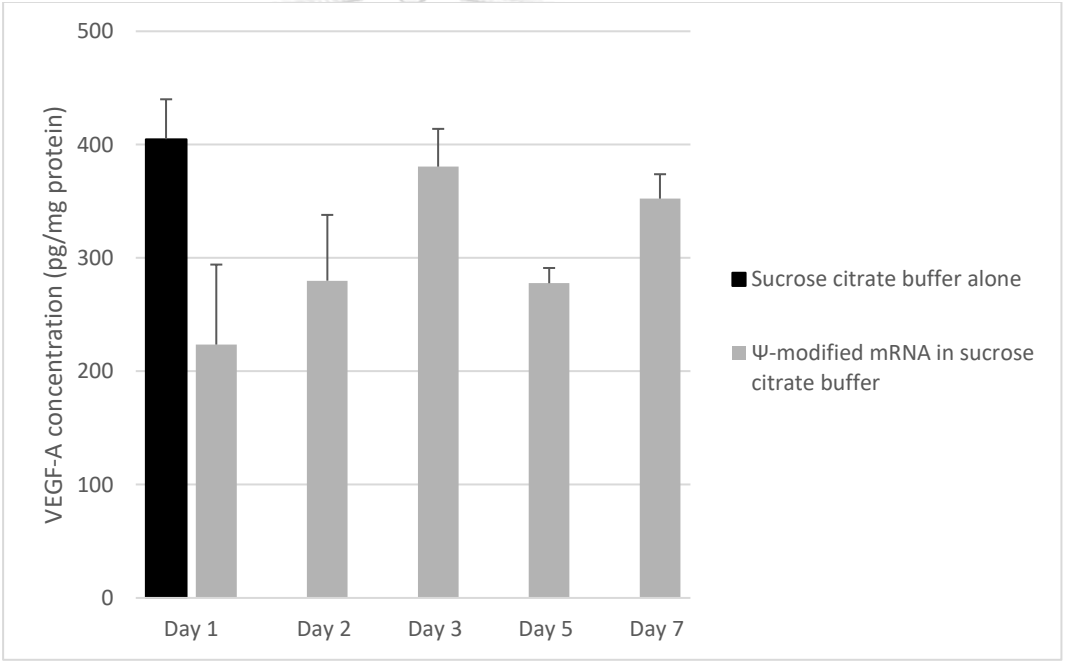


Figure 4 Kinetics of VEGF-A protein expression in rat gingival tissue after injection with pseudouridine-modified mRNA encoding PDGF-BB

(N = 3 for Day 1, 2, 3 and 5; N = 2 for Day 7, Data are presented as mean ± SD)

Pro-inflammatory cytokines stimulation

We evaluated the effects of intragingival injection of Ψ-modified mRNA encoding PDGF-BB in sucrose citrate buffer on the gingival levels of IL-6 and TNF-α. TNF-α and IL-6 were not detected at all time points in Ψ-modified mRNA encoding PDGF-BB in sucrose citrate buffer (Fig.5, Supplementary Appendix; Table 5-8). This suggested that intragingival injection of Ψ-modified mRNA encoding PDGF-BB did not alter gingival levels of both TNF-α and IL-6, since the levels of both proinflammatory cytokines in control group were also below the detection limit of the ELISA.

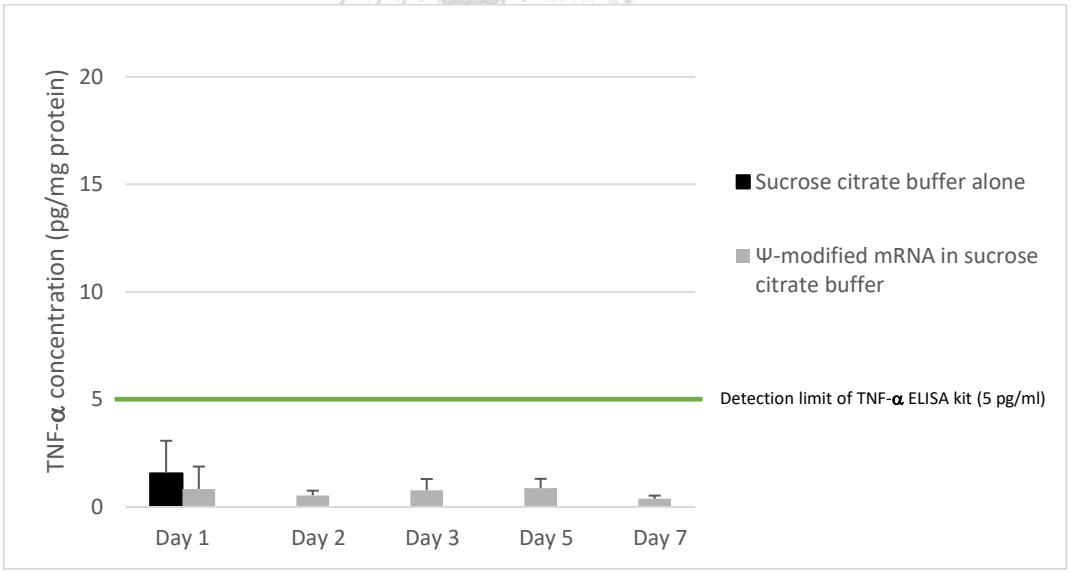


Figure 5 Kinetics of TNF-α protein expression in rat gingival tissue after injection with pseudouridine-modified mRNA encoding PDGF-BB
(N = 3 for Day 1, 2, 3 and 5; N = 2 for Day 7, Data are presented as mean ± SD).

CHAPTER V

DISCUSSION AND CONCLUSION

This study is the first pre-clinical study utilizing mRNA for periodontal regeneration. The keys to delivery of growth factor for therapeutics are to deliver a transient, robust signal at a precise time and target site. Although recombinant PDGF-BB offers a great therapeutic potential, the major drawbacks are the high cost and short half-life. At the periodontal defect, the half-life of recombinant growth factors are significantly reduced due to the proteolytic breakdown and rapid dilution by gingival crevicular fluid and saliva (Anusaksathien & Giannobile, 2002). In contrast to recombinant protein, the pseudouridine-modified mRNA encoding PDGF-BB in this study provided a transient, strong signal directly to the target site, which resulted in a high production of PDGF-BB protein that could retain around the target tissue.

The present study demonstrated that direct intragingival injection of modified mRNA encoding PDGF-BB in sucrose citrate buffer successfully resulted in PDGF-BB protein expression at the injection size. The highest PDGF-BB expression was at 24 hours, which was significantly higher compared to control. The superior protein level was observed until day-3 post-injection. Our findings are in line with the previous studies examining the kinetics of mRNA *in vivo*. Zangi *et al.* showed that cardiac injection of 100 μg modified mRNA encoding Luciferase (Luc) in lipid nanoparticle (RNAiMAX) resulted in an immediate Luc expression that reached high level after 3 hours, peaked at 18

hours and returned to baseline at 144-150 hours (Zangi et al., 2013). Sultana *et al.* studied the kinetic expression of modified mRNA encoding Luc in mice model and found that cardiac injection of the modified mRNA in sucrose citrate buffer yielded an increased Luc expression, significantly above baseline within 10 minutes. The highest expression was at 24 hours and returned to the basal level at 96 hours (Sultana et al., 2017). Carlsson *et al.* revealed that injection of 15 μg modified mRNA encoding Luc in sucrose citrate saline into cardiac muscle could provide rapid Luc expression within 30 minutes. The expression peaked at 24 hours, persisted throughout 4 days and became negative by day-7 post-injection. However, intradermal injection of this mRNA could prolong the skin expression to more than 216 hours. In skeletal muscle, protein expression lasted more than 30 days (Carlsson et al., 2018). Thus, delivery of mRNA into different tissues might need further optimization in order to obtain the most effective protein production. Nevertheless, the transient controlled expression may be more important, as prolonged exposure of PDGF could lead to adverse effect on osteoblastic cell collagen production, differentiation and bone formation (Yu, Hsieh, Bao, & Graves, 1997).

To minimize the number of animals used in the study, control group was conducted only at one time point. The amount of PDGF-BB in group receiving sucrose citrate buffer alone at day-1 was used as a control or baseline level of PDGF-BB in rat gingiva to eliminate the bias from cross-reactivity of human PDGF-BB ELISA kit with rat

PDGF-BB. Single intragingival injection of pseudouridine-modified mRNA encoding PDGF-BB in sucrose citrate buffer significantly increased the level of PDGF-BB protein production from baseline. This difference indicated the newly synthesized protein in gingival tissues after the mRNA administration.

Within the limitation of this study, the cell sources of PDGF-BB as a result of the delivered mRNA were not identified. Nevertheless, we hypothesized that secreted PDGF-BB could stimulate periodontal tissue healing and regeneration of teeth adjacent to the site of mRNA administration. PDGF-BB is a potent growth factor for periodontal regeneration since its chemotactic and mitogenic effects on periodontal ligament and bone precursor cells have been clearly demonstrated(Ojima, Mizuno, Kuboki, & Komori, 2003; Ozaki et al., 2007). PDGF-BB is synthesized primarily by platelets, also fibroblasts, endothelial cells and macrophages, and has a high affinity to β-receptors which abundantly express on a surface of cells resided in periodontal tissue and at the periodontal defect. In addition to fibroblasts and perivascular smooth muscle cells, β-receptors can be found on endothelial cells, platelets, T-cell and macrophages(Heldin & Westermark, 1999). Further study regarding the cellular uptake of delivered mRNA and secretion of PDGF-BB protein could provide additional insights for the future application of mRNA in periodontal regeneration.

Although VEGF production could be induced by PDGF, the patterns of expression between these two proteins were different. VEGF level peaks at a later timepoint after PDGF delivery. In periodontal lesion after stimulating with recombinant PDGF-BB, the level of VEGF was found to immediately increase, but peaked at day- 12 to 15 (Cooke et al., 2006). In the present study, the levels of VEGF-A protein were assessed until day-7 after PDGF-BB delivery. Thus, the significant increase of VEGF-A expression might not be observed.

The result in this study demonstrated low toxicity and immunogenicity of pseudouridine-modified mRNA encoding PDGF-BB in sucrose citrate buffer with intragingival injection. Although the gingival levels of IL-6 and TNF-α were below the detection limit of the ELISA, the results were similar to the basal levels of both proinflammatory cytokines. The larger amount of protein sample could possibly increase the sensitivity of ELISA. The gingival tissues harvested from rat were, however, limited. Other assays, such as a PCR test, might be performed in further study to examine the difference in proinflammatory cytokines gene expression. Nevertheless, this preliminary data suggested that mRNA formulation used in this study did not provoke the local immune response. The results were in accordance with previous data showing that pseudouridine-modified mRNA could limit TLRs recognition due to the changes in its secondary structures (Kariko et al., 2008). Moreover, using sucrose citrate buffer as a delivery vehicle may benefit in reducing immunogenicity. Sucrose increases the

viscosity of modified mRNA solution, thus prevents the accumulation of single-stranded modified mRNAs in the mixture. This accumulation might inhibit double-stranded modified mRNA translation, which could result in an immune activation via TLR-3 (Alexopoulou, Holt, Medzhitov, & Flavell, 2001). Together, these data support that modified mRNA in sucrose citrate buffer could be an effective approach for a transient localized mRNA delivery to gingival tissue *in vivo*. This preliminary study provides a set of data encouraging the potential of mRNA encoding PDGF-BB as therapeutic approaches in the field of tissue regeneration.

In conclusion, the present study provides *in vivo* evidence that intragingival injection of pseudouridine-modified mRNA encoding PDGF-BB in sucrose citrate buffer transiently yields rapid and robust PDGF-BB protein production and does not stimulate local tissue inflammation. These preliminary results suggest the potential of mRNA-based therapy application in periodontal regeneration. However, safety, efficacy and effectiveness of the mRNA should be evaluated, and larger sample size should be employed in further study.

APPENDIX

Table 1 Kinetics of PDGF-BB protein expression

	Day 1	Day 2	Day 3	Day 5	Day 7
Test 1	8270.36	253.24	67.95	87.27	84.12
Test 2	6385.77	202.38	77.84	91.78	67.61
Test 3	6002.83	118.39	58.84	89.20	
Mean ± SD	6886.32 ± 1213.81*	191.34 ± 68.10	68.21 ± 9.50*	89.2 ± 2.32	75.87 ± 11.67

PDGF-BB concentration in pg/mg protein

*p<0.05; Kruskal-Wallis with pairwise comparison

Table 2 PDGF-BB concentration 1-day post-injection

	Control	Test
	80.49	8270.36
	48.60	6385.77
	36.14	6002.83
Mean ± SD	55.08 ± 22.87*	6886.32 ± 1213.81*

PDGF-BB concentration in pg/mg protein

*p<0.05; Mann-Whitney U Test

Table 3 Kinetics of VEGF-A protein expression

	Day 1	Day 2	Day 3	Day 5	Day 7
Test 1	270.06	338.32	344.95	289.16	367.56
Test 2	142.35	222.22	410.97	262.90	337.06
Test 3	258.27	279.24	385.78	281.04	
Mean ± SD	223.56 ± 70.58	279.93 ± 58.05	380.57 ± 33.32	277.70 ± 13.44	352.31 ± 21.57

VEGF-A concentration in pg/mg protein

p>0.05; Kruskal-Wallis with pairwise comparison

Table 4 VEGF-A concentration 1-day post-injection

	Control	Test
	442.70	270.06
	398.58	142.35
	373.06	258.27
Mean ± SD	404.78 ± 35.23	223.56 ± 70.58

VEGF-A concentration in pg/mg protein

p>0.05; Mann-Whitney U Test

Table 5 Kinetics of TNF-α protein expression

	Day 1	Day 2	Day 3	Day 5	Day 7
Test 1	2.04	0.69	0.19	1.30	0.27
Test 2	0.35	0.29	1.13	0.90	0.48
Test 3	0.13	0.64	1.03	0.44	
Mean ± SD	0.84 ± 1.04	0.54 ± 0.22	0.78 ± 0.52	0.88 ± 0.43	0.38 ± 0.15

TNF-α concentration in pg/mg protein

p>0.05; Kruskal-Wallis with pairwise comparison

Table 6 TNF-α concentration 1-day post-injection

	Control	Test
	3.31	2.04
	0.80	0.35
	0.58	0.13
Mean ± SD	1.56 ± 1.52	0.84 ± 1.04

TNF-α concentration in pg/mg protein

p>0.05; Mann-Whitney U Test

Table 7 Kinetics of IL-6 protein expression

	Day 1	Day 2	Day 3	Day 5	Day 7
Test 1	<0.010	<0.010	<0.010	<0.010	<0.010
Test 2	<0.010	<0.010	<0.010	<0.010	<0.010
Test 3	<0.010	<0.010	<0.010	<0.010	
Mean ± SD	-	-	-	-	-

IL-6 concentration (pg/mg protein)

p>0.05; Kruskal-Wallis with pairwise comparison

Table 8 IL-6 concentration 1-day post-injection

	Control	Test
	<0.010	<0.010
	9.62	<0.010
	<0.010	<0.010
Mean ± SD	-	-

IL-6 concentration (pg/mg protein)

p>0.05; Mann-Whitney U Test

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