PROTEIN EXPRESSION AFTER GINGIVAL INJECTION OF MESSENGER RNA ENCODING PLATELET-DERIVED GROWTH FACTOR-BB IN LIGATURE-INDUCED RAT PERIODONTITIS

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การแสดงออกของโปรตีนภายหลังการฉีดเมสเซนเจอร์อาร์เอ็นเอที่เข้ารหัสเพลทเลทดีไรฟ์ โกรทแฟกเตอร์-บีบี ในเหงือกของหนูแรทที่เป็นโรคปริทันต์อักเสบจากการเหนี่ยวนำด้วยเส้นไหม

น.ส.พิมพ์พร มีขันทอง

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พิมพ์พร มีขันทอง : การแสดงออกของโปรตีนภายหลังการฉีดเมสเซนเจอร์อาร์เอ็นเอที่เข้ารหัสเพลทเลทดีไรฟ์ โกรทแฟกเตอร์-บีบี ในเหงือกของหนูแรทที่เป็นโรคปริทันต์อักเสบจากการเหนี่ยวนำด้วยเส้นไหม. (PROTEIN EXPRESSION AFTER GINGIVAL INJECTION OF MESSENGER RNA ENCODING PLATELET-DERIVED GROWTH FACTOR-BB IN LIGATURE-INDUCED RAT PERIODONTITIS) อ.ที่ปรึกษาหลัก : ศ. ทพญ. ดร.รังสินี มหานนท์, อ.ที่ปรึกษาร่วม : อ. ทพญ. ดร.วิชญา วิศิษฐ์รัศมีวงศ์

ปัจจุบัน นวัตกรรมเอ็มอาร์เอ็นเอ จัดเป็นรูปแบบยาชนิดใหม่ที่มีความปลอดภัยและมีประสิทธิภาพ วัตถุประสงค์ของงานวิจัยนี้เพื่อศึกษาผลจากการอักเสบต่อการแสดงออกของโปรตีนและความสัมพันธ์ของระดับโปรตีนที่ผลิตออกมากับความเข้มข้นของเอ็มอาร์เอ็นเอ ภายหลังฉีดเอ็มอาร์เอ็นเอที่เข้ารหัสเพลตแลทดีไรฟ์โกรทแฟคเตอร์-บีบีในลิปิดนาโนพาร์ทิเคิล ที่เหงือกหนูแรทที่เป็นโรคปริทันต์อักเสบจากการเหนี่ยวนำด้วยเส้นไหม โดยผูกไหม 3-0 ล้อมรอบฟันกรามบนซ้ายซี่ที่สองเป็นเวลา 2 สัปดาห์แล้วนำไหมออก ส่วนฟันกรามบนขวาซี่ที่สองไม่ได้ผูกไหม เมื่อดูภายใต้กล้องสเตอริโอไมโครสโคป พบการละลายของกระดูกในด้านที่ผูกไหมเมื่อเทียบกับด้านที่ไม่ผูกไหม ซึ่งแสดงให้เห็นว่าการผูกไหมเหนี่ยวนำให้เกิดโรคปริทันต์อักเสบ อย่างไรก็ตาม ไม่พบความแตกต่างของระดับสารสื่ออักเสบ (อินเตอร์ลิวคิน-1เบต้า ทูเมอร์เนคโครซิสแฟคเตอร์-อัลฟา และอินเตอร์ลิวคิน-17เอ) และปริมาณเซลล์อักเสบระหว่างด้านที่ผูกไหมและไม่ผูกไหม เป็นไปได้ว่ากระบวนการอักเสบนั้นเกิดก่อนช่วงเวลาที่เก็บตัวอย่างหลังจากนำไหมออก ทำการฉีดด้วยเอ็มอาร์เอ็นเอที่เข้ารหัสเพลตเลทดีไรฟ์โกรทแฟคเตอร์-บีบีในลิปิดนาโนพาร์ทิเคิลด้วยความเข้มข้นที่ต่างกัน (3 ไมโครกรัม 10 ไมโครกรัม และ 30 ไมโครกรัม) สารละลายฟอสเฟตดุลเบกโก และลิปิดนาโนพาร์ทิเคิล ที่เหงือกด้านเพดานทั้งด้านซ้ายและขวา หลังจากฉีดเอ็มอาร์เอ็นเอไปแล้ว 24 ชั่วโมง พบระดับโปรตีนเพลตเลทดีไรฟ์โกรทแฟคเตอร์-บีบีสูงทั้งด้านที่ผูกไหมและไม่ผูกไหม รวมถึงโปรตีนที่แสดงออกนั้นมีแนวโน้มสูงตามระดับความเข้มข้นของเอ็มอาร์เอ็นเอที่ฉีด นอกจากนี้ยังพบการแสดงออกของสารสื่ออักเสบ ได้แก่ อินเตอร์ลิวคิน-1เบต้า และอินเตอร์ลิวคิน-17เอ จึงสรุปได้ว่าการนำส่งเอ็มอาร์เอ็นเอที่เข้ารหัสเพลตเลทดีไรฟ์โกรทแฟคเตอร์-บีบีในลิปิดนาโนพาร์ทิเคิลในเหงือกหนูแรทที่เป็นโรคปริทันต์อักเสบจากการเหนี่ยวนำด้วยเส้นไหมไปแล้ว 24 ชั่วโมง สามารถพบระดับโปรตีนเพลตเลทดีไรฟ์โกรทแฟคเตอร์-บีบีสูง ถึงแม้ว่าจะไม่พบลักษณะอักเสบเฉพาะที่ในโมเดลนี้ โมเดลนี้ยังสามารถใช้ศึกษาผลทางการรักษาของเอ็มอาร์เอต่อไป

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KEYWORD: ligature-induced periodontitis, LNPs, mRNA, PDGF-BB, periodontitis

Pimphorn Meekhantong : PROTEIN EXPRESSION AFTER GINGIVAL INJECTION OF MESSENGER RNA ENCODING PLATELET-DERIVED GROWTH FACTOR-BB IN LIGATURE-INDUCED RAT PERIODONTITIS. Advisor: Prof. RANGSINI MAHANONDA, D.D.S., M.D.Sc., Ph.D. Co-advisor: WICHAYA WISITRASAMEEWONG, D.D.S., M.Sc., D.M.Sc.

As a new class of medicines, mRNA platform has proven to be safe and effective. The objective of this study was to investigate the effect of local inflammation on platelet derived growth factor-BB (PDGF-BB) expression after gingival injection of PDGF-BB mRNA in lipid nanoparticles (LNPs) in ligature-induced rat periodontitis and to examine the dose-dependent effect of gingival delivery of mRNA. 3-0 silk was placed around maxillary left second molar for two weeks and removed, while the corresponding molar on the right was non-ligated. Under stereomicroscope, a significant bone loss at ligature sites was observed as compared to the non-ligature sites, indicating established periodontitis. However, there were no differences in inflammatory cytokine levels (IL-1β, TNF-α and IL-17a) and cellular infiltrates between the two sites. The local inflammation may occur at earlier time point in this ligature model. Different doses of PDGF-BB mRNA (low (3 µg), medium (10 µg), high (30 µg)), DPBS (control) and LNPs alone were injected into palatal gingiva upon ligature removal. After 24 hour of mRNA delivery, we found high levels of translated PDGF-BB protein at both ligature and non-ligature sites, demonstrating a trend of dose response. In addition, some amount of inflammatory cytokine including IL-1β, and IL-17a was detected. In conclusion, this study of 24 hour-local delivery of PDGF mRNA-LNPs into gingiva using ligature-induced rat periodontitis model results in high translated PDGF protein. Even though, local gingival inflammation was not detected in this established periodontitis model, this model may be useful for future testing of mRNA therapeutic effects.

Field of Study: Periodontics Student's Signature .....

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CHAPTER I

INTRODUCTION

Background and Rationale

For the past few decades, intensive research based on tissue engineering strategies for periodontal tissue regeneration has been the use of stem cells, scaffolds, and signaling molecules. Many growth factors such as platelet-derived growth factors-BB (PDGF-BB), insulin-like growth factors (IGF), fibroblast growth factors (FGF), bone-morphogenetic protein (BMP) have shown potential to regenerate the lost periodontal tissues (Bartold et al., 2000). Recombinant human PDGF-BB (rhPDGF-BB), GEM 21S® (Osteohealth, USA), has become the first growth factor product, which was clinically approved by U.S. FDA in 2005 for periodontal regeneration. However, one of its limitations in clinical application is the relatively short half-life of growth factors *in vivo*, which typically ranging from several hours to days. As a result, supraphysiologic doses or several administrations are required, and such high doses of growth factors may cause undesirable side effects and increase the cost of therapy (Carragee et al., 2011). Gene therapy is considered as an alternative approach to address the drawbacks of protein delivery. The gene therapy involves a delivery of DNA or mRNA encoding protein of interest into the cells, thus allowing cell transfection and protein translation to occur. Hence, your own body makes the desired protein by himself (Magadum et al., 2019).

In 2021, mRNA-based technology has been emerged in the field of medicines by the first mRNA vaccine against SARS-CoV-2, which was approved by the U.S. FDA (Baden et al., 2021) . This mRNA vaccine uses nucleoside-modified mRNA encoding a spike protein antigen, which is encapsulated with lipid nanoparticles (LNPs) . It is known that nucleoside modified mRNA translated more protein than unmodified mRNA by suppressing inflammatory response, which can interfere with protein translation (Karikó et al., 2008). Encapsulation of mRNA with LNPs can protect mRNA from degradation by extracellular ribonucleases mRNA and make mRNA more stable (Hou et al., 2021). Hence, mRNA-LNPs technology has been recognized as the most advanced platform in medicine. Now more than a billion doses of mRNA vaccine have been used around the world. Thus, the mRNA vaccine is both safe and effective (Pardi et al., 2018). Likewise, the same mRNA technology platform could be applied for mRNA-based therapeutics.

In the field of mRNA-based regenerative medicine, Zangi et al., (2013) showed that the use of modified mRNA encoding human vascular endothelial growth factor-A (VEGF-A mRNA) in a mouse myocardial infarction model could enhance the formation of new blood vessels in infarction area and the survival rate increased as compared to controls (no mRNA administration). The phase 2 study showed safety and improved heart function after direct injections of VEGF-A mRNA into the hearts of 11 patients with coronary diseases during open heart surgery, suggesting a promising clinical outcome of VEGF-A mRNA treatment for heart tissue regeneration

(Anttila et al., 2020). Currently, the phase 3 clinical study of VEGF-A mRNA in a larger number of heart failure patients are ongoing (Collén et al., 2022).

Our research group has studied the potential use of mRNA encoding growth factors for periodontal regeneration. We demonstrated high protein expression after 24 hour-transfection *in vitro* of primary human periodontal ligament cells and primary human gingival fibroblasts with modified mRNA encoding PDGF-BB (Surisaeng et al., 2020). Furthermore, we demonstrated *in vivo* that direct injection of modified PDGF-BB mRNA in healthy rat gingiva induced high protein translation (Bhongsatiern et al., 2020). Therefore, the present study investigated protein translation following gingival injection of PDGF-BB mRNA in rat periodontitis. A ligature-induced periodontitis in rats was selected as a study model since it mimics the pathogenesis of periodontitis in human caused by dental plaque accumulation around the ligature (Graves et al., 2008). Different mRNA doses were also tested. Findings from this study will provide information regarding the use of ligature-induced periodontitis in rats for further investigation of PDGF-BB mRNA therapeutic efficacy for periodontal regeneration.

Objectives

1. To investigate the effect of local inflammation on PDGF-BB protein expression after gingival injection of N1-methylpseudouridine-modified mRNA encoding PDGF-BB in LNPs in ligature-induced periodontitis model in rat

2. To examine the dose-dependent effect of gingival injection of N1-methylpseudouridine-modified mRNA encoding PDGF-BB in LNPs on PDGF-BB protein expression in inflamed gingiva

Hypothesis

The inflammation in gingiva has negative effect on PDGF-BB protein expression after gingival injection of mRNA encoding PDGF-BB in LNPs.

Field of research

in vivo study of mRNA encoding PDGF-BB in LNPs

Limitation of research

This study is an *in vivo* study in periodontitis model, thus increasing sample size and therapeutic efficacy of mRNA should be employed in further study.

Application and expectation of research

The result from this study provides data which will help to study the potential and therapeutic effects of PDGF-BB mRNA for periodontal regeneration.

Keywords

ligature-induced periodontitis, LNPs, mRNA, PDGF-BB, periodontitis

CHAPTER II

REVIEW LITERATURE

Periodontal treatment

The ultimate goal of periodontal treatment is to remove inflammation-mediated plaque bacteria and to regenerate the lost periodontal tissues and restore periodontal health (Ivanovski, 2009). Nevertheless, non-surgical therapy and the majority of surgical periodontal procedures usually result in long junctional epithelium or connective tissue attachment which often leads to a reduced periodontium with compromised function and esthetics. Current treatment for periodontitis is to remove plaque bacteria by scaling, root planing and periodontal surgery. While these treatments can result in elimination of periodontal inflammation, however regeneration of lost periodontal tissues does not occur, especially periodontal ligament, the cementum and the alveolar bone (Caffesse & Echeverría, 2019). Regenerative surgical procedures, such as guided tissue regeneration (GTR) or bone grafting, are the first treatment of choice whenever possible. These regenerative therapies have the potential to restore the damaged periodontium and demonstrate promising results in sites with intrabony and furcation defects (Murphy & Gunsolley, 2003). Nevertheless, their outcomes are not predictable and their efficacy are still in questioned due to several limitations. Several studies suggested that the complete regeneration is limited (Avila-Ortiz et al.,

2015; Kao et al., 2005); therefore, the new therapeutic approaches utilizing the concept of "tissue engineering" was introduced in the field of periodontal tissue regeneration (Bartold et al., 2000). These regenerative approaches include the use of stem cells, growth factors, scaffolds and gene therapy (non-viral and viral vector) (Cochran et al., 2015).

Growth factors

Growth factors are natural proteins that regulate the cellular events involved tissue repair and regeneration. Intracellular signaling pathways are induced after the growth factors bind to specific cell membrane receptors of the target cells. This results in the activation of genes that could alter cellular activity and phenotype. Experimental studies have shown that growth factors have the potential to enhance tissue regeneration by a series of events including cell chemo-attraction, differentiation and proliferation (Kaigler et al., 2006). Many growth factors including PDGF-BB, IGF, FGF and BMP have shown potential to regenerate the lost periodontal tissues in periodontitis patients (Liang et al., 2020).

Platelet-derived growth factor (PDGF)

PDGF is a polypeptide growth factor that secreted by activated platelet, activated macrophage, fibroblasts, endothelial cells (Dereka et al., 2006). PDGFs are consist of two polypeptide chains and classified in five isoforms such as PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD. PDGFs bind to PDGF receptor α and β

to stimulate chemotaxis and proliferation of periodontal ligament fibroblasts, cementoblasts and osteoblasts (Fredriksson et al., 2004; Kaigler et al., 2011). Among these PDGF isoforms, PDGF-BB is the most dominant isoform which stimulates mitogenesis and chemotactic effect of periodontal ligament cells (Boyan et al., 1994).

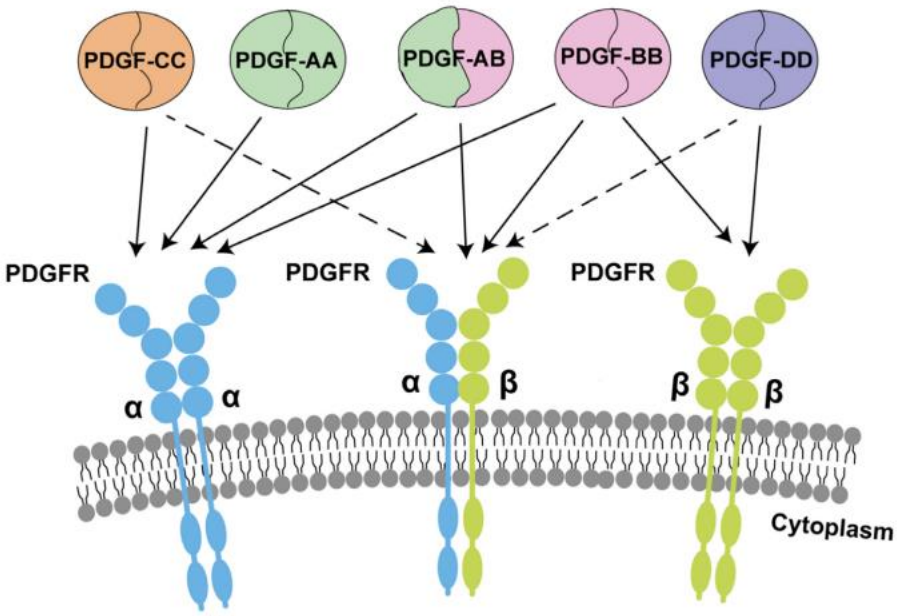

Figure 1. The difference isoforms of PDGF and different PDGF receptor at transmembrane (Evrova & Buschmann, 2017)

PDGF has been one of the most thoroughly studied growth factors in periodontics. Lynch and coworkers (1989) first demonstrated that the use of purified human PDGF combined with recombinant IGF-I revealed markedly new bone and cementum formation in beagle dogs with natural occurring periodontitis. In the initial phase I/ II clinical study, the application of combined recombinant human (rh) PDGF-BB and rhIGF-1 in a methylcellulose gel in moderate to severe periodontitis patients resulted in a significant bone fill compared to conventional surgery

(Howell et al., 1997). When a single growth factor rhPDGF-BB was applied with osteoconductive scaffold (demineralized freeze dried-bone allografts) which prevent soft tissue collapse, it was found a significant improvement in probing depth reduction, clinical attachment gain and bone fill in class II furcation defects and intrabony defects (Nevins et al., 2003). The first growth factor product approved by US FDA (2005) for periodontal regeneration is GEM 21S® (Osteohealth, USA), consisting of rhPDGF-BB combined with beta-tricalcium phosphate (β-TCP). It is recommended for treatment of intrabony defects, furcation defects and gingival recession.

In spite of their attractive properties for tissue regeneration, several studies showed inconclusive clinical efficacy of recombinant growth factor application in periodontal regeneration (Darby & Morris, 2013). Half-life of growth factors *in vivo* is relatively short, usually ranging from several hours to days, 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 (Carragee et al., 2011). It should be pointed out that the production of recombinant growth factors is relatively complicated, required a complex production and purification system and may encounter abnormal posttranslational modification which could affect bioactivity and stability of the proteins (Palomares et al., 2004).

Gene therapy

The gene therapy is an alternative approach to improve tissue regeneration. By transplanting the nucleic acids, either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) encoding growth factors into the cells and the cells act like a growth factor factory. The gene therapy could provide a more sustained biological effects than the topical protein application. Various gene delivery methods are available through the use of non-viral and viral vectors. The non-viral vectors include naked plasmid DNA (pDNA), liposome-DNA complexes and polymer-DNA complexes (Yin et al., 2014). Viral vectors used for gene transfer include retrovirus, lentivirus, adenovirus, adeno-associated virus and herpes simplex virus (Elangovan & Karimbux, 2010).

Giannobile and co-workers (2001) demonstrated the first use of growth factor gene delivery to cementoblasts. Recombinant adenovirus encoding PDGF-A (Ad-PDGF-A) was more effective in stimulating proliferation of cementoblasts than rhPDGF-AA. Not only Ad-PDGF-A, but also Ad-PDGF-B showed a potential use in tissue regeneration. It was found that the delivery of Ad-PDGF-B significantly stimulated proliferative response of human gingival fibroblasts when compared to that of Ad-PDGF-A (Anusaksathien et al., 2003). This same research group demonstrated a direct *in vivo* Ad-PDGF-B gene transfer with collagen matrix successfully enhanced alveolar bone formation and cementogenesis in rat alveolar bone defects (Chang et al., 2009; Jin et al., 2004).

Besides viral vector, plasmid DNA gene delivery was used particularly in bone regenerative area. Huang et al (2005) investigated bone regeneration in a rat cranial defect using pDNA encoding BMP-4 complexed with polyethylenimine (PEI) encapsulated in poly (lactic-co-glycolic acid) (PLGA) scaffolds. They found significant bone formation was observed compared to scaffold alone (Huang et al., 2005). In contrast, PEI-pDNA encoding PDGF-B polyplexes in collagen scaffold delayed bone regeneration and induced inflammatory cell infiltration in the lateral to the defect area (Plonka et al., 2017).

The major challenge of gene therapy using viral vector and pDNA for clinical application in particular for the treatment of periodontitis, a non-life-threatening diseases is a safety issue. For encoding protein expression, DNA has to integrate into host genome, thus, poses the risk of mutagenesis. Unlike DNA, mRNA does not enter nucleus, hence there is no risk of mutagenesis. In addition, protein expression of delivered mRNA is transient and naturally degraded which normally lasts for days (Pardi et al., 2015). Therefore, the translated protein could achieve its desired biological function. Altogether, mRNA demonstrates a good safety profile. Unlike viral vector therapy, the use of mRNA is not restricted by the preexisting anti-vector immunity. Regarding manufacturing, mRNA is scalable and cost-effective (Sahin et al., 2014).

mRNA-technology platform

mRNA is now a new and attractive technology in medicine. COVID-19 mRNA vaccine is the first product using mRNA technology platform, which was approved by FDA to prevent pandemic COVID-19 in 2020 (Baden et al., 2021). Application of mRNA in gene therapy was first described in 1990 when intramuscular injection of mRNA in mice resulted in local production of an encoded reporter protein (Wolff et al., 1990). However, given the fragility of mRNA, it was abandoned for many years while researchers were in favor of the more stable DNA. In recent years, the limitations of mRNA have been addressed by; 1) complexation with cationic liposomes or polymers, thereby preventing RNase degradation and improving mRNA delivery into target cells , 2) translation efficiency of mRNA has been greatly improved by engineering of sequences (5'cap, 5' and 3' UTR and the length of the poly (A)) that have been known to stabilize mRNA, and of importance the use of chemical modifications of nucleosides (Sahin et al., 2014).

Immune activation of mRNA is suppressed by nucleoside-modified mRNA

mRNA can activate innate immune responses leading to undesired inflammation, therefore limiting the use of mRNA as therapeutic agent. *In vitro* synthesized mRNAs are recognized via innate sensing receptors such as toll-like receptor (TLR)3, TLR7/8 and RIG-I like receptors, MDA5, and NOD2. The binding of *in vitro* synthesized mRNA to their receptors results in innate immune activation and

release of inflammatory cytokine including interferon α which could inhibit protein translation (Pollard et al., 2013). Several approaches are being used to overcome these problems and one of them is the use of mRNA containing modified nucleosides (Karikó et al., 2005; Karikó et al., 2008). Recent observations showed that nucleoside modification with pseudouridine or N1-methypseodouridine reduced innate immune responses and simultaneously enhanced protein expression (Andries et al., 2015; Karikó et al., 2008). At present, modification with N1-methylpseudouridine was demonstrated to be best to induce high protein expression and suppress innate inflammatory reaction (Andries et al., 2015). Interestingly, one of the crucial components of mRNA COVID-19 vaccine invented by Moderna and Pfizer/BioNtech is N1-methylpseudouridine-modified mRNA encoding SARS-CoV-2 spike protein. This mRNA COVID-19 vaccine is the first mRNA product, approved by US FDA for emergency use to prevent pandemic COVID-19 in 2020 (Baden et al., 2021).

mRNA delivery system

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 it must be made more resistant against degradation and more efficient for translation. Encapsulation of mRNA by lipids (Mintzer & Simanek, 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 advanced studied RNA delivery system is the use of lipid nanoparticles (LNPs) which has consistently shown promising results and used in construction of mRNA COVID-19 vaccine (Baden et al., 2021). The structure of LNPs is consist of phospholipids, cholesterol, ionizable cationic lipids and PEGylated lipids for support, stabilization, complexation of negatively charged mRNA molecules, facilitating endosomal escape, and reducing nonspecific endocytosis by host immune cells, respectively (Kauffman et al., 2015; Kulkarni et al., 2018). LNPs are potential mRNA delivery system due to protect mRNA degradation, enhance protein translation, decrease immune response and ease of design and manufacture (Kulkarni et al., 2018)

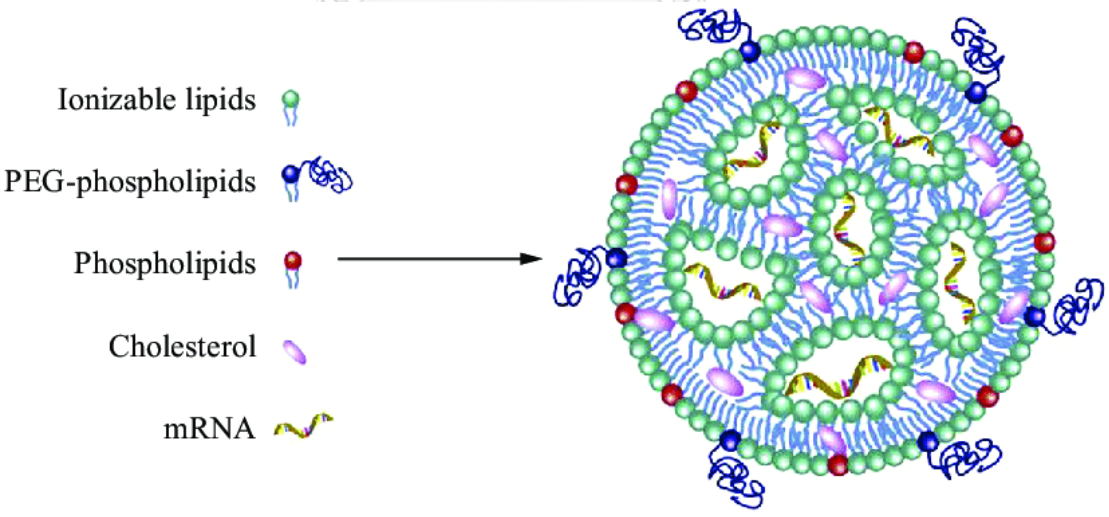

Figure 2. Schematic structure of mRNA-lipid nanoparticle complex (Forchette et al., 2021)

Nucleoside-modified mRNA as a novel technology in tissue regeneration

Recently, the use of modified mRNA as a therapeutic agent has received a significant attention as an alternative technology to stem cell, growth factor and DNA/viral vector therapies in regenerative medicine. While the mRNA technology platform has not yet been studied in the field of periodontal tissue regeneration, mRNA therapeutics encoding growth factors have been evaluated and demonstrated promising findings in various models of soft and hard tissue regeneration such as myocardial infarction (Zangi et al., 2013), diabetic wound healing (Sun et al., 2018), and calvarial and femoral bone defects (Elangovan et al., 2014).

The most advanced research in regenerative medicine is the potential application of human vascular endothelial growth factor-A (VEGF-A) mRNA for heart tissue regeneration. Zangi et al. (2013) showed that the use of modified VEGF-A mRNA in a mouse myocardial infarction model could enhance the formation of new blood vessels in infarction area and the survival rate increased as compared to controls (no mRNA administration). The phase 2 study showed safety and improved heart function after direct injections of VEGF-A mRNA into the hearts of 11 patients with coronary diseases during open heart surgery, suggesting a promising clinical outcome of VEGF-A mRNA treatment for heart tissue regeneration (Anttila et al., 2020). Currently, the phase 3 clinical study of VEGF-A mRNA in a larger number of heart failure patients is ongoing (Collén et al., 2022).

Given the potential of mRNA-based therapy in tissue regeneration, our research team at the Excellent Center for Periodontal Disease and Dental Implant propose to develop nucleoside-modified mRNA encoding PDGF-BB for periodontal regeneration. We currently collaborate with Professor Drew Weissman and Assistant Professor Norbert Pardi (University of Pennsylvania, USA) for the synthesis of N1-methylpseudouridine-modified mRNA encoding PDGF-BB formulated with LNPs. Our early *in vitro* study showed high protein expression after 24 hour-transfection of primary human periodontal ligament cells and primary human gingival fibroblasts with modified mRNA encoding PDGF-BB (Surisaeng et al., 2020). In addition, previous *in vivo* study (rats) showed that direct injection of PDGF-BB formulated in LNPs into healthy gingiva induced high protein expression (Mahanonda R, unpublished data).

Effect of inflammation on the translational efficiency of mRNA

Inflammation could affect mRNA translation and the therapeutic effect. There was an indirect evident that shown the effects of inflammatory cytokine to protein translational process. Excessive amount of type I interferon could inhibit the translation of mRNA due to its upregulation of eIF-2α kinase and ′-5′-oligoadenylate synthetase (OAS) expression (de Haro et al., 1996; Pardi et al., 2018). So far, there has been limited information regarding the translational efficiency of mRNA under inflammatory environment.

Ligature-induced rat periodontitis

Animal models are necessary to investigate the cause and effect relationship or mechanism of novel agents. There are many animal species to study as animal models such as rats, dogs or non-human primates. Rodent models are widely used in experimental studies because of most convenient, inexpensive, and versatile model. As known that periodontitis is initiated by bacteria in dental plaque, then the host response is occurred and cause the inflammation around periodontium. If the inflammation is continued, it is lead to periodontal breakdown.

There are several experimental methods which induce the periodontal breakdown such as oral gavage model, *A. actinomycetemcomitans* feeding model, ligature model and airpouch/chamber model (Graves et al., 2008). Ligature-induced periodontitis is commonly used to mimic periodontitis by placing ligature around teeth which causes plaque accumulation and ulceration of sulcular epithelium and connective tissue (de Molon et al., 2018; Graves et al., 2008). To compare between oral gavage model and ligature model, ligature model is more effective to lead to inflammation and alveolar bone loss than using oral gavage model (de Molon et al., 2016). Abe and Hajishengallis demonstrated the procedure of ligation in mice model to study the proper site to evaluate bone loss by using silk 5-0 to pass the interproximal contact of second molar and loop around the teeth. Suture was performed in triple-knot at palatal site. They found that bone loss was significantly

progressed from day 5 to day 8 post-ligation (Abe & Hajishengallis, 2013). In addition to alveolar bone loss, high expression of inflammatory cytokines such as IL-1β, TNF-α, and IL-6 were observed in gingival crevicular fluid of ligature-induced periodontitis in mice (Matsuda et al., 2016).

The ligature model are used to create inflammatory environment to evaluate the efficacy of material in periodontal regeneration potential. For example, Ni and his colleagues investigate the preventive and therapeutic effect of gold nanoparticles in rat periodontitis. They use 4-0 silk ligation around upper second molars to induce periodontitis and inject gold nanoparticle at palatal gingiva every three days and all rats are sacrificed at day 14. They found that the ligature successfully cause bone loss around teeth and local delivery of gold nanoparticle could increase newly-formed periodontal attachment and inhibit osteoclast activity and inflammatory response (Ni et al., 2019).

In this study, ligature-induced rat periodontitis model was used to evaluate protein expression after gingival injection of PDGF-BB mRNA. This model was previously established by our research group using 3-0 silk ligated around maxillary second molar teeth for 14 days (Wichienrat W, unpublished data). So far, there has been limited information regarding the translational efficiency of mRNA in inflammatory disease. Findings from this study would be crucial to determine if

ligature-induced periodontitis in rats is a suitable model for testing PDGF-BB mRNA therapeutic efficacy for periodontal regeneration.

CHAPTER III

MATERIALS AND METHODS

Construction of N1-methylpseudouridine - modified mRNA encoding PDGF-BB (PDGF-BB mRNA)

The nucleotide sequence of human PDGF-BB was designed by Professor Rangsini Mahanonda and Dr. Sathit Pichyangkul, and the N1-methylpseudouridine - modified mRNA encoding PDGF-BB encapsulated with LNPs was synthesized by Assistant Professor Norbert Pardi (University of Pennsylvania, USA) (Pardi et al., 2015).

Ligature-induced experimental periodontitis in rats

Animal care and experimental procedures were approved by the Ethics committee at Faculty of Tropical Medicine-Institute Animal Care and Use Committee at Mahidol University (FTM-IACUC) and the Ethics committee of the Faculty of Dentistry, Chulalongkorn University. Sprague-Dawley male rats (6 weeks 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 the beginning of the experiment. Figure 3A demonstrated experimental design of ligature-induced rat periodontitis. Rats were anaesthetized by intraperitoneal injection with Zoletil (40 mg/kg) and Xylazine (5 mg/kg). Induction of experimental periodontitis in rat, silk ligatures (3-0 silk threads, Johnson & Johnson, New Brunswick, NJ, USA) were placed on the maxillary left second molar for 2 weeks (Figure 3B).

Suture was tied firmly with a double-knot on the buccal side of the maxillary left second molar. The maxillary right second molar was left non-ligature (Figure 3B). The animal was fed with regular diet, received postoperative care, and checked the ligature at 1 week post-ligation.

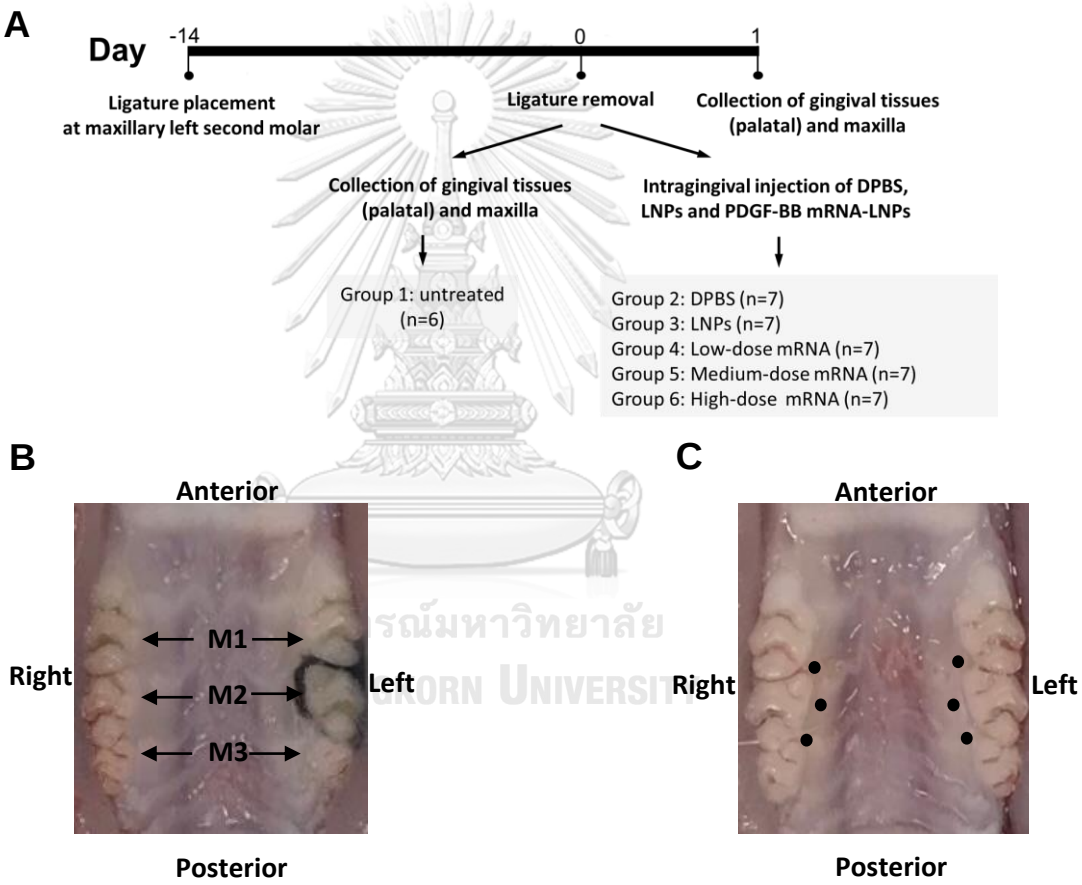

Figure 3. Overview of the experimental design. (A) Diagram of ligature-induced rat periodontitis and the intragingival delivery of DPBS, LNPs, and PDGF mRNA-LNPs. (B) In each rat, ligature was placed around maxillary left second molar while maxillary right second molar was not ligated. (C) Black dots indicate the injection sites. (DPBS =

Dulbecco's phosphate-buffered saline, LNPs = lipid nanoparticles, PDGF mRNA = N1-methylpseudouridine - modified mRNA encoding platelet derived growth factor-BB encapsulated with LNPs).

Administration of DPBS, LNPs and N1-methylpseudouridine mRNA encoding PDGF-BB in LNPs

All ligatures were removed after ligation for two weeks. The animals were randomized into six groups (six to seven animals per group) as demonstrated in Figure 3A. The first group was immediately sacrificed and served as untreated group. Group 2 to 6 were received different substances by intragingival injections. The injection was performed at six sites (Figure 3C) with the volume of 6 µl solution per site, which contained DPBS (group 2, control); LNPs only (group 3); 3 µg PDGF mRNA (group 4, low-dose mRNA); 10 µg PDGF mRNA (group 5, medium-dose mRNA); and 30 µg PDGF mRNA (group 6, high-dose mRNA). A total volume of 36 µl solution was given to each rat. All animals (group 2-6) were sacrificed at 24 hours after administration since a previous *in vivo* study of kinetic protein expression following local delivery of pseudouridine-modified PDGF mRNA in healthy rat, the peak of PDGF-BB protein translation was observed in gingival tissues 24 hours after intragingival injection (Bhongsatiern et al., 2020).

All rats were harvested for maxillae. In each group, four rats were collected gingival tissues (palatal side) for measurement of protein production, while the

remaining maxillae were used for alveolar bone measurement. The other two to three rats in each group were dissected maxillae for micro-computerized tomography (micro-CT) imaging and subsequently for histological analysis.

Measurement of alveolar bone level

To measure alveolar bone level with stereomicroscope, rat maxillae were dissected and defleshed in 5% sodium hypochlorite for 7 days. These samples were pictured under stereomicroscope (Olympus SZ61; Olympus Corporation, Tokyo, Japan) and analyzed alveolar bone level by ImageJ 1.52a software program (National Institutes of Health, USA).

To assess alveolar bone level with microCT analysis, two to three maxillae per group were dissected and fixed with 10% formaldehyde for 2 days. Then, the maxillae were scanned under micro-CT system (Micro-CT μ35 scanco; SCANCO medical, Brüttisellen, Switzerland) and generated 3D images of the maxillae. All images of maxillae were analyzed at buccal and palatal site.

To identify alveolar bone loss, the mean distances from cementoenamel junction (CEJ) to alveolar bone crest (ABC) were measured at five sites on each surface (distobuccal or distopalatal line angle of maxillary first molar, mesiobuccal or mesiopalatal line angle, mid-buccal or mid-palatal, distobuccal or distopalatal line angle of maxillary second molar, mesiobuccal or mesiopalatal line angle of maxillary third molar).

Measurement of protein production

Gingival tissues at palatal side were collected with sulcular incision at mesiopalatal line angle of maxillary first molar to distopalatal line angle of maxillary third molar and horizontal incision was made approximately 2-3 mm below gingival margin. The collected gingiva was weighed and homogenized in RIPA with protease inhibitor cocktail (Sigma, St. Louis, MO, USA). The homogenates were then centrifuged at 16,000 rpm for 15 minutes at 4°C. The amount of total proteins was measured by BCA protein assay kit (PierceTM BCA Protein Assay; Thermo scientific, Co., Ltd., Rockford, IL USA). The level of PDGF protein and inflammatory cytokines (IL-1β and TNF-α) were measured using enzyme-linked immunosorbent assay (ELISA) kits (Quantikine® ELISA; R&D System, Inc., Minneapolis, MN, USA). The level of IL-17a was quantified by ELISA kits (LEGEND MAX™ ELISA; BioLegend Inc., San Diego, CA, USA). According to the manufacturer, the sensitivity of the IL-1β, TNF-α and IL-17a was < 5 pg/mL and < 4 pg/mL, respectively.

Histological evaluation

After micro-CT scanning, the maxillae were decalcified in 10% ethylenediaminetetraacetic acid solution at 4°C for two weeks. Decalcified maxillae were dehydrated and embedded in paraffin. The serial section of specimens was performed in 7 μm thickness in mesio-distal direction and stained with hematoxylin and eosin (H&E) to analyzed inflammatory cell infiltration.

Inflammatory infiltration was evaluated in 3 specific regions: 1). epithelial region near sulcus/periodontal pocket; 2). connective tissue near sulcus/periodontal pocket; 3). furcation. The severity of the inflammatory process was classified in each region using polymorphonuclear leukocyte and mononuclear cell inflammation scoring, as described previously (de Molon et al., 2014), 0 = absence of inflammatory cells, + = mild inflammation (some inflammatory cells), ++ = moderate inflammation (remarkable inflammatory cells), +++ = severe inflammation (predominance of inflammatory cells).

Statistical analysis

All data were analyzed by the statistical software SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Data between non-ligated site and ligated site were compared by Paired T- test. Differences among groups were analyzed by ANOVA followed by multiple comparisons with Bonferroni's post hoc test. The statistically significance was considered as *P-value* < 0.05.

CHAPTER IV

RESULTS

Ligature-induced periodontitis model in rats

In group 1 (untreated group), after two weeks of ligature placement, the mean alveolar bone loss of ligature site was 0.7 ± 0.04 mm (0.63 ± 0.05 mm on buccal sides and 0.78 ± 0.07 mm on palatal sides). Whereas the mean alveolar bone loss of non-ligature sites was 0.5 ± 0.05 mm (0.35 ± 0.02 mm on buccal sides and 0.66 ± 0.08 mm on palatal sides) (Figure 4A - 4C). There was significantly more bone loss at the ligature sites than at the non-ligature sites, indicating an established periodontitis model with periodontal bone loss in rats (Appendix: supplementary table 1).

In group 1 (untreated group), both non-ligature and ligature sites showed low level of inflammatory cytokine IL-1β (< 50 pg/mg protein) and IL-17a (<150 pg/mg protein), with no significant differences between the two sites (Figure 2D). TNF-α was not detected (Appendix: supplementary table 2).

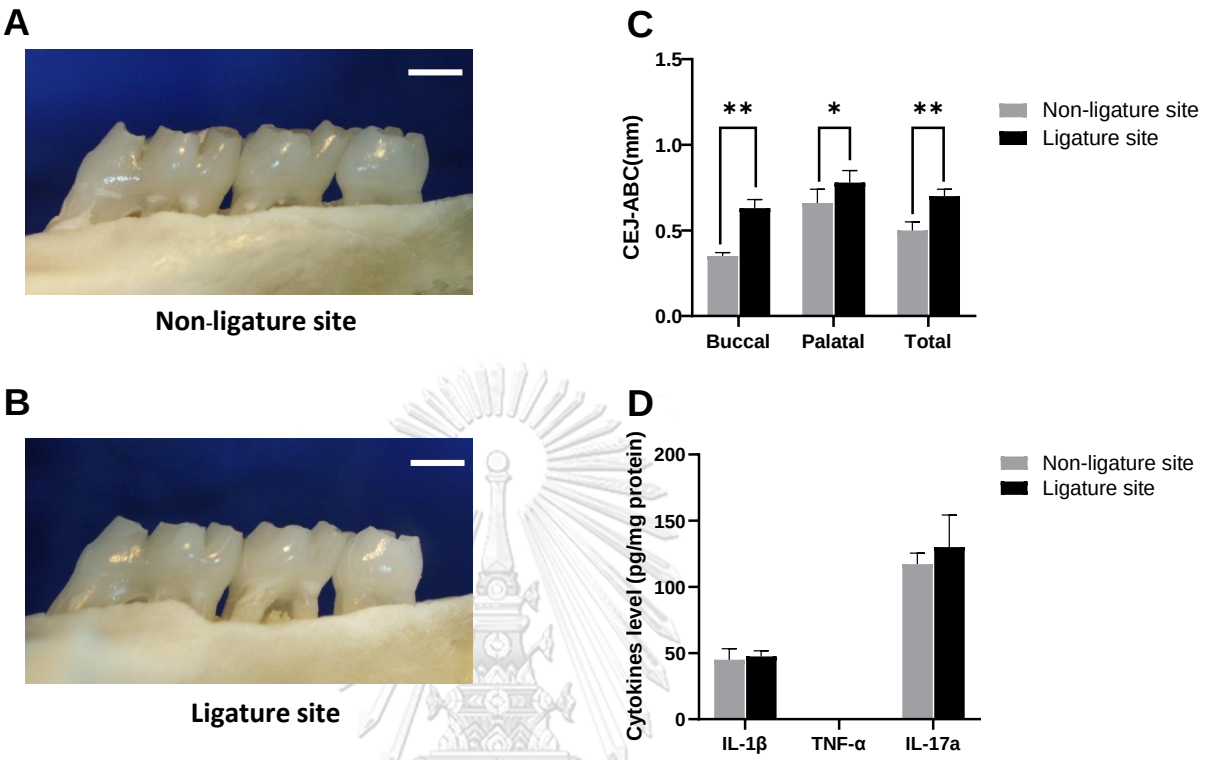

Figure 4. Bone loss and cytokine production after 14 days of non-ligature and ligature sites in group 1 (untreated group). Representative stereomicroscope images from the buccal surfaces of (A) non-ligature, and (B) ligature sites (20x magnification; scale bar = 1 mm) (n=4). (C) The distance (mm) was from cementoenamel junction (CEJ) to alveolar bone crest (ABC), the linear measurement under stereomicroscope and micro-CT. Data shown are mean ± SE of the CEJ-ABC distance from the buccal side, palatal side, and total (the sum of buccal and palatal sides) (n = 6; *$p < 0.05$; ** $p < 0.001$, compared between non-ligature and ligature sites; Paired T- test) and (D) inflammatory cytokine levels of IL-1β, TNF-α and IL-17a in gingival tissues (palatal

site). Data shown are mean ± SE (n = 4). (IL-1β = interleukin-1 beta, TNF-α = Tumor necrosis factor-alpha, IL-17a = interleukin-17a)

H&E stained sections corresponded to the 3D micro-CT imaging (Figure 5A, 5D). The histological findings of the ligature sites showed ulcerated epithelium, crestal bone loss and the presence of inflammatory infiltrates (Figure 5E,5F), while non-ligature sites showed intact sulcular epithelium and some cellular infiltrates (Figure 5B,5C). According to the severity of inflammatory process, both sections were ranked as mild inflammation (de Molon et al., 2014).

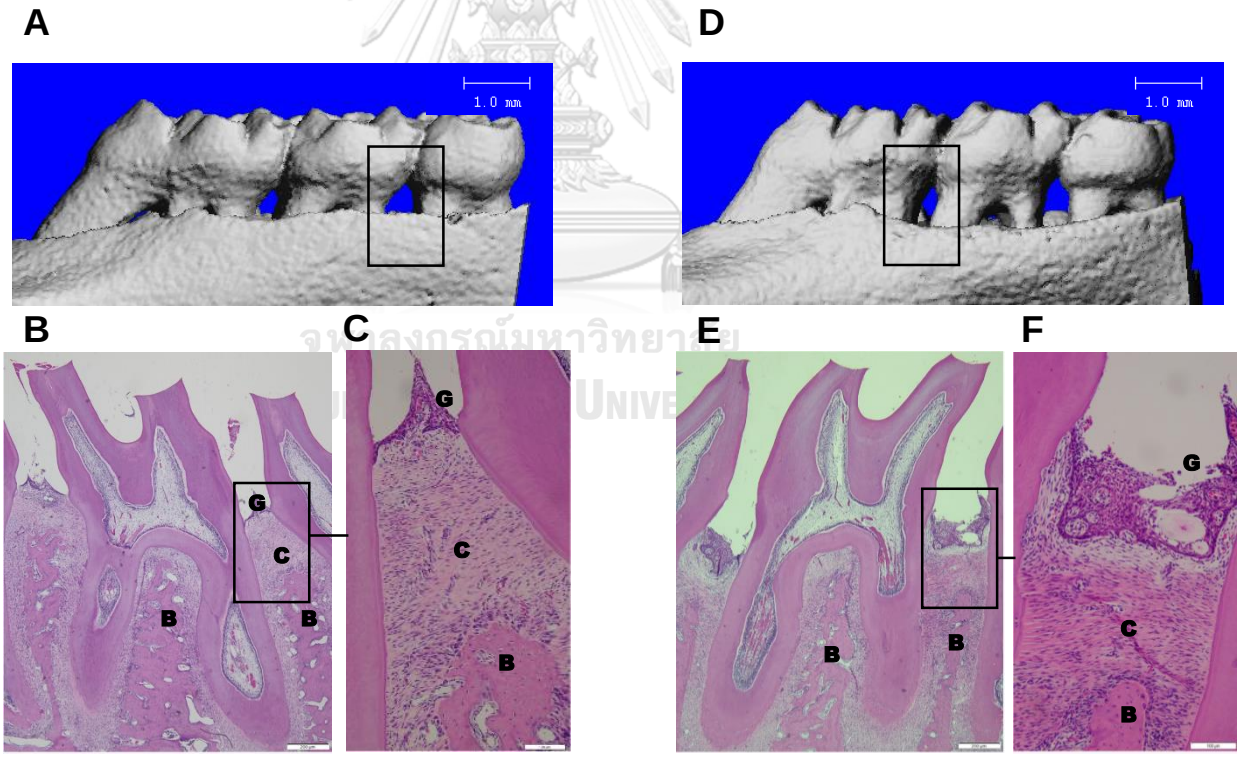

Figure 5 Representative of micro-CT images and histological findings after 14 days of (A) non-ligature and (D) ligature sites in group 1 (untreated group). The corresponding H&E-stained sections of (B&C) showed intact sulcular epithelium and some

inflammatory cell infiltrates whereas (E&F) showed ulcerated epithelium and the presence of inflammatory cell infiltrates in ligature site. (B&E; original magnification x4; C&F; original magnification x20; B= alveolar bone; G= gingival epithelium; C= connective tissue)

Expression of PDGF and inflammatory cytokines after local delivery of PDGF mRNA-LNPs in rat periodontitis model

After 24 hours of gingival injection of PDGF-BB mRNA, clinical findings at the injected sites showed no erythema or swelling. The expression levels of translated PDGF protein were tended to increase as mRNA dose increased at both non-ligature and ligature sites (group 4, 5, 6) (Figure 6A) (Appendix: supplementary table 3). At each mRNA dose, there was no statistically significant difference in protein production between non-ligature and ligature sites. Negligible amount of PDGF protein was detected in untreated group (group 1, ranging from 31.8 ± 8.83 to 35.24 ± 15.58 pg/mg protein), DPBS control (group 2, ranging from 19.26 ± 2.01 to 24.13 ± 3.93 pg/mg protein) and LNPs (group 3, ranging from 18.79 ± 1.83 to 21.96 ± 2.3 pg/mg protein) (Figure 6A). At the non-ligature sites, the mean PDGF protein was 51,883.91 ± 7,415.45 pg/mg protein at the high-dose mRNA; 24,666.22 ± 5,782.33 pg/mg protein at the medium-dose mRNA; and 10,912.54 ± 1,893.94 pg/mg protein at the low-dose mRNA group. It was found that the medium-dose mRNA and high-dose mRNA groups resulted in significantly higher protein production than untreated,

control and LNPs groups. In addition, the translated protein in high-dose mRNA group was significantly higher than in the low-dose mRNA and medium-dose mRNA groups. At the ligature sites, the mean PDGF protein was 48,012.66 ± 16,063.13 pg/mg protein at the high-dose mRNA; 41,134.63 ± 10,430.55 pg/mg protein at the medium-dose mRNA; and 15,918.79 ± 7,681.11 pg/mg protein at the low-dose mRNA. It was found that the mean protein levels only in the high-dose mRNA-LNPs group was significantly higher than control and LNPs groups.

In addition to PDGF protein production, the levels of inflammatory cytokines including IL-1β, TNF-α and IL-17a were assessed after injection of PDGF mRNA (Appendix: supplementary table 4). TNF- α production was undetected in all groups whereas IL-1β production was observed with the mean ranging from 72.08 ± 13.24 to 1,458.44 ± 180.51 pg/mg protein. The IL-1β level was higher in LNPs and high dose of mRNA groups compared to the other groups, however the difference was not statistically significant (Figure 6B) Furthermore, the level of IL-17a at non-ligature sites and ligature sites was below 100 pg/mg protein (Figure 6C).

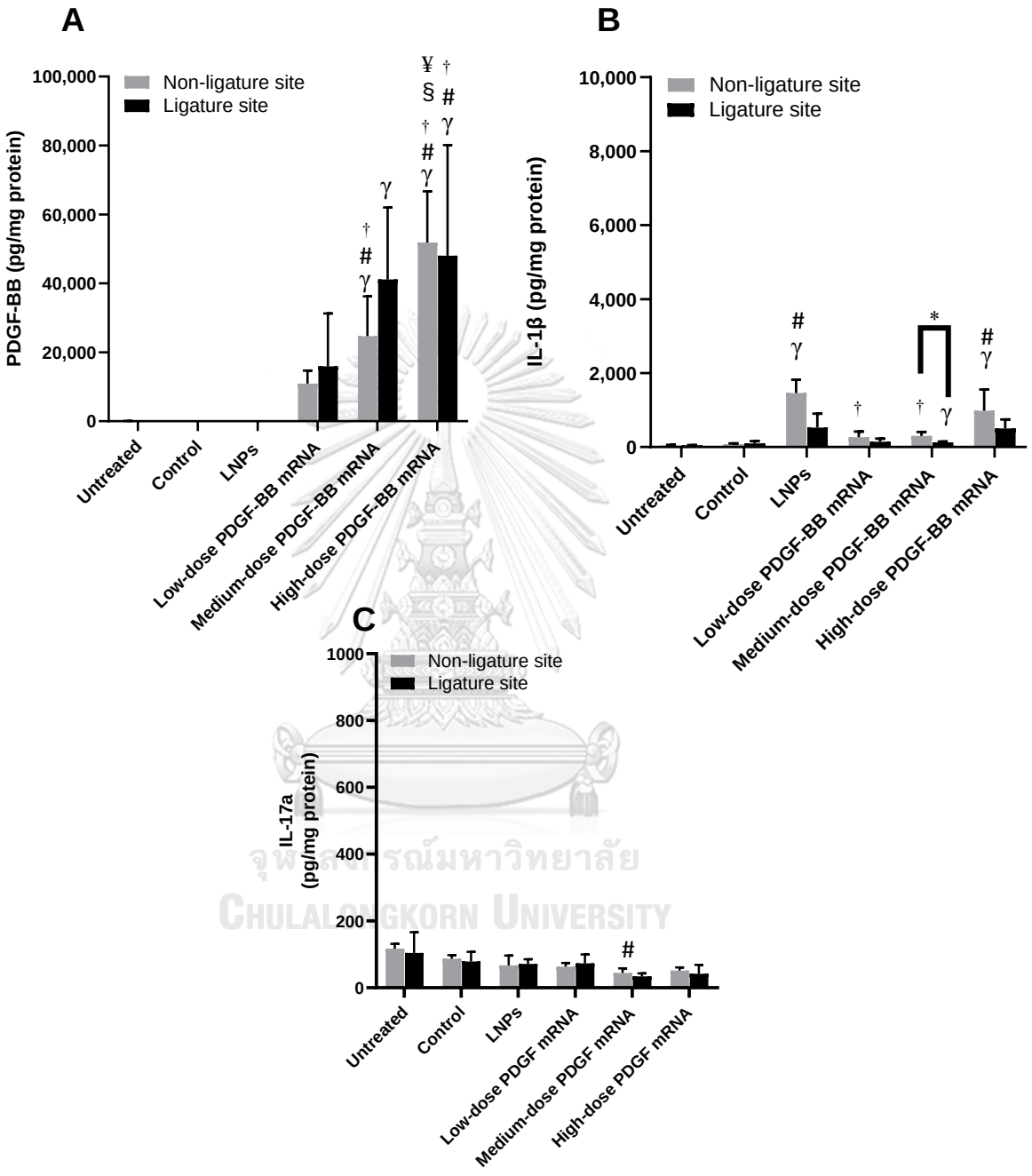

Figure 6. Production of (A) PDGF protein, (B) IL-1β and (C) IL-17a in palatal tissues after gingival injection with DPBS control, LNPs and PDGF mRNA (low, medium, and high doses) in non-ligature and ligature sites. Data shown are mean ± SE *(*p<0.05*,

compared between non-ligature and ligature sites; $^{\gamma}p<0.05$, compared to untreated group, $^{\#}p<0.05$, compared to control group; $^{\dagger}p<0.05$, compared to LNPs group; $\S p<0.05$, compared to low-dose mRNA group; $^{¥}p<0.05$, compared to medium-dose mRNA group; one way ANOVA and Bonferroni's post hoc tests), DPBS = Dulbecco's phosphate-buffered saline, LNPs = lipid nanoparticles, PDGF mRNA = N1-methylpseudouridine - modified mRNA encoding platelet derived growth factor-BB encapsulated LNPs).

Histological findings

In addition to inflammatory cytokine expression, H&E staining was performed on mesiodistal sections (3 samples in each group) to investigate local inflammation. Unfortunately, the cutting plane of individual histological section was not parallel and was not be in the same plane. Few sections were broken. It appeared to be only one section per group available for investigation (Appendix: supplementary figure 7 and 8). From this limited H&E data, we did not observe any foreign substances of mRNA formulation such as LNPs in the tissues. In all the sections, some inflammatory infiltrates were found at epithelium, connective tissue near the sulcus/periodontal pocket and furcation at ligature and non-ligature sites. No severe inflammation was detected (Appendix: supplementary table 5).

CHAPTER V

DISCUSSION AND CONCLUSION

This study is the first study to explore the potential application of mRNA encoding PDGF-BB for periodontal regeneration in periodontitis. Local delivery of PDGF-BB mRNA in LNPs in ligature-induced rat periodontitis demonstrated high production of PDGF-BB protein. Although, the translated protein showed a dose response trend, the differences in protein levels among different mRNA doses were not statistically significant.

The ligature model was used to establish periodontitis in the present study since it is a well-known approach to induce periodontitis and has been used in many relevant studies for testing efficacy of biological reagents (Lin et al., 2021). This model is reported to be similar to human periodontitis in various aspects, as the alveolar bone resorption depends on bacterial plaque and inflammation of gingival tissue. Numerous studies of ligature-induced periodontitis in rodents showed alveolar bone loss, which is a hallmark of periodontitis (Graves et al., 2008; Graves et al., 2011; Lin et al., 2021). In this study, the ligature-induced periodontitis model was successfully established in rats by ligation, as the alveolar bone loss at ligature sites was significantly greater than at non-ligature sites on both buccal and palatal surfaces. Our results supported previous studies that used a similar technique with 3-0 silk for 2-week ligation (Liu et al., 2010). However, the level of inflammatory

cytokine and the presence of inflammatory infiltrates were not different between ligature site and non-ligature site. This could possibly be explained by the timing of tissue collection for cytokine and histological analysis. Our study was conducted on day 14 after ligation, which was later than some other previous studies (de Molon et al., 2018; Marchesan et al., 2018; Wu et al., 2020). It was likely that local inflammation may occur at earlier time point in our ligature model. Wu and colleagues (2020) observed intense infiltration of inflammatory cells at day 7-9 with no significant bone loss (Marchesan et al., 2018; Wu et al., 2020). IL-1β and TNF-α gene expression was also significantly elevated as early at day 1-3 (de Molon et al., 2018; Marchesan et al., 2018). Those studies used real-time PCR to measure mRNA expression, whereas our study used ELISA, a reliable and appropriate method for investigating the protein levels of secreting cytokines.

Periodontitis results from host immune response to dysbiotic plaque. High levels of inflammatory cytokines such as IL-1α, IL-1β, TNF-α, IL-6 and interferon-γ (IFN-γ) have been observed in periodontitis tissues and other cytokines such as IFN-α, IL-8 in plasma of periodontitis patients (Baker et al., 1999; McGee et al., 1998; Wright et al., 2008). In this study, we hypothesized that local inflammation in ligature-induced rat periodontitis may reduce PDGF-BB protein expression after gingival injection of PDGF-BB mRNA. Preliminary data from de Haro et al. (1996) demonstrated that activation of protein kinase R and 2'-5'-oligoadenylate synthetase

led to the inhibition of protein translation (de Haro et al., 1996). Such activation may be induced by IFN-α (Pardi et al., 2018). From our findings, we did not observe the local inflammation in the ligature model at the time periodontitis bone loss occurred. To see the effect of local inflammation, we need to have conducted the experiment earlier, around the 3-7 day ligation. Nevertheless, we foresee the potential use of our ligature model with markedly periodontitis bone loss for evaluating mRNA therapeutic effect regarding periodontal regeneration. Long term observation for new periodontal tissue formation may be required.

The mRNA formulation used in this study was N1-methylpseudouridine-modified mRNA encapsulated with LNPs. In comparison to our previous study with pseudouridine-modified mRNA, N1-methylpseudouridine-modified mRNA encapsulated with LNPs resulted in higher protein translation and expression at the same time point (24 hours following intragingival injection) (Bhongsatiern et al., 2020). This could be explained by the use of the modified nucleobase N1-methylpseudouridine that has been shown to effectively decrease intracellular innate immune signals and, thus, improve mRNA stability (Andries et al., 2015). In addition to the mRNA based modification, LNPs were employed to protect mRNA from RNase degradation and promote intracellular entry (Kowalski et al., 2019). Because of their ease of manufacture and ability to improve mRNA translational capacity, LNPs are widely used as a carrier for mRNA, for example, in mRNA COVID-19 vaccine. The structure of LNPs was consist of phospholipids, cholesterol, ionizable

cationic lipids and PEGylated lipids for support, stabilization, complexation of negatively charged mRNA molecules, facilitating endosomal escape, and reducing nonspecific endocytosis by host immune cells, respectively (Kauffman et al., 2015; Kulkarni et al., 2018; Semple et al., 2022).

However, previous pre-clinical studies reported that cationic lipid component in LNPs could activate an inflammatory response via NF-**κ**B activation, and the production of TNF-α, IL-1β, IL-6 and IFN-γ (Lonez et al., 2014; Ndeupen et al., 2021). In this study, we found that LNPs and high-dose mRNA groups had higher level of IL-1β in compared to other groups, however, we did not observed clinical swelling or erythema at the injected gingiva. Although, LNPs-induced inflammatory response could serve as effective adjuvant for an mRNA-LNPs vaccine, this inflammation may be unfavorable for tissue regeneration. Therefore, development of new types of noninflammatory delivery molecules that protect therapeutic mRNA from degradation and facilitate its cellular uptake would be required to address the issue of LNP-induced immune system activation.

Safety is a major concern when using mRNA as a therapeutic option in patients with periodontitis. mRNA dosage used in our experiment ranging from 3 - 30 μg PDGF-BB mRNA/animal, which is considered lower than previous study by Zangi et al. (2013). They injected 200 μg VEGF mRNA/animal into myocardium of mouse myocardial infarction model. Their results showed improved heart function,

no adverse effects and enhanced long term survival (1 year) (Zangi et al., 2013). Of importance, it is becoming clear that COVID-19 nucleoside- modified mRNA-LNP vaccines have shown a strong safety and efficacy profile (>90%) and to date, 12.68 billion doses of these vaccines have been used around the world (Hogan & Pardi, 2022; Ritchie et al., 2021). The success of mRNA vaccine is likely to facilitate the development of other mRNA-based therapeutic products. However, a future long-term study investigating safety and therapeutic efficacy of PDGF-BB mRNA in small and large animals are required.

Conclusion

This study of 24 hour-local delivery of PDGF mRNA-LNPs into gingiva using ligature-induced rat periodontitis model results in high translated PDGF protein. Even though, local gingival inflammation was not detected in this established periodontitis model, this model may be useful for future testing of mRNA therapeutic effects.

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APPENDIX

Supplementary table 1. The distance from CEJ to alveolar bone crest at 2 weeks post-ligation in untreated group

Untreated group (N=6) / Surface	Mean alveolar bone loss ± SE (mm.)		Significant
	Non-ligature sites	Ligature sites	
Buccal	0.35±0.02	0.63±0.05**	$p<0.001$
Palatal	0.66±0.08	0.78±0.07*	$p<0.05$
Total	0.5±0.05	0.7±0.04**	$p<0.001$

Data was presented as mean ± SE (N=6 in untreated group).

**$p<0.001$ and *$p<0.05$ indicate a significant difference between non-ligatured sites and ligature sites (Paired T-test)

Supplementary table 2. Mean proinflammatory cytokine level in untreated group

Groups (N=4)	Proinflammatory cytokine level (pg/mg protein)						Significant
	IL-1β		TNF-α		IL-17a		
	Non-ligature	Ligature	Non-ligature	Ligature	Non-ligature	Ligature	
Untreated group	45±8.27	47.55±4.3	0.12±0.02	0.1±0.01	117.1±8.33	129.99±24.29	$p>0.05$

Data was presented as mean±SE (N= 4 in untreated group).

Supplementary table 3. The level of PDGF-BB at gingiva after local delivery substances

Groups (N=4/group)	**Mean PDGF-BB level ± SE (pg/mg protein)**	
	Non-ligated site	**Ligated site**
Untreated	35.24±15.58	31.8±8.83
Control	24.13±3.93	19.26±2.01
LNPs	21.96±2.3	18.79±1.83
Low-dose mRNA	10,912.54±1,893.94	15,918.79±7,681.11
Medium-dose mRNA	24,666.22±5,782.93$^{\#,\dagger,\gamma}$	41,134.63±10,430.55$^{\#,\gamma}$
High-dose mRNA	51,883.91±7,415.45$^{\#,\dagger,\gamma,\S,¥}$	48,012.66±16,063.13$^{\#,\dagger,\gamma}$

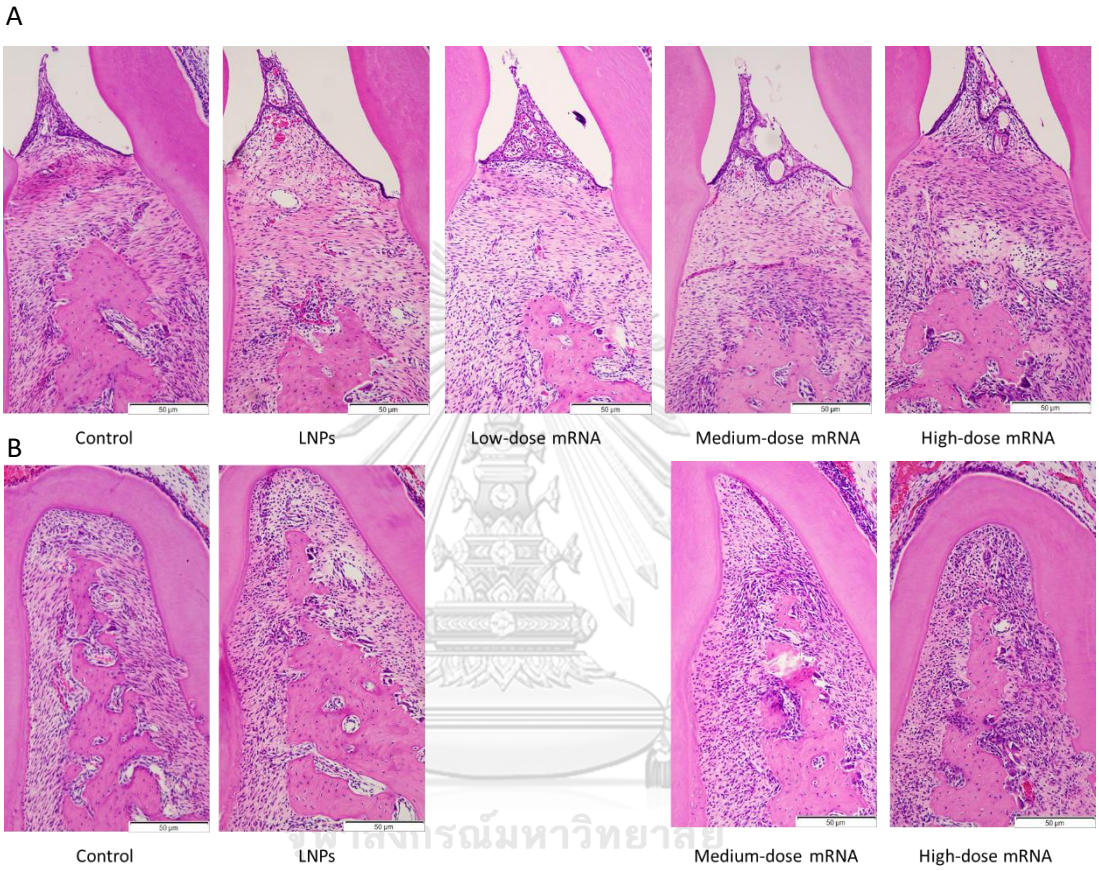
Data shown are mean ± SE (N=4 in each group). *$^{*}p<0.05$*, compared between non-ligature and ligature sites; $^{\gamma}p<0.05$, compared to untreated group, $^{\#}p<0.05$, compared to control group; $^{\dagger}p<0.05$, compared to LNPs group; $^{\S}p<0.05$, compared to low-dose mRNA group; $^{¥}p<0.05$, compared to medium-dose mRNA group; one way ANOVA and Bonferroni's post hoc tests

Supplementary table 4. The level of proinflammatory cytokines at gingiva after local delivery substances

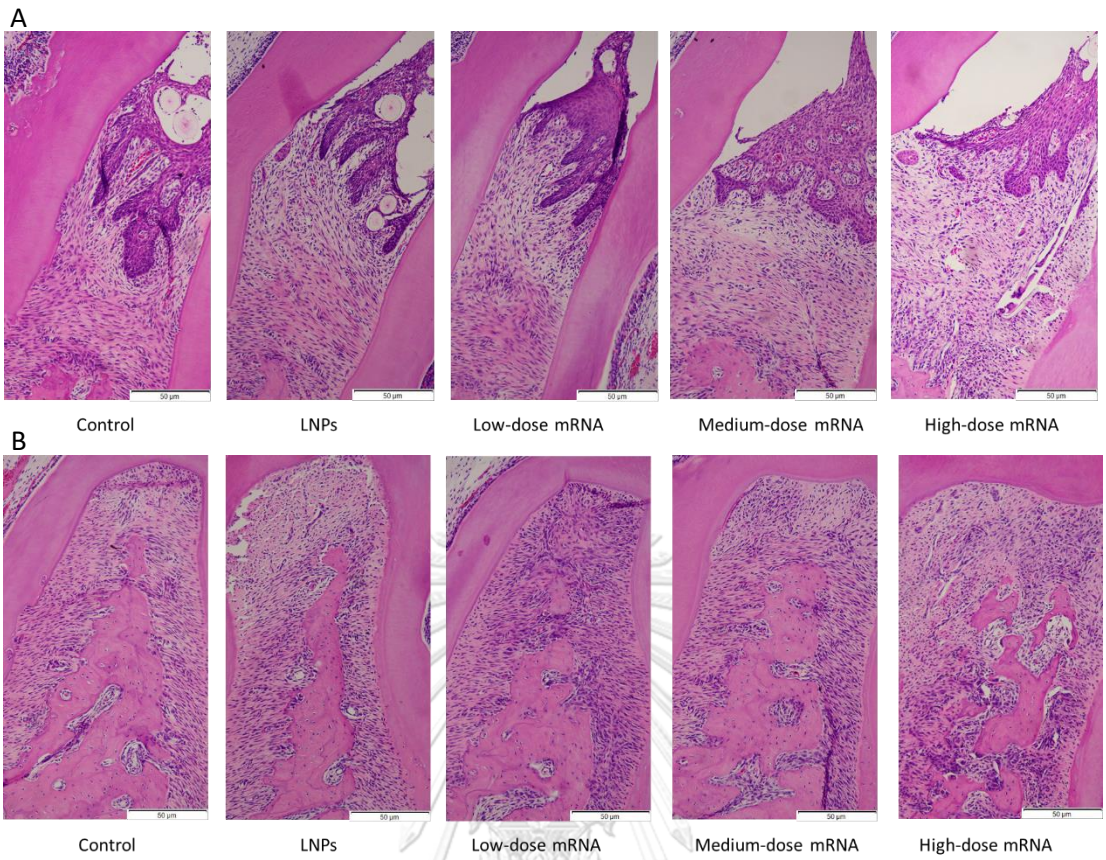
Groups (N=4/group)	**Proinflammatory cytokine level (pg/mg protein)**					
	IL-1β		**TNF-α**		**IL-17a**	
	Non-ligated	**Ligature**	**Non-ligated**	**Ligature**	**Non-ligated**	**Ligature**
Control	72.08±13.24	98.77±29.94	0.08±0.01	0.06±0.00	87.03±5.19	78.74±14.3
LNPs	1,458.44±180.51$^{\#,\gamma}$	526.97±187.49	0.11±0.01	0.08±0.01	67.09±14.67	71.17±7.05
Low-dose mRNA	267.65±75.35†	146.25±41.21	0.09±0.00	0.1±0.02	63.32±5.52	73.21±13.14
Medium-dose mRNA	300.78±53.88*,†	126.09±11.91$^{\gamma}$	0.12±0.02	0.08±0.01	43.99±7.02$^{\#}$	34.39±13.14
High-dose mRNA	981.32±288.39$^{\#,\gamma}$	505.07±121	0.10±0.02	0.08±0.00	51.36±4.86	53.64±4.79

Data shown are mean ± SE (N=4 in each group). *$^{*}p<0.05$*, compared between non-ligature and ligature sites; $^{\gamma}p<0.05$, compared to untreated group, $^{\#}p<0.05$, compared to control group; $^{\dagger}p<0.05$, compared to LNPs group; $^{\S}p<0.05$, compared to low-dose

mRNA group; ¥$p<0.05$, compared to medium-dose mRNA group; one way ANOVA and Bonferroni's post hoc tests



Supplementary figure 7. Representative histological sections following gingival injection of substances at non-ligature sites. (A) gingival sulcus (B) furcation at maxillary second molar



Supplementary figure 8. Representative histological sections following gingival injection of substances at ligature sites. (A) gingival sulcus (B) furcation at maxillary second molar

Supplementary table 5. Histological descriptive analysis of inflammatory infiltration after local injection of DPBS, LNPs and mRNA encoding PDGF-BB

Site / group	Control group	LNPs group	Low-dose mRNA group	Medium-dose mRNA group	High-dose mRNA group
Non-ligature site					
Epithelial region near sulcus	+	+	+	+	+
Connective tissue near sulus	0	+	+	++	++
Furcation area	+	+	NA	++	++
Ligature site					
Epithelial region near sulcus	+	+	+	++	++
Connective tissue near sulus	+	++	+	+	++
Furcation area	+	++	+	+	+

0 = no inflammatory cells; + = mild inflammation (a some inflammatory cells); ++= moderate inflammation (remarkable inflammatory cells); +++ = severe inflammation (predominance of inflammatory cells); NA = not applicable.

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