Micro-computed Tomography Analysis of Bone Regeneration Using mRNA Encoding Bone Morphogenetic Protein-2 with Implant Placement in Rat Femur

Nopparada Lawtrakulngam[1], Philaiporn Vivatbutsiri[2], Noppadol Sa-Ard-Iam[3], and Jaijam Suwanwela[*, 1]

[1] Department of Prosthodontics, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand
[2] Department of Anatomy, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand
[3] Immunology Research Center, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand
[*]Corresponding author, E-mail: jaijam1220@gmail.com

Abstract

Bone morphogenetic protein-2 (BMP-2) has a potential for enhancing bone regeneration and osseointegration around implant materials. However, the drawback of BMP-2 protein delivery is instability. Therefore, high doses of BMP-2 need to be used clinically, which results in higher costs and more side effects. Although gene-based therapy, in the form of mRNA BMP-2, can overcome these limitations. Recent clinical studies on mRNA BMP-2 regarding bone regeneration around implant materials are lacking. This study aims to investigate the ability of N1-methylpseudouridine-modified mRNA encoding bone morphogenetic protein-2 (m1Ψ-BMP-2 mRNA) to enhance bone regeneration after implant placement in rat femur defects. Twelve implant titanium wires were placed into 6 Sprague-Dawley (SD) rats. The femur defects were randomly filled with 15 µg m1Ψ-BMP-2 mRNA (n = 4), 4 µg recombinant human bone morphogenetic protein-2 (rhBMP-2) (n = 4), or Dulbecco's phosphate-buffered saline (dPBS) (n = 4). The animals were sacrificed 6 weeks after implantation. The percentages of bone volume fraction (%BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and bone-to-implant contact (BIC) were evaluated using micro-computed tomography imaging (micro-CT). One-way ANOVA was used to analyze the data between the three groups to compare BMP-2 effectiveness. All statistical analysis was performed at a significant level of 5% (p-value < 0.05). Bone volume and trabecular micro-architecture parameters were significantly greater in the m1Ψ-BMP-2 mRNA group than the dPBS group. In conclusion, these results reveal a positive effect of m1Ψ-BMP-2 mRNA that results in increased bone formation at the peri-implant area compared to rhBMP-2 and dPBS.

Keywords: *Bone regeneration, mRNA, Osseointegration, Bone morphogenetic protein-2, Micro-computed Tomography, Dental implant*

1. Introduction

Today, our general health and well-being are significantly impacted by our oral health. Without adequate care, dental problems can lead to discomfort, an abscess, and even tooth loss. Between 1990 and 2010, there was a considerable decrease in both the prevalence and incidence of severe tooth loss. However, according to the 2015 Global Burden of Disease study, 276 million individuals worldwide still suffered from significant tooth loss (Kassebaum et al., 2017). More than 85.3% of the Thai population aged 35 and older had tooth loss (Bureau of Dental Health, 2018). Consequently, tooth loss could be regarded as a vulnerability in need of improvement. One of the most common replacement procedures is a dental implant. In comparison to other fixed prostheses, it has a variety of advantages, including preserving the jawbone, protecting surrounding teeth, and promoting good oral hygiene. However, the main issues that must be considered are the amount of alveolar bone, the healing process, and the time required to achieve osseointegration.

Osseointegration was first defined by Branemark et al. (1969) as a direct interface between the bone and metallic implants without intermediate soft tissue layers. This process requires about three to six months, depending on the quality of the socket bone and the location of the implant. The success of an implant is determined by osseointegration, which controls implant stability. There are a variety of factors

that affect implant stability, including the surface characteristics of the implant and the bone quality. Improving the quality of the bone with bioactive substances such as bone morphogenetic proteins (BMPs) is one method of reducing treatment time. The process of implantation damages tissue and interrupts blood vessels. Following the implant placement, the injured bone undergoes osseointegration, which can be separated into four phases: hemostasis, inflammatory, proliferative, and remodeling phases. The BMPs regulate the inflammatory and proliferative phases, which drive the differentiation of osteoblast progenitor cells into osteoblasts, which in turn stimulate the formation of new blood vessels and bone regeneration.

BMPs are osteoinductive substances that induce undifferentiated mesenchymal stem cells and other immature cells to develop into osteoblasts and form bone in targeted tissues. They are multifunctional cytokines discovered by Urist (1965). BMPs play crucial roles in several treatment strategies, such as bone induction, bone repair, and inducing osseointegration (Wang et al., 2014). There are currently several distinct forms of BMPs in the human genome. rhBMP-2 has been approved for use as an autograft alternative for several interbody spinal fusion operations by the U.S. FDA since 2002. It was authorized in 2007 as an alternative to autogenous bone transplant for the augmentation of the alveolar ridge and maxillary sinus following tooth extraction. Several studies also demonstrated that BMP-2 can promote bone regeneration and osseointegration. In a previous study, fibrin loaded with BMP-2 in large rat femur defects showed more new bone formation than fibrin without BMP-2 (Koolen et al., 2019). Absorbable collagen sponges containing BMP-2 could enhance fracture healing in patients with open tibial fractures (Govender et al., 2002). Combining BMP-2 with dental implants was an effective osseointegration that accelerated bone healing and increased bond strength at the bone-implant interface (Sykaras et al., 2001). In iliac sheep models, BMP-2 coating on implant surfaces prior to implantation significantly improved osseointegration compared to non-BMP-2 coated implants (Liu et al., 2004). BMP-2 is also beneficial for peri-implantitis problems. An in vivo study showed that BMP-2 has the capacity to generate more new bone formation and re-osseointegration (Schwarz et al., 2011). In addition, BMP-2 can enhance bone deposition in fracture areas and promote distraction osteogenesis (DO) (Ashinoff et al., 2004). However, their unfavorable side effects are inflammatory responses and rapid resorption (James et al., 2016; Shields et al., 2006; Zara et al., 2011).

Recently, gene-based therapy is a technology that has been used to eliminate the limitations of recombinant protein therapies. Messenger RNA (mRNA) is a single-stranded RNA molecule that plays a critical role in protein synthesis by transporting genetic information from DNA in the nucleus to ribosomes in the cytoplasm to produce encoded proteins. By reducing DNA mutations and minimizing contact with the host genome in the nucleus, mRNA promotes fast and high-level protein synthesis with rapid translation, cost-effective benefits, and patient safety (Patel et al., 2019). Elangovan et al. (2015) provided the first evidence of the safety and effectiveness of mRNA-based transfection in bone regeneration. They demonstrated that in rat calvaria bone defects, chemically modified ribonucleic acid (cmRNA) expressing BMP-2 triggered noticeably more bone regeneration than normal plasmid DNA (pDNA) encoding BMP-2. Compared to BMP-2 DNA and rhBMP-2, BMP-2 mRNA significantly increased the stimulation of bone regeneration. Therefore, mRNA could be a more effective gene transfer strategy (Balmayor et al., 2016; Elangovan et al., 2015). In contrast, the limitations of mRNA are its instability and high immunogenicity, which may result in an unfavorable inflammatory response (Karikó et al., 2004). Using modified nucleoside methods can enhance the translational capacity of mRNA, increase its biological stability, and reduce innate immunity. Nucleosides modified by replacing uridine with N1-methylpseudouridine (m1Ψ) can increase protein translation, protein expression, and cellular viability when compared to other modified nucleosides, according to a study by Andries et al. (2015). It can also reduce cytotoxicity and intracellular innate immunogenicity.

In this study, m1Ψ-modified mRNA was employed for a protein delivery application that was safe, simple, and effective. The effect of BMP-2 mRNA on osseointegration at the implant surface has not been studied. The purpose of this study is to assess the effect of BMP-2 mRNA on bone regeneration after implant placement in rat femurs using micro-computed tomography. This suggested research may assist in accelerating regeneration and promoting osseointegration. It enhances the quality and timeliness of peri-implant bone regeneration following the implantation of an implant.

2. Objectives

To compare bone regeneration differences after implant placement with or without mRNA encoding BMP-2 in rat femurs analyzed using micro-computed tomography.

3. Materials and Methods

3.1 Experimental design

Samples were divided into three groups as follows:

Group 1: Implant with dPBS
Group 2: Implant with rhBMP-2
Group 3: Implant with m1Ψ-BMP-2 mRNA

Titanium wires were implanted in 12 rat femurs randomly among three groups on both sides of distal femurs. The rats were sacrificed at 6 weeks after implant placement.

Table 1 Experimental design

	Groups	6 weeks
1	Implant + dPBS (Negative control)	N = 4 (2 rats)
2	Implant + rhBMP-2 (Positive control)	N = 4 (2 rats)
3	Implant + m1Ψ-BMP-2 mRNA (Experiment)	N = 4 (2 rats)
	Total	N = 12 (6 rats)

3.2 Animal model

Six ten-week-old male SD rats (weighing >250 g) were used for this study. These rats were obtained from Nomura Siam International Co., Ltd. (Bangkok, Thailand). The experiment protocol was approved by the Animal Care and Use Committee of Chulalongkorn University Laboratory Animal Center (CULAC) (Ethical Approval Number 2173019). The animals were housed in light and temperature-controlled facilities with a standard rat diet and water ad libitum during the preoperative and postoperative periods.

3.3 Production of mRNA encoding BMP-2

The synthesis of N1-methylpseudouridine-modified mRNA encoding BMP-2 (m1Ψ-BMP-2 mRNA) was kindly provided by Dr. Norbert Pardi, who is an expert in microbiology and mRNA therapeutics from the University of Pennsylvania (Pardi et al., 2017).

For rhBMP-2, INFUSE® Bone Graft (rhBMP-2/ACS) of Medtronic Spinal and Biologics, Memphis, TN, was used.

3.4 Implant design

Ten millimeters of 0.5-mm-diameter grade 5 Eli ASTM F136 medical-grade titanium wires were used for the representation of dental titanium implants. A total of 12 titanium wires were used in this study.

3.5 Surgical procedure and implantation

The rats were acclimated to the new environment for one week before starting the experiment. 11-week-old male SD rats were operated on under conditions of general anesthesia using 2.0–5.0% isoflurane with an induction box. The rats were provided with 12.5 mg/kg of tramadol (Vesco Pharmaceutical, Bangkok, Thailand) as a preoperative analgesic drug. For maintenance of anesthesia, the operation used 0.5–3% Isoflurane as an inhaled anesthetic agent using a nose cone.

A subcutaneous injection of 2 mg/kg of lidocaine (LOCANA 2%) on the incision line was performed for local anesthesia. A vertical incision, 10-mm long, was made at the knee joint of the hindlimbs. The distal femurs were exposed after dislocating the patella and joint tissue. A 0.6-mm circumferential hole was created by a 25-gauge needle from the femoral intercondylar fossa, parallel to the long axis of bone. After preparation, the hole was irrigated and then packed with sterile cotton gauze to stop bleeding. The hole defects were divided into three groups at random (Table 1). The 50 µl of 15 µg of m1Ψ-BMP-2 mRNA, 50 µl of 4 µg of rhBMP-2, or 50 µl of dPBS were injected by a 27-gauge insulin needle, respectively. Following that, the titanium implants were inserted into the hole on both sides at the distal femurs. Bone wax W810 (Ethicon®, NJ, USA) was used to close the opening site. The knee joints were repositioned. The flap was closed and sutured with 4-0 nylon. Drops of 0.5% bupivacaine were locally applied at the incision line. The aseptic technique was applied in all procedures.

3.6 Specimen collection

At 6 weeks after implantation, rats were sacrificed by CO_2 inhalation at 30-70% displacement rate of chamber volume. The 6 rats (12 femurs) were harvested, cleared of all soft tissue, and disinfected with 2% chlorhexidine and 70% ethanol. Thirteen millimeters of the knee joint were cut with a carborundum disc and then immediately immersed in 10% neutral buffered formalin overnight and kept at 4°C to preserve biological tissue for micro-computed tomography analysis.

3.7 Micro-computed Tomography Imaging Analysis

The specimens were then rinsed with PBS before being analyzed with micro-CT imaging (µCT 35, Scanco Medical AG) in a high-resolution scanning mode with a 20-mm-diameter cylindrical holder. The following micro-CT settings were used: 70 kVp, 114 µA, 8W, and a voxel size of 6 µm. The specimens were scanned at the region of interest, which is 8-10 mm from the insertion point (Figure 1). The %BV/TV, Tb.N, Tb.Th and BIC were analyzed.

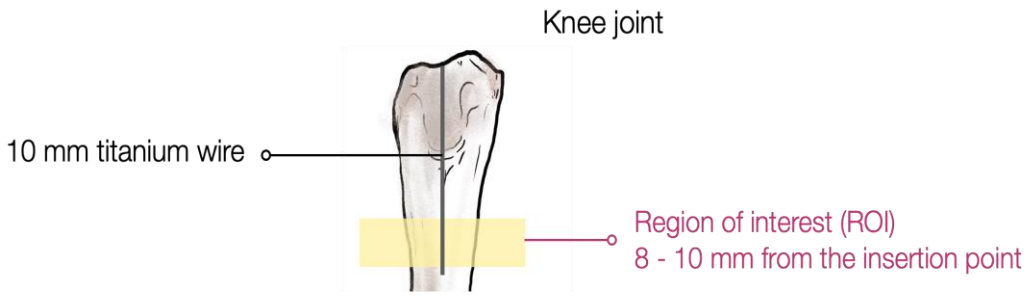

Figure 1 Region of interest (ROI) for micro-computed tomography analysis

3.8 Statistical analysis

The data were analyzed using SPSS version 22.0 (SPSS Inc, Chicago, Illinois, USA). The data was a normal distribution. To compare the differences of BMP-2 effectiveness, the data with normal distribution and equal standard deviation between three groups was analyzed with one-way ANOVA followed by Post Hoc Tukey's Honestly Significant Difference. All statistical analysis was performed at a significant level of 5% (p-value < 0.05).

4. Results and Discussion

4.1 Results

Three-dimensional micro-CT images showed bone formation in the m1Ψ-BMP-2 mRNA group was greater than the other two groups (Figure 2).

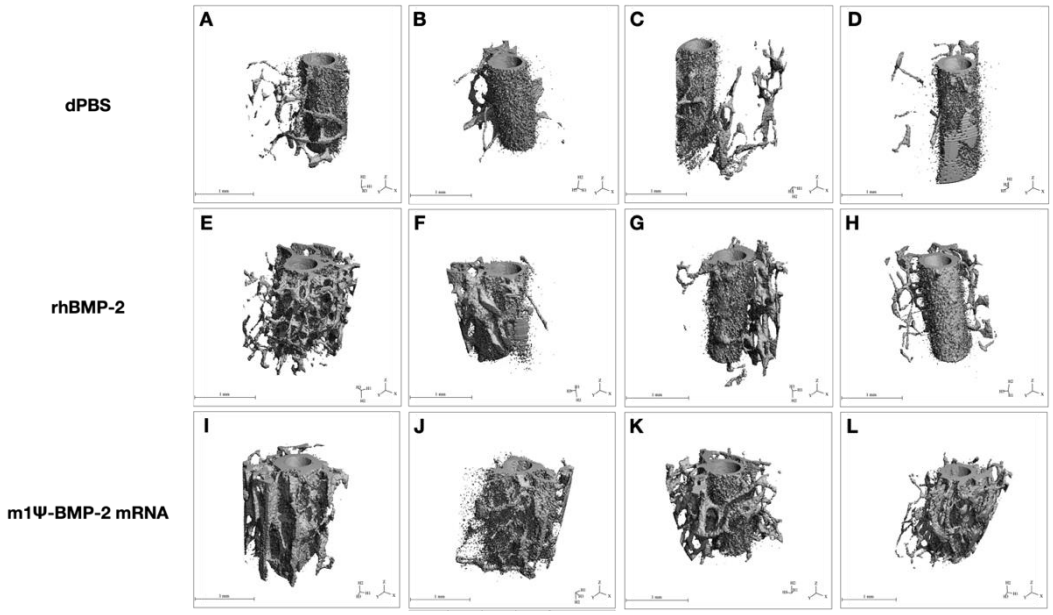

Figure 2 Representative 3D micro-computed tomography images of bone volume at 6 weeks after implantation in the ROI. The 3D images perform dPBS group (A, B, C, D), rhBMP-2 group (E, F, G, H) and m1Ψ-BMP-2 mRNA group (I, J, K, L)

As shown in Table 2, the volume of bone formation in the BMP-2-added groups was observed to be especially high in the m1Ψ-BMP-2 mRNA group at 6 weeks after implantation. The amount of bone formation was rather low in the dPBS group. Bone volume and trabecular micro-architecture parameters, which are %BV/TV, Tb.N, and BIC, were significantly greater in the m1Ψ-BMP-2 mRNA group than the dPBS group. While there was no significant difference between the m1Ψ-BMP-2 mRNA and rhBMP-2 groups.

Table 2 Comparison of bone formation and trabecular micro-architecture at 6 weeks after implantation within the region of interest (ROI)

Groups	Substance	Micro-computed tomography analysis			
		BV/TV (%)	Tb.N (1/mm)	Tb.Th (mm)	BIC (%)
1	dPBS	5.52 ± 1.23 [a]	2.21 ± 0.34 [a]	0.0529 ± 0.0064 [a]	14.05 ± 1.92 [a]
2	rhBMP-2	8.41 ± 2.74 [ab]	2.96 ± 0.45 [ab]	0.0580 ± 0.0039 [a]	18.91 ± 6.11 [ab]
3	m1Ψ-BMP-2 mRNA	12.06 ± 3.63 [b]	3.19 ± 0.42 [b]	0.0692 ± 0.0135 [a]	31.60 ± 13.17 [b]

The data is presented as the mean ± SD. The different letters (a and b) in each column indicate the statistically different comparisons after one-way ANOVA ($P < 0.05$) within the subgroups (BV/TV = bone volume fraction; Tb.N = trabecular number; Tb.Th = trabecular thickness; and BIC = bone to implant contact)

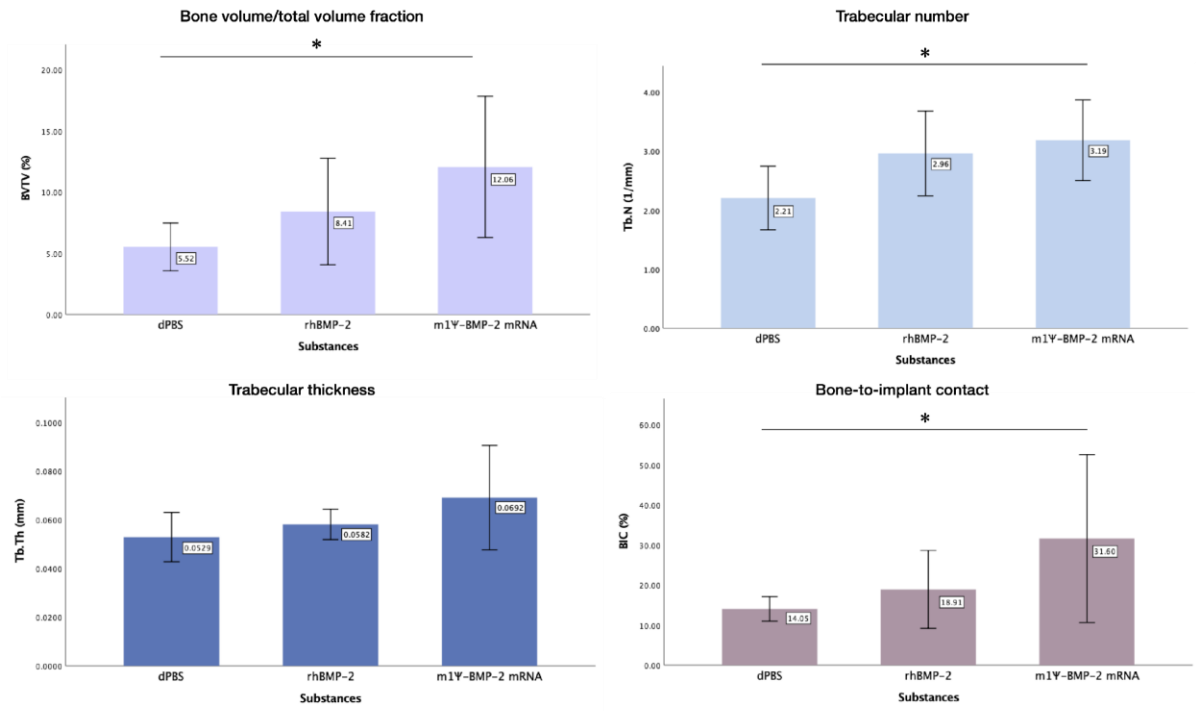

Figure 3 Comparison of bone formation and trabecular micro-architecture at 6 weeks after implantation within the region of interest (ROI). BV/TV = bone volume fraction; Tb.N = trabecular number; Tb.Th = trabecular thickness; and BIC = bone to implant contact. * Indicates significant difference, $P < 0.05$

4.2 Discussion

BMP-2 can enhance bone regeneration and osseointegration around implant materials. Several previous studies successfully demonstrated its efficacy in both in vitro and in vivo models (Ashinoff et al., 2004; Einhorn & Gerstenfeld, 2015; Govender et al., 2002). mRNA technology has been utilized to alleviate the shortcomings of conventional recombinant protein treatment, such as instability and inflammation caused by the use of supraphysiologic protein dosages. In this study, m1Ψ-BMP-2 mRNA was used to promote bone regeneration in rat femur defects determined by micro-CT between m1Ψ-BMP-2 mRNA group and control groups after implant placement.

Micro-CT is a technique that employs X-rays to generate three-dimensional (3D) pictures from two-dimensional (2D) trans-axial projections. Micro-CT utilized the same technique as medical CT, which was developed in the 1970s. However, it operates on a smaller scale and has a greater resolution than medical CT, which can operate at a resolution of one micron (Boerckel et al., 2014). It is the gold standard for evaluating the 3D form and microstructure of bone in the tiny models used for the current quantitative analysis of bone regeneration data using criteria similar to those of histomorphometry. Also, micro-CT is the least invasive technique and requires less time to analyze than other techniques since the material requires less preparation. Micro-CT was used to evaluate the amount of bone mineralization and new bone formation in the rat femur (Kim et al., 2021; Zeitoun et al., 2019). However, a limitation of micro-CT is the scattering from metal implant materials that causes image artifacts and mistaken data (Liu et al., 2012). The location for dependable micro-CT bone analysis to evaluate the bone around implant areas should be at least 24 μm away from the implant surface, according to Butz et al. (2006), who found a good association between BV/TV and the histological bone structure in a zone stretching from 24 μm to 240 μm.

In this study, the quantity of regenerated bone tissue was evaluated using a micro-CT scan. The %BV/TV, Tb.N and Tb.Th in the m1Ψ-BMP-2 mRNA and rhBMP-2 groups were greater than the control group, respectively. The %BV/TV and Tb.N in the m1Ψ-BMP-2 mRNA group were significantly greater than those in the control group, correlating with previous studies indicating that the BMP-2-added group was effective in the bone tissue regeneration as determined by the trabecular bone microarchitecture and that mRNA-based therapy promotes tissue regeneration more effectively than protein and DNA-based therapies (Balmayor et al., 2016; Elangovan et al., 2015; Geng et al., 2021; Nosho et al., 2020; Schwarz et al., 2018). Although, %BV/TV and Tb.N in the m1Ψ-BMP-2 mRNA group showed a significant difference with the dPBS group, there was no significant difference between the rhBMP-2 and dPBS groups due to the instability and rapid resorption of protein during interaction with tissues (El Bialy et al., 2017). Tb.Th showed an increasing trend in the rhBMP-2 and m1Ψ-BMP-2 mRNA groups, while the difference between the groups was not statistically significant based on previous research (De La Vega et al., 2022; Yao et al., 2022). Although Tb.N and Tb.Th are related to the quantitative morphology of trabecular bone, the measurements may create errors because they were determined indirectly from a fixed-structure model, either a rodlike or platelike structure. In contrast, the actual form may consist of rods mixing with plates, with the precise composition varying by location (Bouxsein et al., 2010). Moreover, increasing the number of sample sizes and duration of the experiment may lead to better outcomes. The BIC levels in the m1Ψ-BMP-2 mRNA group were significantly greater than those in the dPBS and rhBMP-2 groups, corresponding to a few prior studies (Fayed et al., 2021). This study was the first to establish that BMP-2 mRNA resulted in BIC capability when in contact with titanium implants in vivo. According to the experiment's positive control, rhBMP-2 has a favorable effect on bone tissue regeneration. Thus, m1Ψ-BMP-2 mRNA and rhBMP-2 provide comparable outcomes. However, we investigate BMP-2 mRNA since the instability of rhBMP-2 results in increased treatment costs that require a large amount of substance to be effective and long-lasting. Many undesirable effects may result from the supraphysiologic dosage (James et al., 2016; Shields et al., 2006; Zara et al., 2011).

However, in the mRNA group, m1Ψ-BMP-2 mRNA is chosen as the modified-nucleoside mRNA that comprises the LNPs' delivery vehicles. Although LNPs-mRNA stimulate high protein expression and preserve mRNA from degradation, it is known that they induce an innate immune response, which is a disadvantage of regenerative treatments compared to those employed in immunotherapy applications, such as vaccines (Vlatkovic, 2021). Additionally, inflammatory responses in the early stage of bone regeneration may be driven on by BMP-2 injection, according to the study that demonstrated how BMP-2 stimulates inflammation in the inflammatory and proliferative stages (Terheyden et al., 2012). Further studies can be done in the aspect of the inflammatory response after using m1Ψ-BMP-2 mRNA.

Several scaffolds, including collagen sponge and hydrogel, were used in previous experiments to hold substances in place and maintain their release (Geng et al., 2021; Lyu & Lee, 2020; Park et al., 2007; Sykaras et al., 2001). In contrast to these studies, scaffolds were not required since the experimental model site was the rat femur, a long bone consisting of thick cortical bone and porous trabecular bone in the middle, serving as a container for the substances. The porosity of trabecular bone facilitates the diffusion of substances, and mRNA technology enhances long-term protein synthesis outcomes. In our study, substances were injected directly into rat femurs, which is an uncomplicated, valuable, and time-saving procedure. Utilizing the scaffold, however, increases the unnecessary factor. The sort of scaffold chosen must be appropriate for the experimental design and biocompatible with the models. Consequently, this makes the results inaccurate and the interpretation more complex.

There were some limitations to this study. First, due to metallic implant material artifacts, micro-CT is not the greatest technique for assessing quantitative bone development. Although we made assessments in the appropriate position (Butz et al., 2006). To evaluate osseointegration, histomorphometric analysis needs to be correlated to determine the bone volume surrounding the implant. As a result, further investigation will be carried out. Second, the experiment was conducted using a rat model, thus the outcomes differed between rats, and humans. Rats have also been a common choice for human

pharmacology and toxicology research due to their small size, ease of reproduction and similarities in biology and behavior to humans. Although this experiment had a positive effect, it needs to be investigated in greater depth in future human studies.

5. Conclusion

This study revealed an effect of m1Ψ-BMP-2 mRNA on bone regeneration and osseointegration after implant placement in vivo. At 6 weeks, the results indicated that the m1Ψ-BMP-2 mRNA group had significantly greater %BV/TV, Tb.N, and BIC than the dPBS group. While there was no significant difference between the m1Ψ-BMP-2 mRNA and rhBMP-2 groups, further studies can be carried out in the aspect of the inflammatory response after using m1Ψ-BMP-2 mRNA, and investigation in greater depth in future human studies can be conducted.

6. Acknowledgements

This study was supported by the Asia Research Center, Chulalongkorn University (No. 007/2565) and Chulalongkorn University Laboratory Animal Center (CULAC). The authors sincerely thank Professor Dr. Rangsini Mahanonda for providing information on m1Ψ-BMP-2 mRNA. The authors report no conflict of interest related to this study.

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