의학박사 학위논문

Fat Graft with Allograft Adipose Matrix and Magnesium Hydroxide-Incorporated PLGA Microspheres for Effective Soft Tissue Reconstruction

효과적 연부조직 재건을 위한 동종지방기질 및 수산화마그네슘 함유 폴리(락틱-코-글리콜산) 미립구를 이용한 지방이식술

2022년 8월

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Fat Graft with Allograft Adipose Matrix and Magnesium Hydroxide-Incorporated PLGA Microspheres for Effective Soft Tissue Reconstruction

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초 록

연구배경

자가 지방이식은 외상이나 수술 후 연조직 결핍이나 함몰 기형을 교정하기 위해 성형외과 영역에서 널리 사용되는 수술 중 하나로, 공여부(donor site)의 이환(morbidity)을 줄이면서도 충분한 양의 조직을 비교적 손쉽게 확보할 수 있는 좋은 수술 방법이다. 그러나 수용부(recipient site)로의 혈류(vasucular), 대사(metabolic) 및 구조적(structural) 지원이 부족할 경우, 최종적으로 지방 이식의 생존에 영향을 주어 지방괴사(fat necrosis), 낭종형성(oil cyst) 및 석회화(calcification) 등과 같은 합병증을 유발할 수 있고 이는 특히 제한된 공간에 다량의 지방을 한 번에 이식하게 될 경우 더 문제가 될 수 있다. 최근에는 이식된 지방의 생착률을 높이기 위해 platelet rich plasma (PRP), stromal vascular fraction (SVF)이나 조직공학을 이용한 생체재료(biomaterial) 등을 활용한 다양한 연구들이 이루어지고 있다. 이들 중 동종지방기질(AAM) 혹은 poly(lactic-co-glycolic acid) (PLGA)와 같이 세포외기질(extracellular matrix)을 기반으로 한 스캐폴드(scaffold)들은 다양한 활용성을 지니며, 본 연구에서 저자들은 지방이식 시 발생할 수 있는 여러 문제점들을 줄이기 위한 노력의 일환으로 지방이식술 시행 시 이들 스캐폴드를 함께 사용해 보기로 하였고, 더불어 각각의 스캐폴드가 지방이식 시 이식된 지방의 생존률에 어떠한 영향을 미치는지 함께 비교분석해 보기로 하였다. 일반적으로 생체에 이식된 합성 고분자(synthetic polymer)가 생분해(biodegradation)될 때 발생하는 산성 부산물(acidic byproduct)들이 주변 조직을 괴사시키거나 염증을 일으키는 등의 문제를 야기할 수 있다고 알려져 있다. 이에 저자들은 알칼리성 물질인 수산화마그네슘 [MH, $Mg(OH)_2$]이 생분해된 PLGA의 부산물과 반응해 중

화반응을 일으켜 상기 문제들을 예방해 줄 수 있다는 기존의 연구 결과를 토대로 지방이식술에서도 이식된 스캐폴드 본연의 역할을 수행하면서 이식된 지방의 생착률을 높일 수 있을 것이라는 가정 하에 PLGA를 수산화마그네슘[$Mg(OH)_2$]과 결합시킨 합성 PLGA/MH 미립구(microsphere)를 제작해 수산화마그네슘의 역할을 검증하고 더불어 지방이식술 시행 시 PLGA/MH 순기능을 확인해 보기로 하였다.

연구방법

AAM, PLGA, PLGA/MH 각 스캐폴드의 세포독성(cytotoxicity), 혈관신생(angiogenesis), 상처회복(wound healing)에 미치는 영향 등을 실험실에서 분석(*in vitro*)해 보았을 뿐 아니라, 이식된 지방의 생존률에 함께 이식한 이들 스캐폴드가 생체 내에서(*in vivo*) 어떠한 영향을 주는지에 대해서도 쥐를 이용한 동물실험을 통해 함께 확인하였다.

연구결과

실험실 분석에서 AAM과 PLGA/MH 스캐폴드에서는 세포독성(cytotoxicity)이 확인되지 않았고, 상처회복(wound healing)에 있어서도 탁월한 효과를 보인데 반해, PLGA 단독으로 사용한 스캐폴드의 경우에는 스캐폴드가 생분해 되면서 발생한 산성 부산물(acidic degradation byproduct)로 인해 세포의 생존력(cell viability)이 통계적으로 유의하게 감소함을 확인할 수 있었다. 생체 내 실험에서 지방이식만 단독으로 시행한 대조군에 비해 AAM과 PLGA/MH 스캐폴드를 함께 이식한 실험군에서 이식된 지방의 생존률이 높게 나타났고, 특히 PLGA/MH 스캐폴드를 지방과 함께 이식한 군에서는 혈관신생(angiogenesis)과 항염(anti-inflammation)효과가 있음

을 확인할 수 있었다.

연구결론

본 연구를 통해 수산화마그네슘[$Mg(OH)_2$]은 pH를 중화해 줌으로써 PLGA/MH 미립구(microsphere)를 생체 내에 이식할 경우 발생하는 산성 부산물(acidic byproduct)로 인한 염증(inflammation)반응과 이로 인한 세포 독성(cytotixicity)을 예방해 줄 수 있음이 실험적으로 입증되었다. 또한, PLGA/MH 미립구는 신생혈관의 성장을 유도(angiogenesis)하는 등 지방이식술과 함께 사용할 경우 이식된 지방의 생착률을 제고하는데 상당한 도움을 줄 수 있어 임상에서 다량의 지방이식 시 발생할 수 있는 여러 합병증을 예방하는 데 좋은 대안이 될 수 있을 것으로 사료된다.

주요어 : 지방이식, 동종지방기질(AAM), PLGA, PLGA/MH 미립구, 수산화마그네슘[$Mg(OH)_2$], 혈관생성, 지방이식 생착률

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Table of Contents

1. Introduction ····· 1

2. Materials and Methods ····· 5

Materials ····· 5
Fabrication of the PLGA microspheres ····· 5
Scaffold characterization and *in vitro* degradation test ····· 6
Cell cytotoxicity assay ····· 6
In vitro wound healing assay ····· 7
Quantitative real-time PCR ····· 7
Fat preparation ····· 8
Allograft adipose matrix preparation ····· 8
Animal study ····· 8
Histological analysis ····· 9
Statistical analysis ····· 10

3. Results and Discussion ····· 10

Scaffold fabrication and characterization ····· 10
In vitro biocompatibility and anti-inflammatory effect of scaffold ····· 11
Wound healing and angiogenic effect of the scaffold ····· 14
Volume retention effect of the scaffold for fat grafting survival ····· 15
In vivo evaluation of the scaffold for fat grafting survival ····· 17

4. Conclusions ····· 19

References ····· 21

Abstract ····· 26

Acknowledgements ····· 28

List of Abbreviations

Abbreviation	Meaning
PLGA	Poly(lactic-co-glycolic acid)
AAM	Allograft adipose matrix
MH	Magnesium hydroxide
PLGA/MH	Magnesium hydroxide-incorporated PLGA
PRP	Platelet rich plasma
SVF	Stromal vascular fraction
ADSCs	Adipose-derived stem cells
PLLA	Poly(L-lactic acid)
PGA	Polyglycolic acid
PEG	Polyethylene glycol
TGA	Thermogravimetric analysis
qRT-PCR	Quantitative real time polymerase chain reaction
hDF	Human dermal fibroblast
IL	Interleukin
TNF	Tumor necrosis factor
MMP2	Matrix metalloproteinase-2
VEGF	Vascular endothelial growth factor
αSMA	Alpha smooth muscle actin
ANGPT2	Angiopoietin-2
PPARG	Peroxisome proliferator activated receptor gamma
AP2	Adipocyte protein 2
CD31	Cluster of differentiation 31
IHC	Immunohistochemistry

List of Figures

[Figure 1] ····· 11
[Figure 2] ····· 13
[Figure 3] ····· 13
[Figure 4] ····· 15
[Figure 5] ····· 16
[Figure 6] ····· 18

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1. Introduction

Reconstructive surgery refers to surgical procedures to restore the appearance and function of a damaged part of the body. This includes breast reconstruction, mainly in the area of mastectomies and repair of congenital defects, extremity reconstruction related to trauma, wound defects, tumor resection, and facial reconstructive surgeries. The current global reconstructive market is estimated to exceed $1 billion annually. Additionally, the market for reconstructive surgery is predicted to grow and in the case of Europe, market growth is accelerating. There are lots of market drivers responsible for the growth. But, there are also several market constraints, primarily based on the current technologies available to the market. Therefore, if there were more options in the reconstructive surgery, such as clinically practical adipose tissue engineering technology, additional growth of these markets could be facilitated. These need could lead to significant market driver for adipose tissue grafting. The reconstructive surgery is closely linked to the cosmetic surgery. Whereas reconstruction is done to repair the deficit, cosmetic surgery could enhance the appearance or enhance the status of the body. The global cosmetic procedure market is estimated to be $30 billion with growth rate of 25% annually. Because the cosmetic and reconstructive surgery depends on similar procedures and materials, the new technology developed by using adipose tissue engineering would affect both markets. Autologous fat grafting is a useful procedure for soft tissue reconstruction since it has plenty of advantages, including minimal invasiveness, easy accessibility, and less donor site morbidity[1]. It is not a simple procedure and should be performed only by skilled plastic surgeons. Recently, it has been popular as an alternative to flap surgery or injectable fillers because it has the ability to replace volume without significant donor site morbidity and down the cost. However, their clinical outcomes are often suboptimal, unpredictable, and sometimes produce unpleasant complications, such as oil cyst,

fat necrosis, and calcification which were mainly caused by vascular insufficiency of recipient site and/or large volume bolus fat graft[2,3]. The new cosmetic techniques such as a micro-fat graft (tiny volume fat graft) has been introduced because large volume bolus fat graft, for regenerating soft tissue defects, has frequently resulted in complications like calcification and oil cysts. Furthermore, recent researches have focused to improve the retention rate of transplanted fat with a combination of various additives such as platelet rich plasma (PRP), stromal vascular fraction (SVF)[2,3], hyperbaric oxygen[4], and tissue engineering scaffold[5]. Autologous platelet- rich plasma (PRP) is growth factor rich material that can be generated quickly and cost effectively from a patient's own blood. Over 800 different proteins have been identified to be secreted by platelets into the plasma, which affect a wide range of cells in the body. In terms of improving fat grafting, PRP has multiple potential benefits. The growth factor in PRP allows cells to resist the hypoxic stress experienced within the first few days after fat transfer and promote proper arrangement of transplanted tissue by producing the extracellular matrix, and also promote angiogenesis. Stromal vascular fraction (SVF) consists of multipotent elements including adipose-derived stem cells (ADSCs), endothelial cells, fibroblasts, pericytes, and other immune cells that are easily extracted from adipose tissues. SVF has been applied for treatment of scar-related conditions related to its regenerative potential, including the release of growth factors and activation of dermal angiogenesis[6]. So it also might improve the survival rate in case of fat graft. Although fat grafting and biomaterials have been widely used in clinical practice, problems such as multiple operations because of absorption and rejection reactions are not completely solved. Recently many studies of the combination of injectable biomaterials with fat transplantation show great potential in preclinical studies. Among the new approaches for effective soft tissue reconstruction, allograft adipose matrix (AAM) has been recently investigated as a natural scaffold to promote adipose tissue regeneration[7]. AAM is obtained through

decellularization of allogeneic adipose tissue to retain the structural and biochemical properties of native matrix that would give support of adipogenesis[8-11]. In recent studies, AAM would promote soft tissue reconstruction by preventing the ischemic necrosis inside grafted tissue[9-11]. Giatsidis *et al.* investigated that the combination of external volume expansion method and AAM for soft tissue reconstruction results in high volume retention and tissue preservation by adipogenesis and angiogenesis[9]. Additionally, the various synthetic polymers have been applied as synthetic scaffolds, including poly(L-lactic acid) (PLLA), polyglycolic acid (PGA), poly(lactic-co-glycolic acid) (PLGA), polyethylene glycol (PEG), polyethylene terephthalate, polytetrafluoroethylene, and polyethylene[12-16]. Among them, PLGA and its derivatives were approved by the Food and Drug Administration (FDA) in the fields of medical devices[17]. The PLGA microsphere has been widely used for various tissue regeneration including soft tissue because of its great degradability, biocompatibility, and usability to control shape and size[15]. For a polyester-based synthetic biodegradable polymers formulation to be successfully translated clinically, it must be non-toxic after *in vivo* implantation. However, synthetic polyester polymers generate acidic byproducts while being hydrolyzed at implanting site *in vivo*. The acidic degradation products of polymer can cause inflammation and necrosis of the surrounding tissue[18-20]. To address this issue, magnesium hydroxide [MH, $Mg(OH)_2$] was adapted in this study. In our group's previous studies, MH nanoparticles showed a great pH neutralization effect on acidic decomposition products from polyester polymers in various biomedical fields[14,17,19,21,22]. Magnesium hydroxide is an alkaline ceramic particle used clinically as an antacid. MH is a suitable candidate for neutralizing acidic byproducts in the process of decomposing polyester polymers because it has a pH neutralization ability and biocompatibility superior to those of other alkaline ceramic particles. However,

basically it is challenging to encapsulate MH particle in polymeric microspheres. Because MH particles are hydrophilic, they do not disperse easily in the oil phase, and most of the MH particles escape to the aqueous phase during the manufacturing process. The low encapsulation efficiency of MH particles in the microspheres limits the effective neutralization of acid[23]. In these case, we are able to improve the encapsulation efficiency of MH in polymeric microspheres by modifying the surface of MH with hydrophobic lipids to suppress inflammation through effective pH neutralization. MH particles in the PLGA composite effectively prevents PLGA-induced pathological responses. Furthermore, Mg^{2+} released from MH directly affects the function of endothelial cells, which play a crucial role in maintaing the functional integrity of the vascular wall in both intracellular and extracellular spaces and prevents the cellular stress responses against the degradation products of PLGA and improve endothelial function[23]. In this study, for effective fat graft with natural extracellular matrix-based scaffolds, namely AAM and synthetic biodegradable polymer scaffold (PLGA) were used as regenerative scaffolds[4,5]. We attempted to combine fat graft with regenerative scaffolds to reduce the amount of fat graft for achieving a target volume, and at the same time to increase retention volume of fat graft. We compared the volume retention rate of each scaffold with fat graft, including characterization of AAM, PLGA, and PLGA/MH through *in vitro* degradation test, cell cytotoxicity, and wound healing. In addition, we investigated how these regenerative scaffolds affect fat graft survival and volume retention through an animal study.

2. Materials and Methods

Materials

Poly(D,L-lactide-co-glycolide) (PLGA, lactide:glycolide = 50:50, Mw; 110 kDa) was purchased from Evonik Ind. (Essen, Germany). Magnesium hydroxide [MH, $Mg(OH)_2$] and polyvinylalcohol (PVA,87–90% hydrolyzed, average Mw; 30–70 kDa) were purchased from Sigma-Aldrich (St Louis, USA). Dichloromethane (DCM) was obtained from Daejung Chemicals (Korea). Allograft adipose matrix (AAM) was processed by CG Bio Co. (Seongnam, Korea).

Fabrication of the PLGA microspheres

A water-in-oil-in-water (w/o/w) double emulsion-solvent evaporation method was employed. A solution of PVA (2 w/v% in deionized water) was prepared for the external aqueous phase, and 1 ml DCM containing 20 mg of PLGA was used as internal oil phase. The mixture was homogenized at 800 rpm for 1 min (Silverson, L5M-A) and dried for 150 min to completely evaporate DCM at room temperature. The prepared emulsion was sifted out using sieve to targeted sizes. Microspheres were then washed in deionized water for 4–5 times being centrifuged at 3500 rpm for 15 min removing impurities. Finally, the purified microspheres were put in liquid nitrogen then immediately lyophilized (-80 ℃) for at least 2 days.

The MH-loaded PLGA microspheres were prepared by dispersing MH (30 wt%) in PLGA solution sonicating at an amplitude of 40% power for 30 sec. Making PLGA/MH solution in advance is required to be injected into external aqueous phase. PLGA/MH microspheres were also fabricated following the above process.

Scaffold characterization and *in vitro* degradation test

The prepared microspheres were visualized by optical microscopy (CKX53, Olympus, Japan), and the size was determined with supplied software. The surface morphology of the scaffolds was observed using scanning electron microscopy (SEM; GENESIS-1000, Emcraft, Gwangju, Korea). The thermal property of the scaffolds was analyzed by a thermogravimetric analyzer (TGA 4000, PerkinElmer, Waltham, MA, USA).

To assess the neutralization capacity of the PLGA/MH microsphere, the mass and pH changes were measured in 0.5 ml phosphate buffered saline (PBS) solution (pH 7.4) at 37 °C for 60 days. The change in the pH value of the PBS solution was evaluated using a digital pH-meter (FP20, Mettler-Toledo GmbH, Schwerzenbach, Switzerland).

Cell cytotoxicity assay

Human dermal fibroblasts (hDFs) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, UT, USA) containing 10% fetal bovine serum (FBS; Hyclone) and 1% antibiotic-antimycotic solution (Gibco, Grand Island, NY, USA) at 37°C in a humidified atmosphere with 5% carbon dioxide (CO_2). The viability of the cells was determined using a live-dead viability/cytotoxicity kit (Invitrogen, Thermo Scientific Inc.,Waltham, MS, USA) and the fluorescence images were obtained using LSM880 (Zeiss, Jena, Germany). D-Plus™ cell counting kit 8 (CCK-8) cell viability assay kit was obtained from Dongin LS (Seoul, Korea) following manufacturer's instructions.

In vitro wound healing assay

Human dermal fibroblasts (hDFs) were seeded at a density of 2×10^5 cells/well in 6 cell tissue culture plate and cultured to form a confluent monolayer. The layer of cells was scraped with 1ml micropipette tip straightly to create an artificial wound. The culture plate was washed three times with PBS solution to remove the detached cell debris and then treated with the scaffolds using trans-well inserts in DMEM containing 1% FBS and 1% antibiotic-antimycotic solution for 24h. Wound closure was calculated as a percentage of the initial wound area, quantitated using NIH Image J software.

Quantitative real-time PCR (qRT-PCR)

The gene expressions of an anti-inflammation and angiogenesis *in vitro* were analyzed by qRT-PCR. Total cellular RNA from hDFs was isolated using the AccuPrep® Universal RNA Extraction Kit (Bioneer, Daejeon-si, Korea) following the manufacturer's instructions. The cDNA form isolated RNA was synthesized using PrimeScript RT Reagent Kit (Perfect Real Time, Takara, Japan). The qRT-PCR was performed using each primer and SYBR Green PCR Master Mix (Applied Biosystems, Thermo Scientific Inc., Waltham, MS, USA). The expression of angiogenic and inflammation related genes was calculated with the 18S rRNA as a reference gene using the $2^{-\Delta\Delta Ct}$ method. The gene expressions of adipocyte and angiogenesis *in vivo* were assessed by qRT-PCR same as *in vitro* evaluation.

Fat preparation

The study was performed after approval by the CHA University Bundang CHA Medical Center Internal Review Board (CHAMC 2020-03-013-003). Lipoaspirate (approximately 50 cc) was obtained by manual liposuction (Colemann technique) in the patient who underwent abdominoplasty and processed in a sterile fashion. The lipoaspirate was centrifuged at approximately 1800 g for 3 min to separate the adipose tissue from the oil part and the stromal vascular fraction: oil component was discarded to obtain the processed adipose tissue.

Allograft adipose matrix preparation

Subcutaneous adipose tissues were obtained from donated human adipose tissue of woman who underwent abdominoplasty. Allograft adipose matrix (AAM) was processed as follows. The tissue was placed in isopropanol (Junsei, Japan) and treated using a shaking incubator at 37°C for 12 h twice. The tissue was washed three times with PBS solution for 30 min each. This was followed by treatment with 1% SDS solution (Sigma-Aldrich) for 4 h at room temperature and washed overnight while replacing the PBS solution several times. After treatment in 0.1% peracetic acid solution (Sigma-Aldrich) for 2 h, it was washed three times for 30 min with distilled water. Samples were lyophilized and pulverized with a laboratory blender (Waring Products Co., USA) to obtained ECM powder. The powder and distilled water were mixed in a 1:4 ratio and radiation sterilized[7,23].

Animal study

The animal experimental protocols for the use of animals were approved by the Institutional Animal Care and Use Committee of CHA University (IACUC

approved number 200074) and carried out under the guidelines of the IACUC. A total of eight male 8-week-old BALB/c nude mice (Orient Bio Co., Seongnam, Korea) were used for this study and allowed to acclimatize for 2 weeks before the experiments. We allocated fat graft and regenerative scaffolds into four groups, including fat graft alone (group 1), fat graft with AAM (group 2), fat graft with PLGA/MH (group 3), and AAM alone (group 4). Processed lipoaspirate and/or rehydrated scaffolds (total 0.4 ml injection/each site) were injected into the subcutaneous layer of equally allocated four sites on each dorsum of 8 athymic nude mice under inhalation anesthesia using 2% isoflurane (Terel solution, Hana Pharm) gas. The grafted materials were harvested after 8 weeks, and each specimen volume was evaluated.

Histological analysis

Grafts from BALB/c nude mice were harvested 8 weeks after the experiment. Grafted tissue samples were fixed in 4% paraformaldehyde for a minimum of 48 h. The samples were processed using the standard method and prepared for paraffin tissue slides. The paraffin sections of 5 μm thickness were stained with hematoxylin and eosin (H&E) and immunohistochemistry (IHC) using anti-perilipin-1 antibody (Abcame, ab3526, 1:200 dilution) and anti-CD31 antibody (Abcam, ab28364, 1:200 dilution). The secondary antibody (Alexa 555 Invitrogen, A21428) was incubated at room temperature for 2 h and nuclear staining was performed using DAPI. Fluorescent signals from graft tissue were visualized using a Nikon Microscopy (ECLIPSE 50i, Nikon Inc., Tokyo, Japan) and slide scanner (Zeiss Axio scan). Images were acquired using an Olympus DP71 digital fluorescence microscopy.

Statistical analysis

All experiments were repeated at least three times. The results are shown as the means ± standard error of the mean (SEM). Statistically significant differences were evaluated by one-way ANOVA using GraphPad Prism 7.0 software (GraphPad Software, Inc., CA). p values < 0.05 were regarded as statistically significant.

3. Results and Discussion

Scaffold fabrication and characterization

Degradable polymer microspheres have been widely used in the fields of medical devices and medical applications. Among them, polymeric microspheres have been utilized for numerous biomedical applications such as tissue engineering and regeneration[26], drug or cell delivery[15], and cancer therapy[27]. When the polymeric microspheres are used as a biomaterial safely in clinic, the particle size of the microspheres should be consistently controlled. If the particle size is too small (less than 40 μm), the microspheres can cause capillary embolism[28]. As shown in Figure 1, the surface of the biodegradable scaffolds was analyzed through the scanning electron microscopy (SEM). Based on representative SEM images, both of microspheres were observed with uniform size and smooth surface. And the AAM was full of the fibers of extracellular matrix. The amount of MH encapsulated in the PLGA microspheres was analyzed using thermal gravimetric analyzer (TGA). The content of MH in PLGA/MH microspheres was 21%. Therefore, the initial weight loss of the PLGA/MH was shown faster than the PLGA group. To assess an acid neutralization of MH enclosed PLGA microspheres, pH value was evaluated during degradation for 60 days (Figure 1D). In day 1, pH value slightly increased

in the PLGA/MH microspheres group due to initial release of MH in the surface. However, during polymer degradation, it performed great neutralization against acidic byproducts, lactic acid and glycolic acid.

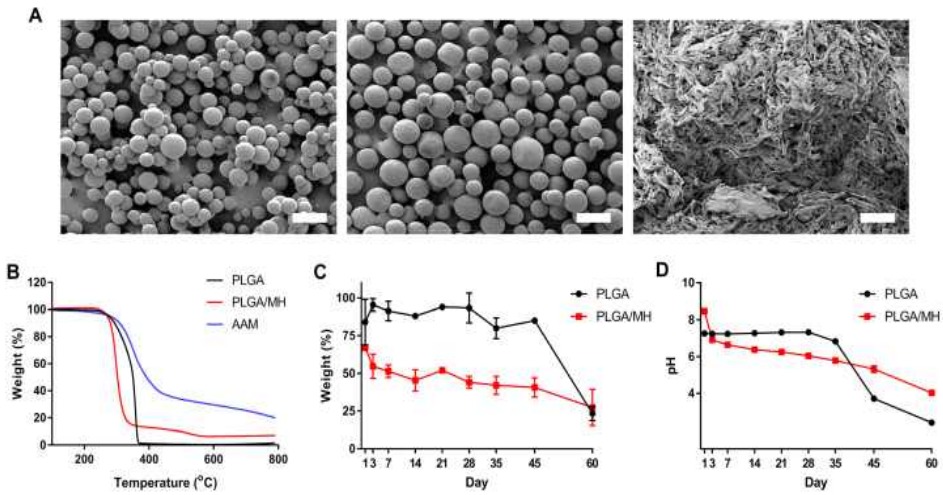

Fig.1 Scaffold characterization. A representative scanning electron microscopy (SEM) images of the PLGA microsphere. PLGA/MH microsphere, and AAM (scale bar = 50μm). **B** thermal gravimetric analysis (TGA) thermograms of each scaffolds. **C** mass and **D** pH during in vitro degradation in PBS solution at 37℃ for 60 days.

In vitro biocompatibility and anti-inflammatory effect of the biodegradable scaffold

To investigate cytotoxicity of the scaffolds *in vitro*, in Figure 2A, calcein AM and ethidium homodimer 1 (EthD-1) stainings were conducted with hDF at 24 h. Because of its well-known biocompatibility of the PLGA and AAM, the EthD-1 positive cells indicating dead cells were observed rarely in all the microspheres even the PLGA only group. However, the morphology of hDFs was changed

abnormally. In Figure 2B, the cell viability was quantified by CCK-8 with same time point. In the only PLGA microspheres treated group, the cell viability significantly decreased than control due to its acidic degradation products ($p < 0.01$). Additionally, inflammation has been always considered as a critical challenge to develop the effective biomaterials. Quantitative real-time PCR (qRT-PCR) was conducted to determine the expression of pro-inflammatory cytokine genes by the biodegradable scaffolds using hDFs (Figure 3). The effect of the scaffolds was assessed with indirect cell culture system using trans-well. As a result, the PLGA microspheres increased the expression of pro-inflammatory cytokine genes, interleukin-6 (IL-6) and interleukin-1β (IL-1β) compared to normal hDFs as a control group. However, the PLGA/MH microspheres restricted or approximated the expression levels of IL-6, IL-1β, and tumor necrosis factor-α (TNF-α) compared to control. Especially, the expression of IL-1β significantly increased in the PLGA microspheres compared to control ($p < 0.001$) and statistically significant decreased in the PLGA/MH ($p < 0.05$). There were no statistical differences of the pro-inflammatory gene expressions in the AAM group compared to control. Consequently, AAM was not significantly related to the inflammatory response, and microspheres fabricated only with PLGA induced inflammation, but PLGA microspheres containing MH can greatly attenuate inflammatory response.

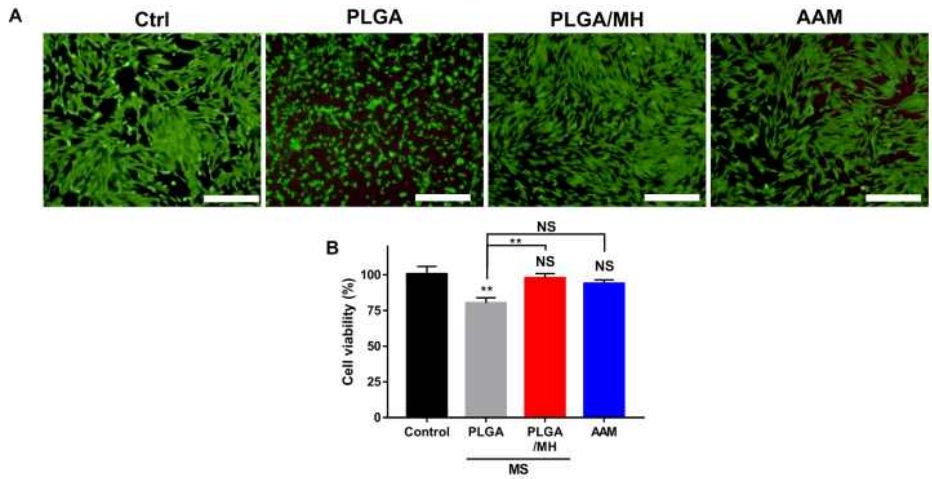

Fig2. Biocompatibility of the scaffolds. A live-dead assay images at 24 h (scale bar = 500μm). **B** cell viability of the hDFs onto each scaffold at 24 h *in vitro*. The differences were considered significant when NS= not significant ($p \geq 0.05$), *p <0.05, and **p<0.01 (n≥3)

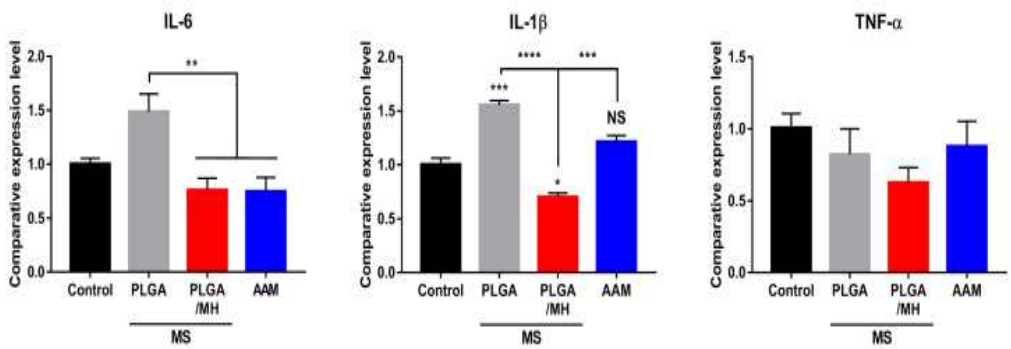

Fig3. Anti-inflammatory effects on the scaffolds using hDFs. Gene expressions of hDFs related to inflammatory mediator levels: IL-6, IL-1β, and TNF-α. The differences were considered significant when NS= not significant ($p \geq 0.05$), *p <0.05, **p <0.01, ***p <0.001, and ****p <0.0001 (n≥3)

Wound healing and angiogenic effect of the biodegradable scaffold

To increase transplanted adipose tissue survival, various studies have been attempted to develop new grafting systems with sufficient angiogenic activity[30,31]. We also hypothesized that these new vessels would grow into grafted site during scaffolds degradation to assist retention and prevent apoptosis of the grafted fat. As shown in Figure 4, the biological ability of the scaffolds was investigated in aspects of angiogenesis and wound healing for grafted fat survival. Based on wound healing analysis, the wound closure rates also highly increased from 31.56 (in the control) to 53.98% in the PLGA/MH microspheres, and slightly increased to 39.14% in the AAM compared to control. The qRT-PCR was assessed to determine the expression of angiogenesis related genes on the scaffolds with hDFs. The expression levels of matrix metalloproteinase-2 (MMP2) slightly increased in the PLGA/MH and the AAM groups, and contrastively, significantly decreased in the PLGA microspheres. Vascular endothelial growth factor (VEGF) is known to be mainly involved blood vessel formation. The VEGF expressions notably increased in the PLGA/MH ($p < 0.0001$) and the AAM ($p < 0.001$). Interestingly, in the PLGA/MH group, all the angiogenic related mRNA expressions were upregulated; MMP2, VEGF, alpha smooth muscle actin (αSMA), and angiopoietin-2 (ANGPT2). Therefore, on the same side of Figures 2 and 3, the PLGA only microspheres would be insufficient candidate as an implanting scaffold for fat graft.

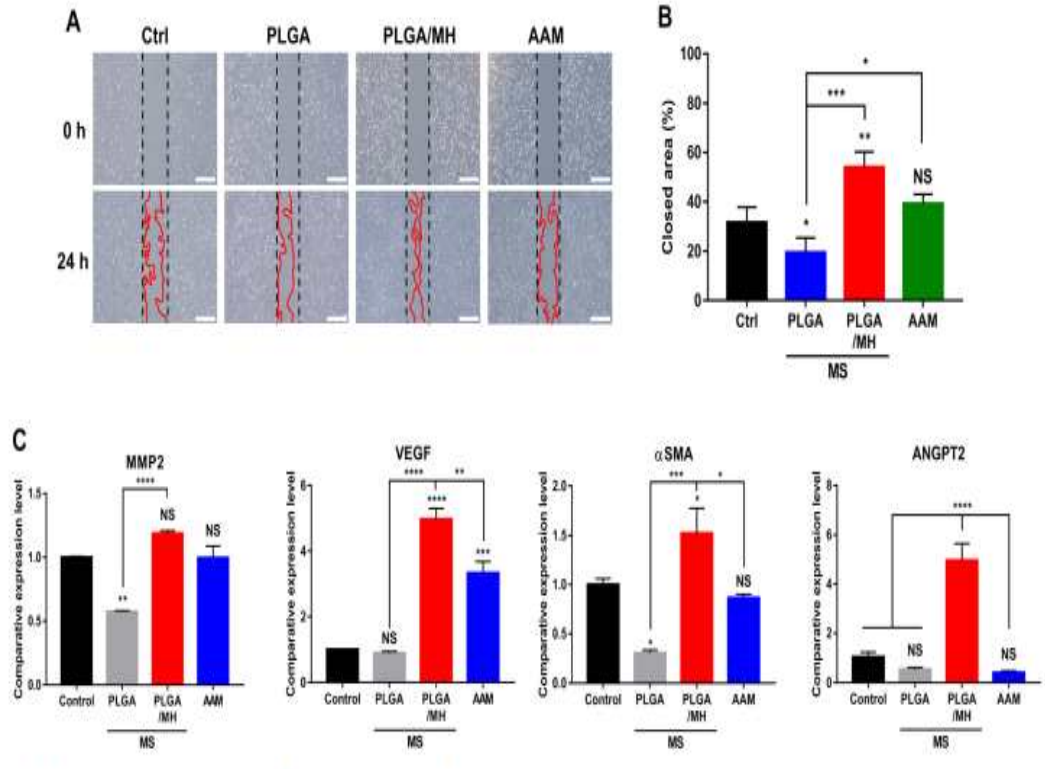

Fig4. Angiogenic effects on the scaffolds using hDFs. A, B Observation of wound healing effect using optical microscopic image and quantification (scale bar = 500μm). **C** Gene expression of hDFs onto the scaffolds related to angiogenesis: MMP2, VEFG, αSMAS, and ANGPT2. The differences were considered significant when NS= not significant ($p \geq 0.05$), *p <0.05, **p <0.01, ***p <0.001, and ****p <0.0001 (n≥3)

Volume retention effect of the biodegradable scaffolds for fat grafting survival

To investigate the actual volume retention ability, we conducted fat implantation with biodegradable scaffolds into the dorsum of mouse. Figure 5A shows the schematic illustration of *in vivo* implantation. After 8 weeks grafting, the only fat group was mostly absorbed. In the groups II and III, implant retention rate was

similar and the highest implant retention rate displayed in the group IV (AAM only group). In gross examination, specimens of groups I and II looked like fat tissues and those of group III exhibited a whitish yellow fat tissue. Watery contents were observed in the implanting site of group II (fat with AAM). On the other hand, the specimens of group IV demonstrated whitish mass surrounded vessels without identified capsule formation (Figure 5B). The average percentage of retention volume is 12.5% in the only fat group, 37.5% in the fat with AAM group, 40% in the fat with the PLGA/MH microsphere group, and 50% in the only AAM group. Especially, the AAM and the microsphere group showed high volume retention compared to the only fat grafted group (Figure 5C).

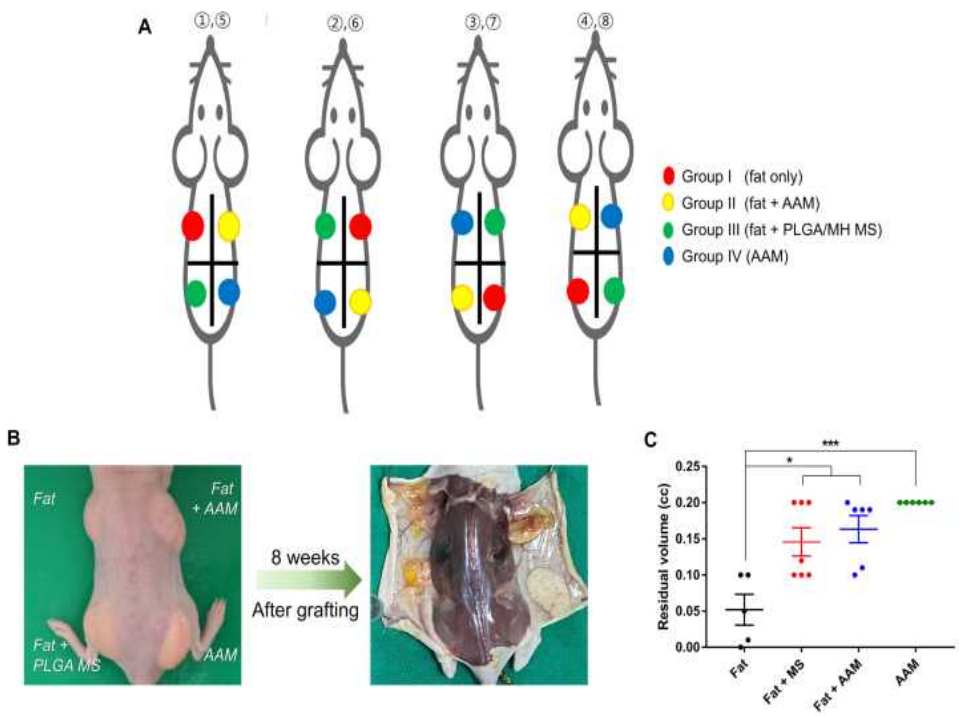

Fig5. A Schematic illustration of in vivo implantation, **B** the representative gross appearance of the biodegradable scaffolds, and **C** residual volume for 8 weeks after grafting. *p <0.05, and ***p <0.001 (n≥3)

In vivo evaluations of the biodegradable scaffolds for fat grafting survival

In order to analyze the residual tissue, histological analysis was conducted by H&E and IHC stains. Based on H&E images, lipid droplet-like structure was observed in Groups I, II, and III (the fat with AAM or PLGA/MH groups). On the other hand, the AAM only group was full of fibers same as Figure 6A and lipid droplet was not observed. In addition, perilipin-positive cells were distributed throughout the inside of the tissue in the group III (fat with PLGA/MH group). And a very small proportion of perilipin-positive cells were observed in the group II (fat with AAM group) because of its high-density fiber. To explore the effects of the scaffolds on neovascularization into grafted tissue, cluster of differentiation 31 (CD31) immunostaining was evaluated. The expression of CD31 (indicated with star mark*) was significantly elevated in the group III (fat with PLGA/MH) grafted tissue. In parallel, mRNA expression levels of adipocyte related genes peroxisome proliferator activated receptor gamma (PPARG) and adipocyte protein 2 (AP2) demonstrated higher in the group III (fat with PLGA/MH) (Figure 6B-D) than the fat with AAM and the AAM only. Besides, we observed the upregulated expression of the angiogenic gene expressions, VEGF and αSMA in the group II. The pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) were also statistically downregulated in the group III (fat with PLGA/MH group). Overall, on the basis of *in vivo* evaluations, although the AAM has great volume retention ability, the PLGA/MH microspheres have not only capability of volume retention, but also anti-inflammation effect and angiogenic ability. These prominent advantages could support to increase fat grafting rate in clinic.

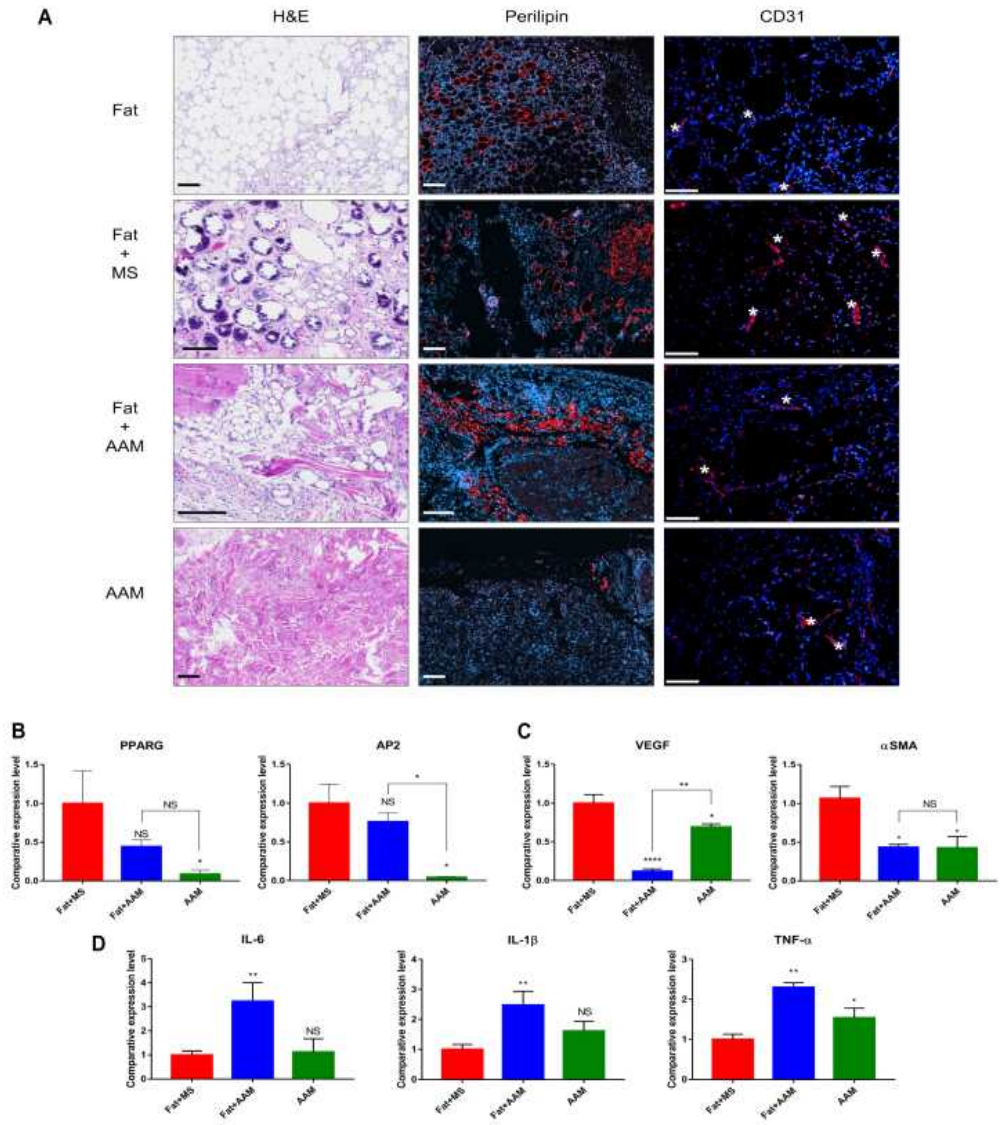

Fig6. *In vivo* evaluation for adipogenesis and angiogenesis of the biodegradable scaffolds. **A** Histological analysis using H&E (scale bar = 200μm) and immunofluorescence stain for perilipin (red, scale bar = 200μm) and CD31 (red, scale bar = 50μm). **B-D** Gene expressions related to adipocyte: PPARG, AP2; related to angiogenesis: MMP2, VEFG, αSMAS, and ANGPT2; and proinflammation cytokine: IL-6, IL-1β, and TNF-α. The differences were considered significant when NS= not significant ($p \geq 0.05$), *p <0.05, **p <0.01, ***p <0.001, and ****p <0.0001 (n≥3)

4. Conclusions

Fat grafting is one of most popular techniques to supplement the volume of soft tissue. For example, aging face, small soft tissue defects can be well corrected by fat grafting. Small volume fat grafts in the well vascularized area are well survived, but large volume fat grafts or fat grafts in the poor vascularized area have frequently shown oil cysts, calcifications within the grafted fat tissue. Recently, AAM filler was developed and used for soft tissue augmentation. Several studies have demonstrated fat regeneration within implanted AAM materials[7,32,33]. It sounded like a fancy material replacing fat grafting. In the contrary of those results, our study, unfortunately showed that fat regeneration was rarely found in the groups of fat graft combined with AAM and AAM only implantation. AAM showed outstanding ability to maintain the implanted tissue volume as a filler to augment the soft tissue, but lack of capability to regenerate the fat tissue. Besides AAM, in the group of fat graft combined with the PLGA/MH, increased volume retention was demonstrated compared to the fat only group, and the PLGA/MH microsphere further increased their vascularity to assist long-term survival (volume retention) of autologous adipose tissue grafts in a murine model. This study accessed the role of additive materials (PLGA/MH microspheres and AAM) in fat grafting. The PLGA/MH and AAM increased volume retention in comparison with only fat grafted group, and the PLGA/MH microspheres further attenuated inflammation and enhanced their vascularity to assist volume retention of autologous adipose tissue grafts in a murine model for 8 weeks. It could prevent to heading toward apoptosis of the grafted tissue. This study has limitation that the result showed a little bit short outcomes to evaluate the regeneration of the fat tissue. Therefore, our further study will be explored for extremely long-term evaluation of PLGA/MH microspheres *in vivo* with the retention volume, vascularization, and apoptosis on the grafted tissue. Taken together, this study demonstrates that the PLGA/MH microspheres can be a great additive biomaterial

for fat graft. This study demonstrates that additive scaffolds have a potential to increase the volume retention of fat graft with increase of vascularity or volume augmentation effect. Therefore, for the soft tissue defects required large volume supplement, fat graft with additive biocompatible scaffold such as the AAM or PLGA/MH seems to be more effective and less complicated than large volume fat graft only.

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Abstract

Fat Graft with Allograft Adipose Matrix and Magnesium Hydroxide-Incorporated PLGA Microspheres for Effective Soft Tissue Reconstruction

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Autologous fat grafting is one of the most common procedures used in plastic surgery to correct soft tissue deficiency or depression deformity. However, its clinical outcomes are often suboptimal, and lack of metabolic and architectural support at recipient sites affects fat graft survival leading to complications such as cyst formation and calcification. Extracellular matrix (ECM)-based scaffolds, such as allograft adipose matrix (AAM) and poly (lactic-co-glycolic acid) (PLGA), have shown exceptional clinical promise as regenerative scaffolds. AAM is composed of the extracellular matrices of adipose tissue through decellularization. Additionally, PLGA is one of the most commonly used biopolymers owing to its biodegradability

for tissue regenerations. Magnesium hydroxide (MH), an alkaline ceramic, has attracted attention as a potential additive to improve biocompatibility. Magnesium hydroxide (MH) could neutralize the acidic microenvironment induced by the acidic decomposed products of PLGA, thereby suppressing undesirable inflammatory reactions. We attempted to combine fat graft with injectable regenerative scaffolds: natural (AAM) and synthetic (PLGA/MH microspheres). We investigated the volume changes and viability of injected fat graft in relation to the effects of biomaterials. In this study, a comparison of the volume retention effect and angiogenic ability *in vivo* between autologous fat grafting, injectable natural (AAM), and synthetic (PLGA/MH microsphere) biomaterials will provide a reasonable basis for fat grafting. And by using PLGA/MH microsphere, we could overcome the disadvantages of conventional PLGA scaffolds and effectively improve fat retention rate with complex biological functions.

Keywords : Fat graft, Allograft adipose matrix, PLGA/MH microsphere, Magnesium hydroxide[$Mg(OH)_2$], Angiogenesis, fat survival

Student Number : 2015-31208

감사의 글

청운의 꿈을 품고 의과대학에 입학한 지 20여년의 긴 세월이 지나 모든 학위과정을 수료하고, 개원의로 근무하면서 우여곡절 끝에 본 박사 학위 논문까지 잘 갈무리 할 수 있도록 도와주신 모든 분들께 진심으로 감사드립니다. 분당서울대병원 성형외과에서 연을 맺고 지도해 주시면서 오랜 꿈이었던 서울대학교 의과대학 대학원에 진학할 수 있도록 힘이 되어 주시고, 본이 되는 선배로, 실력 있는 의사로, 따뜻한 리더로서의 면모를 보여 주신 존경하는 서울의대 성형외과 백롱민 은사님께 먼저 깊은 감사의 말씀을 드립니다. 또한 이번 논문 준비에 있어 너무나도 큰 도움을 주신 차의과학대학교 한동근 교수님과 황은아 교수님 그리고 교실 연구원분들, 부족한 제가 박사 학위 논문을 마무리 할 수 있도록 이끌어 주신 서울의대 의공학과 이정찬 지도교수님, 논문심사를 도와주신 서울의대 성형외과 허찬영 교수님과 최영빈 교수님께도 감사의 말씀 올립니다.

모든 분들을 일일이 나열 할 수는 없어도 관심으로 지켜봐 주시고 여러 분야에서 도움을 주셨던 많은 분들에 대한 고마운 마음을 소중히 간직하며, 자식을 위해 평생을 눈물로 기도하면서 헌신적인 사랑과 희생으로 돌보아 주신 부모님, 그리고 인생의 동반자로서 묵묵히 곁을 지켜주는 사랑하는 아내(방지혜)와 처가식구들, 그리고 박사학위를 취득하게 된 뜻깊은 2022년 임인년 봄에 함께 찾아와 두 배의 기쁨을 안겨 준 예쁜 천사들(김예현, 김예빈)에게도 감사의 인사를 전합니다. 마지막으로 부족한 저에게 귀한 열매를 맺을 수 있도록 이 모든 것을 허락해 주신 하나님께 모든 감사와 영광을 돌리며 감사의 메시지를 갈음합니다.

2022년 김 대 희 올림