

CONTROLLED DRUG DELIVERY SYSTEM FOR ADIPOSE TISSUE RETENTION

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University of Pittsburgh, 2016

Current materials used for adipose tissue reconstruction have critical shortcomings such as suboptimal volume retention, donor site morbidity, and poor biocompatibility. The aim of this study was to develop and examine a controlled delivery system of dexamethasone (Dex) to generate stable adipose tissue when mixed with disaggregated human fat in an athymic mouse model for up to six months. The hypotheses that the slow release of Dex from polymeric microspheres would enhance both adipogenesis and angiogenesis, resulting in long term adipose volume retention, was tested using two microsphere drug delivery systems. In one treatment group, Dex was encapsulated within single-walled poly(lactic-co-glycolic acid) (PLGA) microspheres (Dex SW MS), and in the second, Dex was encapsulated in a (PLGA) core surrounded by a shell of poly(L-lactic acid) (PLLA). The double-walled polymer microsphere system was developed to create a slower and more sustainable drug delivery process. The Dex loaded microspheres were then mixed with human lipoaspirate. Both single- and double-walled empty microspheres and lipoaspirate-only controls were examined. A treatment group consisted of 3 different combinations of microspheres including a group of single- and double-walled

empty microspheres combined and lipoaspirate only as a control was also examined in the nude mouse model. Samples were analyzed grossly and histologically after 6 weeks and 6 months *in vivo*. Mass and volume were measured; Dex microsphere-containing, dose of 27 mg double-walled microspheres samples demonstrated greater adipose tissue retention ($80\pm12\%$) compared to the control group ($10\pm7.3\%$) at 6 months time point. Histological analysis, including H&E and CD31 staining, indicated increased vascularization ($p<0.05$) within the Dex MS-containing samples. Adipose tissue injected in animals was affected by dexamethasone-loaded microspheres showing an improvement in mass and volume measurements. Histology of the extracted fat shows overall healthy adipose tissue morphology with the great presence of vascularity in the treatment groups. Controlled delivery of adipogenic factors, such as dexamethasone via polymer microspheres, significantly affects adipose tissue retention by maintaining healthy tissue formation and vascularization. The use of microspheres as a vehicle for controlled drug delivery of adipogenic factors therefore presents a clinically relevant model of adipose retention.

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PREFACE

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1.0 INTRODUCTION

1.1 SOFT TISSUE ENGINEERING

1.1.1 Clinical need for soft tissue engineering

Soft tissue defects, whether due to trauma, tumor resection, or congenital malformations, require extensive tissue repair. Standard care includes free tissue transfer or prosthetic components such as silicone or saline implants. Resection of tumors in the head and neck area, as well as trauma or congenital abnormalities, often result in contour defects from loss of soft tissue, which is largely composed of subcutaneous adipose tissue. (1) With breast cancer being one of the most common malignant conditions in the new era-1 in 8 women will develop breast cancer in the United States according to the American Society of Plastic Surgeons (ASPS)-there are over 100,000 breast reconstructive surgeries performed per year following a mastectomy. (2)

Currently, the most common strategy used to repair soft tissue defects in these cases is mainly by replacing lost volume using synthetic or prosthetic materials. A major challenge is the deep tissue destruction and discontinuity that is often a result of trauma experienced during war, considerably facial traumas. The Joint Theater Trauma Registry showed 26% of all service members injured during battle and evacuated over a 6-year period in Iraq and Afghanistan suffered wounds to the cranio-maxillofacial region. (3) While the reconstruction of bone tissue has been achieved to some degree of precision, soft tissue reconstruction, which is responsible for the contours of the human form, falls short. Prosthetic restorations used as filler materials prove to not only be ineffective for soft tissue repair but also dangerous to the patient because of negative host reactions associated with local edema, lymphadenopathy, and scarring.

1.1.2 Soft tissue engineering in plastic surgery

Non-autologous materials are most often recognized as foreign bodies and can be degraded by enzymes and inflammatory cell complexes. Repeated injections are required to maintain volume in even the smallest of defects. Although allergic reactions occur rarely only in 3–5% of restorative surgeries hypersensitivity reactions are frequently observed. (4-5) Allografts, also known as homologous tissue grafts, are not ideal due to the potential for viral transmission or immunogenic and allergic reactions to occur. Autologous fat grafting is another option utilized in reconstructive and augmentative surgery. (6)

Current materials used in restorative tissue surgery possess a number of limitations, including unpredictable outcomes, fibrous capsular contraction, allergic reaction, suboptimal mechanical properties, distortion, migration, and long-term resorption. (7) One promising strategy involves the controlled delivery of adipogenic factors, such as dexamethasone (Dex), within the fat graft. (8-9) Our laboratory has a long history of developing novel biomaterials based on both native matrices as well as synthetic polymers for regenerative medicine applications. (8) By encapsulating adipogenic factors within polymer microspheres, the agents will be released in a local environment in a controlled manner. Previous *in vitro* and *in vivo* studies have demonstrated that the controlled delivery of dexamethasone and other adipogenic drugs via polymer microspheres significantly affected mass and vascularization of the fat graft. (8)

1.2 PROSTHETIC MATERIALS IN PLASTIC SURGERY

1.2.1 Benefits of prosthetic materials

Prosthetic implants are widely used in plastic and reconstructive surgery. These implants are obtained from a large number of bioorganic based or artificial non-physiological materials such as metal or silicone. Implants can be placed permanently or temporarily depending on the condition directed to treat.

Early investigators used materials based on availability and ease of application. Paraffin wax, petrolatum, vegetable oils, lanolin, silicone oil, and beeswax have been used for facial augmentation, but with very limited success rate. Research with the purpose of developing the ideal synthetic implant has been based on some primary characteristics: decreased foreign body reaction, easily manipulated or contoured, retain the shape over time, easily sterilized and not interfered with primary condition such as in malignancy cases.

Table 1. Synthetic materials used in plastic and reconstructive surgery

Synthetic Materials	Type of Material	Most Common Usage
Polytetrafluoroethylene	Gore-Tex, Proplast I and II Teflon	Soft tissue and bone repair
Silicone-based materials	BioPlastique, Injectable silicone Silastic sheets, Silicone, Silicone gel	Soft tissue augmentation
High density polyethylene	Medpor	Facial bone augmentation
Tissue adhesives	Cyanoacrylate	Tendon repair
Polymer mesh	Dacron (Mersilene), Dexon, Prolene Supramid Vicryl	Abdominal wall reconstruction
Dermal fillers	Botox (Botulin toxin), Juvederm (hyaluronic acid), Restylane (non-animal hyaluronic acid)	Soft tissue fillers

These synthetic materials have a wide range of use in many plastic surgery procedures including breast reconstruction, craniofacial surgery, maxillofacial trauma and aesthetic surgery. Table 1 lists number of synthetic materials used in plastic surgery.

1.2.2 Prosthetic materials limitations

The limitations that are associated with the use of the synthetic materials in plastic and reconstructive surgery have lead to the examination of autologous materials. The disadvantages of the synthetic materials are associated mostly with the foreign body reaction. Implants should be manipulated as little as possible with very cautious instrument handling. Insertion of the material is usually implanted as far as possible from the final position of the implant. This is performed for the purpose of avoiding extrusion and infection. The insertion of implant requires opening the pocket in surrounding tissue with adequate size. Dermal fillers are short-term solutions for soft tissue reconstruction, but have limited ability to fill a small defect and re-injections are often required.

1.3 NATURAL MATERIALS IN PLASTIC SURGERY

1.3.1 Benefits of natural materials

The use of natural materials for the purpose of reconstructing soft tissue defects has been on developed in the last few decades. Natural materials are physiological tissue that is obtained from the same patient (autologous), or from another person, genetically similar donor (allogeneic tissue). Xenogeneic tissue is natural tissue donated from a different species such as non-human primates or swine. The advantages of the autologous tissue grafting are that grafts are readily available, and there is no need to identify a human leukocyte antigen (HLA) matched donor. Autologous transplants have a lower risk of life-threatening complications; there is no risk of graft vs. host disease (GVHD) and no need for immunosuppressive therapy to prevent GVHD and graft rejection. Immune reconstitution is more rapid than after an allogeneic transplant and there is a lower risk of opportunistic infections. Graft failure occurs rarely. There are a large number of tissues that are used for transplants in plastic surgery, with most common ones being cartilage and fat tissue.

Cartilage tissue is very commonly used as a natural graft, obtained from patients' ears or ribs. The cartilage tissue can be easily tailored and fixed for the patients' needs and there is no need for additional surgery on the donated tissue site. The disadvantages of using cartilage tissue involve the nature of the cartilage tissue, which includes the challenge to manipulate the tissue once the graft is integrated into the defect site.

1.3.2 Adipose tissue grafting

The patient's own fat or adipose, is commonly used for contouring lost soft tissue. The fat tissue is easily harvested via liposuction from the patient and injected back in the desired part of the face or body. Lipoaspirate is typically harvested from the patient's abdomen, thighs or buttocks. The advantages of using fat tissue for soft tissue reconstructive purposes are similar to those advantages described previously for cartilage; adipose (fat) tissue is easily derived from the same patient. Fat tissue is also very easily manipulated. Recovery from fat grafting is usually minimal with very rare post-operative complications. However, there are several disadvantages that go along with fat tissue grafting. (9)

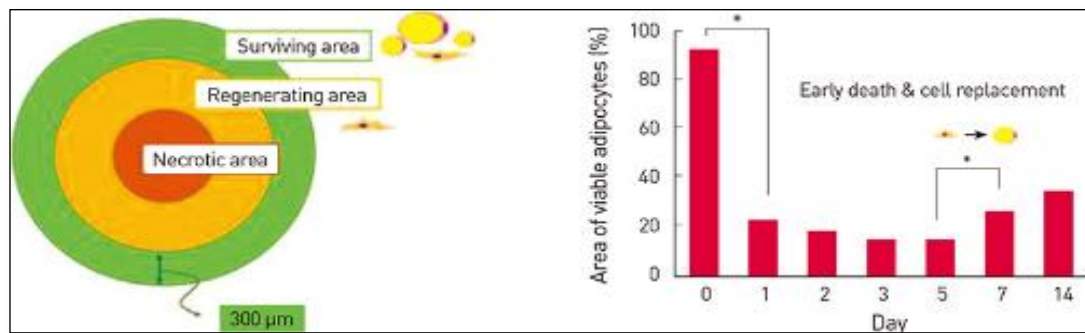


Figure 1. Adipose tissue graft survival (9)

Due to the lack of vascularization in the area where the adipose tissue is injected, a limited volume of fat survives (Figure 1). Formation of blood vessels in the grafted tissue occurs very slowly. The initial lack of vascularization in the grafted tissue causes necrosis of the adipose cells following by reabsorption of necrotized tissue. This lack of early vascularity remains one of the most important reasons why the grafted adipose tissue loses volume over time and needs re-injected.

1.4 DRUG DELIVERY SYSTEMS

1.4.1 History of drug delivery systems

Drug delivery systems are engineered systems that help deliver the drug with a controlled rate in the local area or tissue. New era pharmaceuticals companies have developed advanced ways of targeting certain conditions with different agents. Biomedical engineering research has contributed to the development of different methods of transporting drugs into the body. However, despite this progress, a great number of agents, even those discovered using the advanced molecular biology strategies, have side effects due to the drug interacting with healthy tissues that are not targeted by the drug. Side effects limit the ability to design medications for many diseases such as malignancies and neurodegenerative diseases.

Drug delivery systems have been formulated and optimized for many conditions but mainly for malignancies or other single target types of disease. Most common drug delivery systems include liposomes, pro-liposomes, microspheres, gels, and cyclodextrins. (10)

The 1st generation (1950-1980) of drug delivery systems was based more on the development of oral and transdermal-sustained release. The 2nd generation (1980-2010) of drug delivery systems focused on the development of zero-order release systems, self-regulated drug delivery systems, long-term depot formulations, and nanotechnology-based delivery systems. The optimization of the 2nd generation was focused on studying nanoparticle formulations.(11).

1.4.2 Microspheres as a drug delivery system

Microspheres are composed of biodegradable polymers with the purpose of meeting the requirements needed for drug delivery systems, such as the ability to deliver locally, stable biocompatibility, targeting of specific tissue or cell populations in the tissue, release of the drug in a desired manner, and degradation within a period of time. Between the variety of devices that have been used for controlled release drug delivery, biodegradable polymer microspheres are one of the most common types of drug delivery with several advantages. Microspheres can encapsulate many types of drugs including proteins and growth factors. Microspheres are easily administered through a syringe needle. Microspheres are biocompatible, with minimal side effects in surrounding areas, but most importantly, microspheres are designed to be capable of sustained release for long periods of time.

The fabrication of the microspheres plays an important role in the encapsulation and release of therapeutics. In addition, the type of polymer, the polymer molecular weight, the copolymer composition, and the nature of the drug encapsulated, is important factors affecting the microsphere size, thus controlling the delivery rate.

1.4.3 Clinical applications

The clinical use of microspheres in the last decades has been developed in several applications, such as: 1) fillers and bulking agents, 2) embolic particles, and 3) drug delivery vehicles.⁽¹²⁾ An example of the use of degradable microspheres as drug delivery vehicles in bone-filling formulations or scaffolds include microspheres loaded with bone-morphogenic protein 2 (BMP-2) or vascular endothelial growth factor (VEGF) to improve the formation of new bone in cases of critical size defects. The efficiency of sustained delivery of drugs from microspheres has been studied and applied to numerous malignancy conditions. Microspheres applied for trans-arterial chemo-embolization (TACE) release anti-cancer drugs such as doxorubicin or cisplatin. ⁽¹³⁾ Low molecular weight drugs are typically easily encapsulated in microspheres, although the loading efficiency is still variable; however, high molecular weight bioactive molecules can be difficult to load into microspheres. Therefore, tailored controlled degradation of microspheres will be required to deliver such drugs in a controlled fashion.

1.5 POLYESTERS

1.5.1 Poly(lactide-co-glycolide) PLGA

Biomaterials can be natural or synthetic, and are degraded, either through enzymes or hydrolysis, producing biocompatible, safe products, which are further eliminated via normal metabolic pathways. The biomaterials used in drug delivery systems can be classified as (A) synthetic biodegradable polymers, which includes hydrophobic materials such as hydroxy acids (poly(lactic-co-glycolic acid), PLGA) or polyanhydrides, and (B) naturally occurring polymers, such as complex carbohydrates (hyaluronan, chitosan) or inorganics (hydroxyapatite).

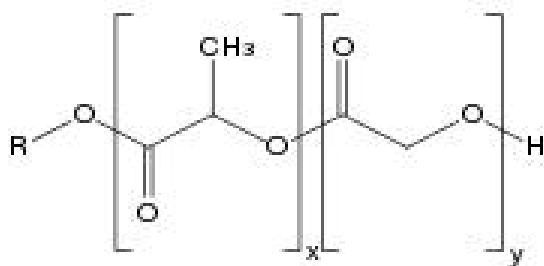


Figure 2. Poly(lactide-co-glycolide) PLGA(87)

The polymer PLGA is a copolymer of poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) (Figure 2). PLGA is the most commonly used polymer in tissue engineering because of the biodegradable properties and low toxicity.

Poly(lactic acid) contains an asymmetric carbon which is described as the D or L. The chemical forms of the polymer PLA are poly(D-lactic acid) (PDLA) and poly(L-lactic acid) (PLLA). PLGA is an acronym for poly(D,L-lactic-co-glycolic acid) where D- and L- lactic acid forms are in equal ratio(87).

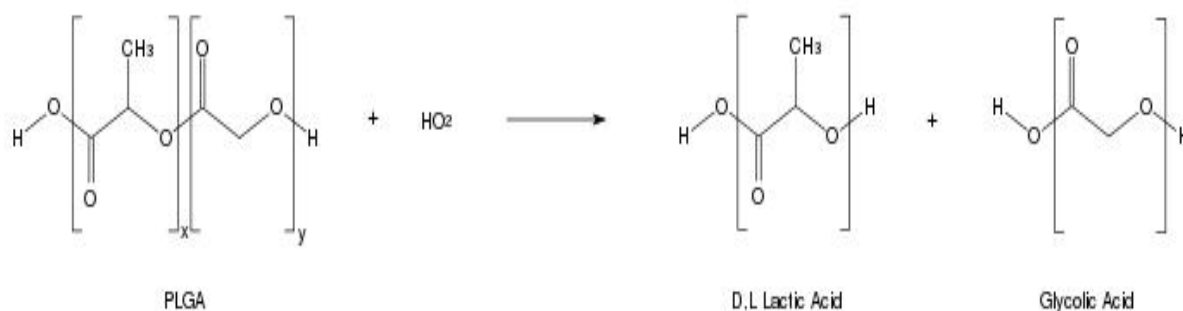


Figure 3. PLGA degradation in the tissue (87)

In the tissue, PLGA degrades by hydrolysis of its ester linkages in the presence of water. PLGA bio-dissolves relatively quickly in the body. PLGA chains are cleaved to monomeric acids that are then eliminated by the Krebs's cycle as CO_2 and in the urine as water (Figure 3).

1.5.2 Poly(L-lactic acid) (PLLA)

Poly(lactic acid) or polylactide (PLA) is a biodegradable polyester widely used not only in medicine but also in everyday life. PLA is often used as a decomposable packaging material, like "plastic" bags. In medical field, PLLA is most commonly used as a building material for biodegradable sutures and soft tissue filler.

In early 2000's, the US Food and Drug Administration approved PLLA for a polymer-based injectable medical device for restoration and/or correction of the signs of facial fat loss in cases with human immunodeficiency virus.(88)

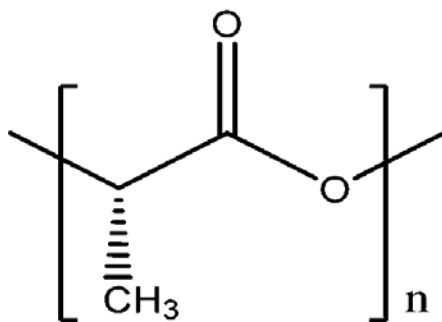


Figure 4. Poly(lactic acid) (PLLA) structure(88)

Poly lactides have a long and stable history of safe use in medical applications, such as pins, plates, screws but also as a drug delivery system for sustained release of drugs. Chemically, the L-isomer of polylactic acid is a biodegradable and biocompatible (Figure 4), which makes PLLA a rather crystalline polymer where as the poly(D,L-lactide) (PDLA) is an amorphous polymer. Similar to PLGA, PLLA is degraded by hydrolytic process with a breakage of ester linkages, resulting in bulk erosion.

1.6 PROJECT OBJECTIVES

1.6.1 Objective 1: Effect of Dexamethasone encapsulated in single-walled microspheres in adipose tissue

Objective #1: To optimize dexamethasone encapsulation in single-walled microspheres (Dex SW MS), optimize the doses of Dex SW MS and test the effect in *in vivo* environment

Hypothesis: Dexamethasone encapsulated in PLGA microspheres, will be released in a controlled manner for duration of 4-6 weeks, affecting the fat in a sustained and local manner.

1.6.2 Objective 2: Fabrication of dexamethasone-loaded double-walled polymer microspheres

Objective #2: To fabricate and optimize dexamethasone encapsulation in double-walled microspheres, test the bioactivity of dexamethasone double-walled microspheres *in vitro*, optimizing the doses of Dex DW MS in *in vivo* testing

Hypothesis: Dexamethasone, protected by the PLGA and PLLA, will be released in a controlled manner for duration of 10-24 weeks, with a minimal burst release effect. Slow and controlled release of dexamethasone within injected fat in a mouse model will enhance adipogenesis in a controlled fashion, therefore increasing adipose tissue retention.

1.6.3 Objective 3: Optimization of combined microspheres doses for prolonged adipose tissue retention

Objective #3: To optimize and evaluate the *in vivo* effect of combining single- and double-walled microspheres for the purpose of prolonged and more efficient fat grafting retention.

Hypothesis: Dexamethasone-loaded microspheres, both single- and double-walled, will release the drug in a controlled fashion with doses being tailored to release by contemplating both forms of microspheres to achieve further stable fat grafting for six months.

2.0 EFFECT OF ADIPOGENIC DRUGS ENCAPSULATED IN SINGLE-WALLED MICROSPHERES IN ADIPOSE TISSUE RETENTION

2.1 INTRODUCTION

Tissue defects from trauma, tumor resection, or congenital malformations require soft tissue repair. Standard care includes tissue flap transfer or prosthetic components such as silicone or saline implants. Autologous fat grafting is a minimally invasive option in plastic and reconstructive surgery. (14) In this technique, the limitations of current restorative and reparative techniques have served as motivation for the development of adipose tissue regeneration as an application area for tissue engineering.

Synthetic materials possess severe limitations, including but not limited to, unpredictable outcome, fibrous capsular contraction, allergic reaction, suboptimal mechanical properties, distortion, migration, and long-term reabsorption. (15) Transplanted fat can have a low survival rate, and the adipose tissue can be quickly resorbed and replaced by fibrous tissue and oil cysts. (16-18) These issues have greatly impacted the widespread adoption of autologous fat as the ideal soft tissue filler.

At present, the exact mechanisms that mediate fat graft survival and resorption remain unclear. One potential mechanism for graft loss is the lack of adequate revascularization within the transplanted fat. Due to the lack of vascularization, ischemia of the tissue occurs, leading to tissue necrosis and graft loss at an early stage. (19,20) In this study, we strived to create a predictable and clinically relevant method of soft tissue retention using pharmacologic interventions to improve autologous fat grafting. Our strategy for soft tissue regeneration involves the controlled, long-term, local delivery of adipogenic factors, such as insulin (Ins) and dexamethasone (Dex), within the fat graft.(8)

This study outlines the design and assessment of encapsulated insulin and Dex in poly(lactic-co-glycolic acid), (PLGA) microspheres (MS) mixed with lipoaspirate, and the effects on both vascularization and fat retention *in vivo*, using a combined drug therapy approach. We sought to determine whether encapsulation of these adipogenic factors and the subsequent localized delivery within fat grafts, resulted in enhanced adipose retention and vascularization as confirmed by immune-histological analysis of the explanted tissue.

2.1.1 Microspheres

2.1.1.1 Polymer-based single-walled microspheres as a drug delivery system

Controlled release drug delivery systems are being developed to address many of the difficulties associated with traditional methods of administration. Controlled drug delivery employs devices such as polymer-based disks, rods, pellets, or microspheres that encapsulate drug and release it at controlled rate for long periods of time.

A variety of devices have been used for controlled release drug delivery, biodegradable polymer microspheres are one of the most common types with several advantages. Microspheres can encapsulate many types of drugs including small molecules, proteins, and growth factors. Microspheres are easily administered through a syringe needle. Microspheres are overall biocompatible, and capable of sustained release for long periods of time. The commercial products that are based on polymer microspheres include Lupron Depot and Nutropin Depot. The disadvantages associated with microspheres include difficulty of large-scale manufacturing, inactivation of drug during fabrication, and poor control of drug release rates.

Co-polymer poly(lactic-co-glycolic acid) (PLGA) microspheres have been developed for many years and have been approved by the US FDA for the use of drug delivery, diagnostics and other applications of clinical and basic science research, including cardiovascular disease, cancer, vaccine and tissue engineering. (21) Despite all the advantages of using PLGA microspheres, after a period of slow drug release, degradation of the polyester PLGA leads to reduced polymer chain length and accelerated diffusion and drug release. This is a challenge when using microspheres in situations that require a long-term release of drugs.

2.1.2 Adipogenic factors

2.1.2.1 Dexamethasone

Dexamethasone (**Dex**) (Figure 5) is commonly used synthetic corticosteroid, covering a great range of inflammations and auto-immune diseases. Dexamethasone is administered in different ways, depending on the severity and location of the target tissue.

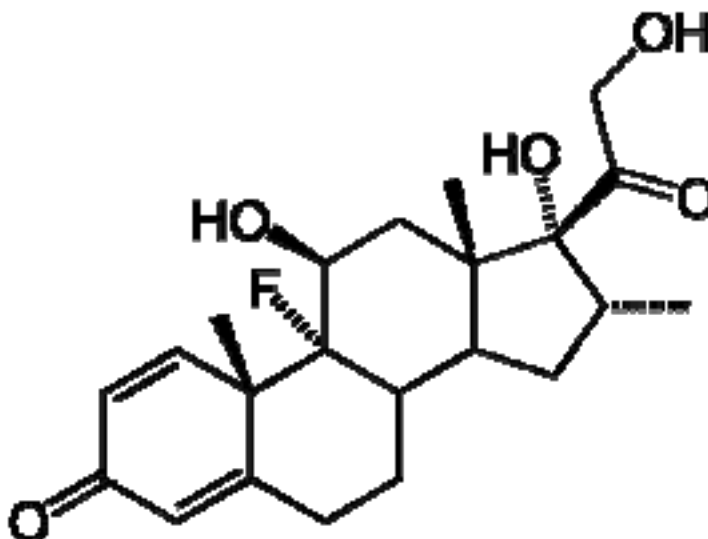


Figure 5. Structure of dexamethasone (22)

Dexamethasone is an important factor in adipogenesis. Dexamethasone as a highly potent synthetic glucocorticoid works through activation of the glucocorticoid receptor, which is a nuclear hormone receptor in the same superfamily as peroxisome proliferator activated receptor- γ (PPAR- γ).

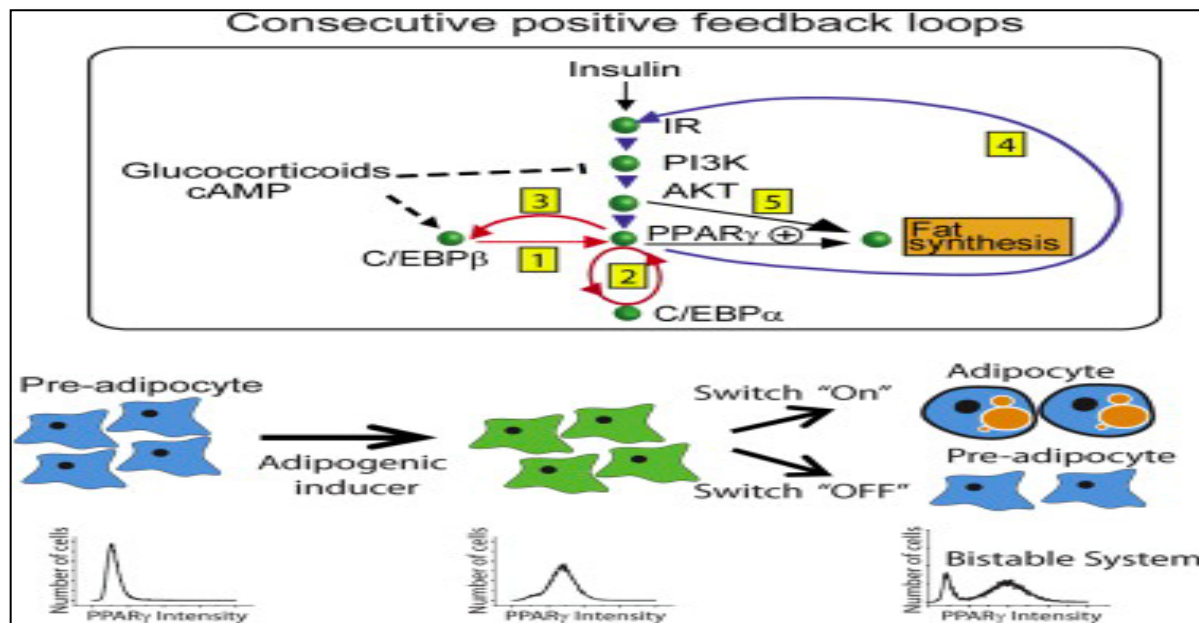


Figure 6. Effect of glucocorticoids in adipose tissue (23)

Dexamethasone induces C/EBP- δ adipogenic activity and reduces the expression of pref-1, a negative regulator of adipogenesis. (22-26) Adipocyte differentiation is multistep process requiring the sequential activation of several groups of transcription factors, including CCAAT/enhancer-binding protein (C/EBP) gene family and peroxisome proliferator activated receptor- γ (PPAR- γ) (Figure 6).

2.1.2.2 Insulin

Insulin is a hormone that is known for regulation of carbohydrates and fat metabolism in the body. The presence of insulin in tissue stops the process of using fat as energy resource. When insulin is absent, cells do not take up glucose and the body begins to use fat

as an energy source or gluconeogenesis (Figure 7), by transfer of lipids from adipose tissue to the liver for mobilization as an energy source. (27) The release of insulin to induce adipogenesis has demonstrated, using *in vitro* and *in vivo* studies, resulting in fat tissue increase, and thus weight increase in couple of weeks. (28-29)

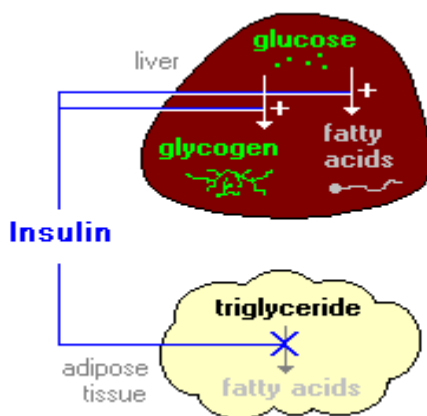


Figure 7. Schematic of the role insulin in adipose tissue (27)

Biodegradable drug delivery systems, such as poly(lactic-co-glycolic acid)-polyethylene glycol (PLGA-PEG) microspheres, have been studied as delivery vehicles for insulin, insulin-like growth factor-1 (IGF-1), and basic fibroblast growth factor (bFGF).

In a subcutaneous rat model, incorporating the growth factors improved autologous free fat graft weight and volume, with the best results observed for either insulin or IGF-1 alone or in combination. The PLGA (75:25) foam was also assessed *in vivo* in combination with IGF-1 and insulin, with fibro-elastic tissue formation at the implantation site at 12 weeks. (30-31)

2.1.3 Specific Aim 1.

The specific aim of this chapter was based on the work for developing sustained and controlled delivery of adipogenic drugs, dexamethasone and insulin. The effect of drug delivery of both adipogenic drugs from single walled PLGA microspheres was tested *in vivo*. Adipogenic drugs loaded in microspheres were expected to be released in a controlled manner for duration of 4-6 weeks, affecting the fat in a sustained and local manner.

To test this hypothesis, dexamethasone and insulin PLGA microspheres were mixed with human disintegrated adipose tissue and injected in athymic mice. Adipogenesis was tested in animals in 2 different time points, 6 weeks and 6 months respectfully. We have also tested the combined dexamethasone and insulin loaded microspheres effect in injected lipoaspirate. Volume and mass measurements of the extracted tissue were calculated followed by histology testing including tissue morphology and presence of blood vessels.

2.2 METHODS

2.2.1 Fabrication and characterization of Dexamethasone encapsulated single-walled microspheres

The protocol for encapsulating Dexamethasone in single-walled PLGA MS has been established in our laboratory. (8) Dexamethasone sodium phosphate PLGA MS (Dex MS) was prepared using a single emulsion/solvent extraction technique. PLGA (75:25) (400 mg) was dissolved in methylene chloride (MC) (4.5 mL).

Dexamethasone phosphate (Dex) (20 mg) was dissolved in methanol (0.5 mL), which was added to the polymer solution. After stirring and the addition of a 600 mg of 2% poly(vinyl alcohol) (PVA) solution, the MS were collected by centrifugation, frozen at - 20 °C, and freeze dried for 12 h (LabConco Freezone 4.5). The loading capacity was determined by using the equation LC = Amount of drug loaded (AD) divided by the amount of polymer and amount of drug loaded (AP+AD). Encapsulation efficiency (EC) was determined by amount of actual drug concentration (Ca) divided by the theoretical concentration (Cth).

$$LC (\%) = \frac{\text{Amount of drug loaded}}{\text{Amount of polymer} + \text{amount of loaded drug}} \times 100$$

Equation 1. Loading capacity of microspheres

$$EC(\%) = \frac{Ca \text{ (Actual concentration)}}{Cth \text{ (Theoretical concentration)}} \times 100$$

Equation 2. Encapsulation efficiency of microspheres

2.2.2 Fabrication and characterization of empty single walled microspheres

Empty MS were prepared and characterized using the same protocol as in the Dex MS, without adding any drugs to the PLGA.

2.2.3 Fabrication and characterization of insulin encapsulated single-walled microspheres

Our previously established protocol was used to encapsulate insulin in PLGA MS.(8) Insulin-loaded PLGA MS (insulin MS) were prepared using a double emulsion/solvent extraction technique. PLGA (75:25) was dissolved in MC (4.5 mL). To form the first emulsion, insulin was dissolved in a phosphate-buffered saline (PBS; 0.2 mL), added to the dissolved PLGA (400 mg), and vortexed to form an emulsion.

The first emulsion was added to a stirring 2.0% PVA solution and stirred. After 2 min, water was added and stirred for 3 h at 500 rpm. The MS were collected by centrifugation, frozen at - 20°C, and freeze-dried for 12 h. The loading capacity was determined by using the equation $LC = De/Sw$, where DE is the amount of drug encapsulated and Sw is the mass of the MS.

2.2.4 Dexamethasone and insulin microsphere characterization

The morphology of Dex and insulin MS was determined by scanning electron microscopy (SEM) microscopy. MS were gold coated using a Cressington 108 Auto (Cressington) followed by usage of JSM 6335F SEM (JEOL) operated at 3.0 kV acceleration for morphology characterization. Particle-size distribution was determined by measuring the diameters of at least 50 MS from SEM images.

2.2.5 Dexamethasone and insulin drug release profile

To determine the release of the drug from the single-walled microspheres, 10 mg of microspheres were placed in Eppendorf tubes and incubated in 1 mL PBS at 37°C. Starting with week 1 and then weekly time points, supernatant was collected and microspheres were replaced with fresh PBS after being vortexed.

The amount of dexamethasone released collected was analyzed using spectrometry. The data collected will be calculated utilizing standard curve. The insulin loaded microsphere drug release was determined by using a commercial kit the FluoroProfile Protein Quantification Kit (Sigma Aldrich). In both drug release studies, the results were compared with PBS only.

2.2.6 Drug dosage for the *in vivo* study

Dex is a synthetic steroid with an anti-inflammatory effect, and is used in different fields of medicine, such as in autoimmune (acquired) disorders of the endocrine system, allergic states, and rheumatologic diseases. Dex can be delivered orally, intramuscularly (I.M.), and in some cases intravenously (I.V.), depending on the severity of symptoms. The official doses used in clinics based on the National Institute of Health (NIH) data, I.V. doses range from 0.5 to 40 mg/kg in life-threatening cases such as unresponsive shock, and the average dose of Dex delivered I.V. or I.M, is typically 10–14 mg/kg, in 24 h.(32)

The doses in the study used in the animals were designed to be easily translated in human models, with drug doses lower than the doses shown above, when maintaining the ratio of animals to humans. The concentration of insulin in the insulin-loaded MS was designed in the same fashion. The physiological levels of insulin in the healthy adult are 24–38 units per day or an average of 8–11 nIU/mL(33) In this murine study, the dosage of insulin encapsulated in MS is lower than the doses above.

2.2.7 Human lipoaspirate processing

Human lipoaspirate was processed from patients undergoing elective surgery and have given informed consent, approved by University of Pittsburgh Institutional Review Board. Briefly, adipose tissue was gently aspirated using a two-holed blunt harvesting cannula attached to 10mL Luer-Lok syringes.

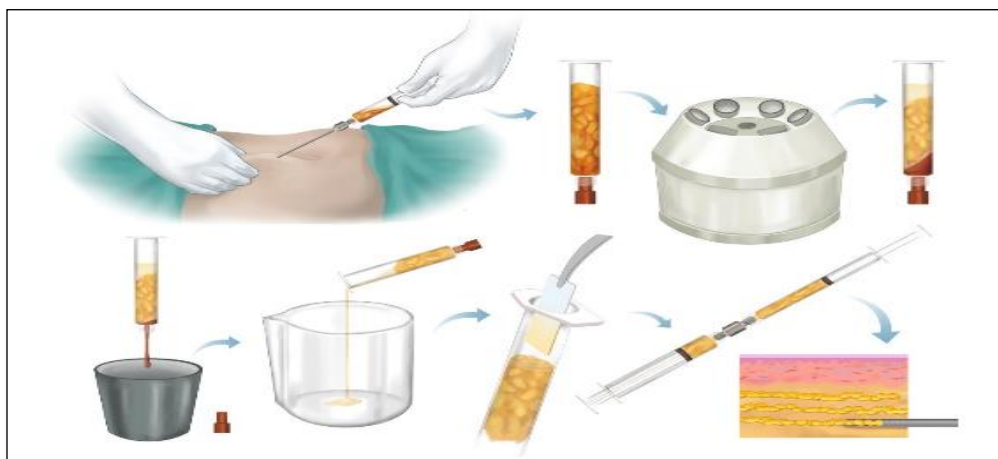


Figure 8. Human lipoaspirate processing (34)

The capped 10mL Luer-Lok syringes was centrifuged at 1200Xg for 3 min. After the upper (oil) and lower (blood and infiltration liquids) layers were removed (Figure 8), the middle layer (purified and processed lipoaspirate) was transferred through Luer-Lok connectors to 1mL syringes. (34) Immediately after fat tissue processing, the lipoaspirate was mixed manually with the specific dose of Dex DW MS homogenously and re-inserted in 1 mL syringes in preparation for animal injections.

2.2.8 Animal surgery: Dex and insulin MS alone

The University of Pittsburgh Institutional Animal Care and Use Committee approved all animal studies. Thirty-five female athymic nude mice (5–10 weeks old; Harlan Laboratories) were equally divided into seven groups with five animals in each group: Dex A= 40 mg MS, Dex B = 80 mg MS, Dex C= 150 mg MS, Insulin A= 10 mg MS, Insulin B = 14 mg MS, Insulin C = 28 mg MS, and Insulin D= 56 mg MS (Table 2). At the time of surgery, animals were weighed and then anesthetized with 12 mg/kg xylazine followed by 80 mg/kg of ketamine. Human lipoaspirate was processed as approved by the University of Pittsburgh Institutional Review Board, obtained by the patients undergoing elective surgery. Briefly, adipose tissue was gently aspirated using a twoholed, blunt harvesting cannula attached to 10-mL Luer-Lok syringes. The capped 10-mL Luer-Lok syringes then were centrifuged at 1200 g for 3 min. After the upper (oil) and lower (blood and infiltration liquids) layers were removed, the lower one-third of the middle layer (purified and processed lipoaspirate) was transferred through the Luer-Lok connectors to 1-mL syringes, known as the Coleman method. (32)

Immediately after sedation, the processed lipoaspirate (300–1000mL lipoaspirate) was injected subcutaneously (fan-injection technique) and bilaterally in the dorsal flanks of nude mice using the 16-gauge infiltration cannula: right side lipoaspirate with MS and right side lipoaspirate only. Immediately following injections, photos were captured for analysis of the adipose retention and were photographed weekly throughout the study. After 5 weeks, the animals were sacrificed in the CO₂ chamber. Adipose explants were analyzed for mass measurements and volume displacement using an Accupyc II 1340 gas Pycnometer (Micrometrics).

Table 2. Dex and insulin MS doses

Dexamethasone MS /1 mL of human lipo	Drug treatment dose	Number of animals	Time points
	40 mg of MS	5	5 weeks
	80 mg of MS	5	5 weeks
	150 mg of MS	5	5 weeks
Insulin MS/ 1mLof human lipo	Drug treatment dose	Number of animals	Time points
	10 mg of MS	5	5 weeks
	14 mg of MS	5	5 weeks
	28 mg of MS	5	5 weeks
	56 mg of MS	5	5 weeks

2.2.9 Animal Surgery: Combined Dex MS and insulin MS study design

In the subsequent study, both Dex MS and insulin MS were combined and examined in the athymic nude mouse model as described above. Seven groups of mice were used in this part of the experiment, each containing nine animals in each group (powered for eight animals with one extra mouse for persistency) for a total of 63 mice, In addition to control groups, different combinations of Dex MS and insulin MS doses, were examined as follows: Group 1 = 50 mg Dex + 90 mg insulin MS; Group 2 = 50 mg Dex + 10 mg insulin MS; Group 3 = 27 mg Dex + 19 mg insulin MS; Group 4 = 27 mg Dex + 0mg Insulin; and Group 5 = 0mg Dex + 10 mg insulin MS. The following two control groups were analyzed: Control 1: Lipoaspirate only and Control 2 = Lipoaspirate + 100 mg Empty PLGA MS (Table 3). The MS were mixed with 0.3mL of human lipoaspirate. Animals were sacrificed after 5 weeks and assessed as described above.

Table 3. Combined drug MS study design

Combined drug MS / 0.3mL of lipo	Drug treatment dose	Number of animals	Time points
Group A	50 mg+90 mg	9	5 weeks
Group B	50 mg+10 mg	9	5 weeks
Group C	27 mg+19 mg	9	5 weeks
Group D	27 mg	9	5 weeks
Group E	10 mg	9	5 weeks
Group F	100 mg Empty	9	5 weeks
Group G	Lipoaspirate	9	5 weeks

2.2.10 Long term Dex MS treatment animals

The six month, long-term time point's animal's surgeries were conducted in the same environment as the six week, short-term animals. The study was conducted to test the long-term drug release from the single-walled dexamethasone-loaded microspheres. A total of 20 animals were injected, divided into 4 groups with 5 animals per group. High dose (50 mg Dex MS) and low dose (27 mg Dex MS) groups were compared with a group of empty microspheres and lipoaspirate only (Table 4).

Table 4. Dexamethasone microsphere long-term animal study

Dexamethasone MS/ 0.3 mL human lipo	Drug treatment dose	Number of animals	Time points
High dose	50 mg Dex MS	5	6 months
Low dose	27 mg Dex MS	5	6 months
Empty MS	50 mg MS	5	6 months
Lipoaspirate	NA	5	6 months

Animals in the short-term time point were injected with 1 mL of lipoaspirate mixed with microspheres on the dorsal part, which was a high volume for rodents. In the long-term dexamethasone MS surgeries, animals were injected with the volume of 0.3 mL, while the dose of the drug remains the same, with the different ratio. Animals were closely monitored for 6 months.

2.2.11 Histological analysis

The samples were fixed in 4% paraformaldehyde, incubated overnight in 30% sucrose (Sigma-Aldrich), and then embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek USA, Inc.), followed by cryosectioning at 18 mm thickness. Hematoxylin (Santa Cruz Biotechnology, Inc.) and eosin (Sigma-Aldrich) (H&E) staining was conducted. To assess vascularization, horseradish peroxidase-based CD31 antibody staining was performed to confirm the presence of blood vessels. Samples were fixed in 10% buffered formalin for 1 h, processed, and embedded in paraffin.

Samples were first deparaffinized and then rehydrated with an ethanol gradient. Antigen retrieval (95 °C citrate buffer for human for 20 min) was then performed and slides were washed. Slides were blocked (sequentially for avidin, biotin, and endogenous enzymes (Dako), proteins (Dako), and 5% rabbit serum (Jackson ImmunoResearch) for human or 3% peroxide and 5% BSA for mouse, and washed with tris buffered saline between blocking steps. The slides were incubated with the CD31 primary antibody (goat anti-human 1:100 for 1.5 h at room temperature, SC-1506; Santa Cruz Biotechnology or rat anti-mouse 1:100 for 2 h at room temperature, ab56299; Abcam) in PBS. Slides were washed and then incubated with a secondary antibody (rabbit anti-goat 1:200 in PBS with 6% rabbit serum for 30 min at room temperature, BA-5000; Vector Laboratories or rabbit anti-rat 1:100 in PBS for 1 h at room temperature, P0450; Dako). Slides were washed and human slides were incubated with an ABC kit avidin–biotin complex (Vector Laboratories) for 15 min at room temperature and washed.

2.2.12 Image analysis

The slides were imaged using an Olympus Provis microscope (Olympus). Blood vessels were counted using ImageJ (NIH). Cylindrical-shaped structures surrounded with endothelial cells were identified as vascular lumens on H&E-stained slides and were subsequently confirmed as blood vessels using CD31 staining.

2.2.13 Blood vessel quantification

Blood vessel lumens were counted using CD31-stained slides, five slides per group, and the average was recorded. In addition, using CD31-stained slides, 20 random cell surface areas were evaluated with Image J starting from the center of the slide and counting five cells in four directions for the purpose of setting the difference in cell areas between the control and treatment groups. Ten random tissue sections were quantified for each sample and means were determined for the aforementioned variables.

2.3 RESULTS

2.3.1 Dexamethasone and insulin loaded single-walled microsphere characterization

Dex MS were examined by SEM (Figure 9a). The MS had an average diameter of ≈ 100 μm . The release of Dex occurred over 34 days (Figure 10a). A controlled release was maintained over this period. After 24 h, $30.4\% \pm 4.4\%$ of total Dex was released. A large burst effect (e.g., $> 80\%$ during the first 24 h) was not observed. After 35 days, $90.0\% \pm 1.5\%$ was released. The yield was $68.3\% \pm 15.1\%$. The loading capacity was 7.3 ± 1.5 mg Dex per mg of MS.

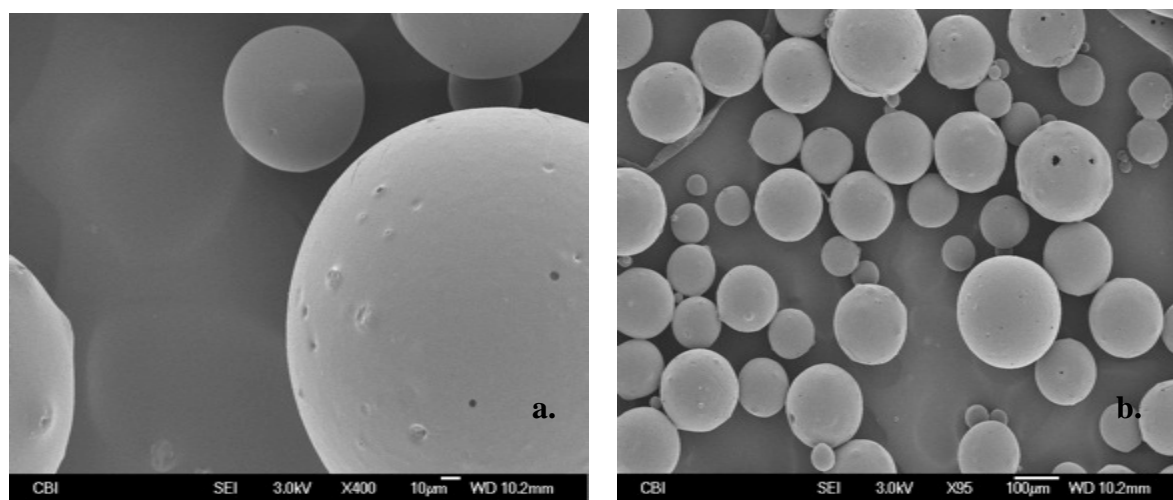


Figure 9. SEM images of a) dexamethasone (Dex) poly (lactic-co-glycolic acid) (PLGA) microspheres (MS) and b) insulin-loaded PLGA MS

In the same manner, insulin MS were examined utilizing SEM (Figure 9b). The average diameter of the insulin MS was 200–300 μm . The release of insulin was observed over 21 days (Figure 10b). The insulin microsphere yield was $40.6\% \pm 11.3\%$. The loading capacity 10.7 ± 2.3 mg insulin per mg of MS.

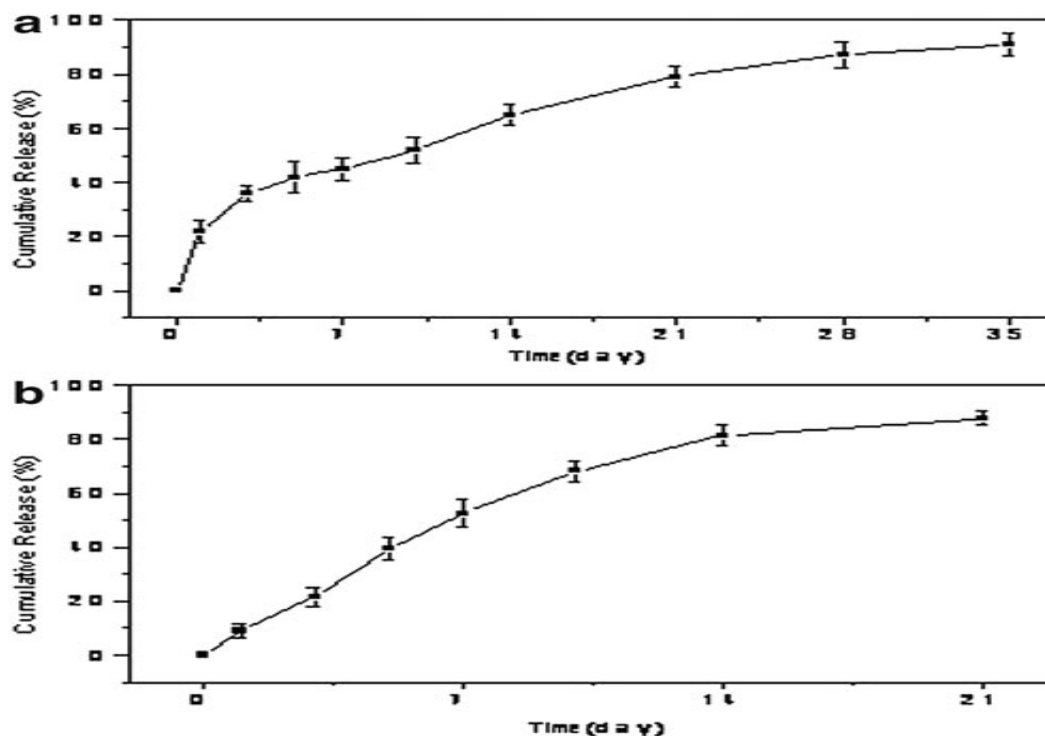


Figure 10. Drug release kinetics: a) dexamethasone loaded microsphere and b) insulin loaded microspheres

2.3.2 Dex MS and insulin MS *in vivo* studies

The animals were sacrificed after 5 weeks. Photographs showing the differences between the treatment groups and the control group are depicted in Figure 11. Dex MS were mixed with 1 mL of lipoaspirate, whereas the control consisted of 1mL of human lipoaspirate (C = control without MS and S = sample with Dex MS). Macroscopic differences were observed within the implants that had MS treatment compared with tissues without treatment.

The results showed that the fat samples extracted from the nude mice with the Dex MS had increases in mass measurements when compared with lipoaspirate injections only (Figure 12). Insulin MS were mixed with 1mL of human lipoaspirate and also compared with lipoaspirate alone (Figure 13). Results indicate that mass increased with increasing insulin concentrations.

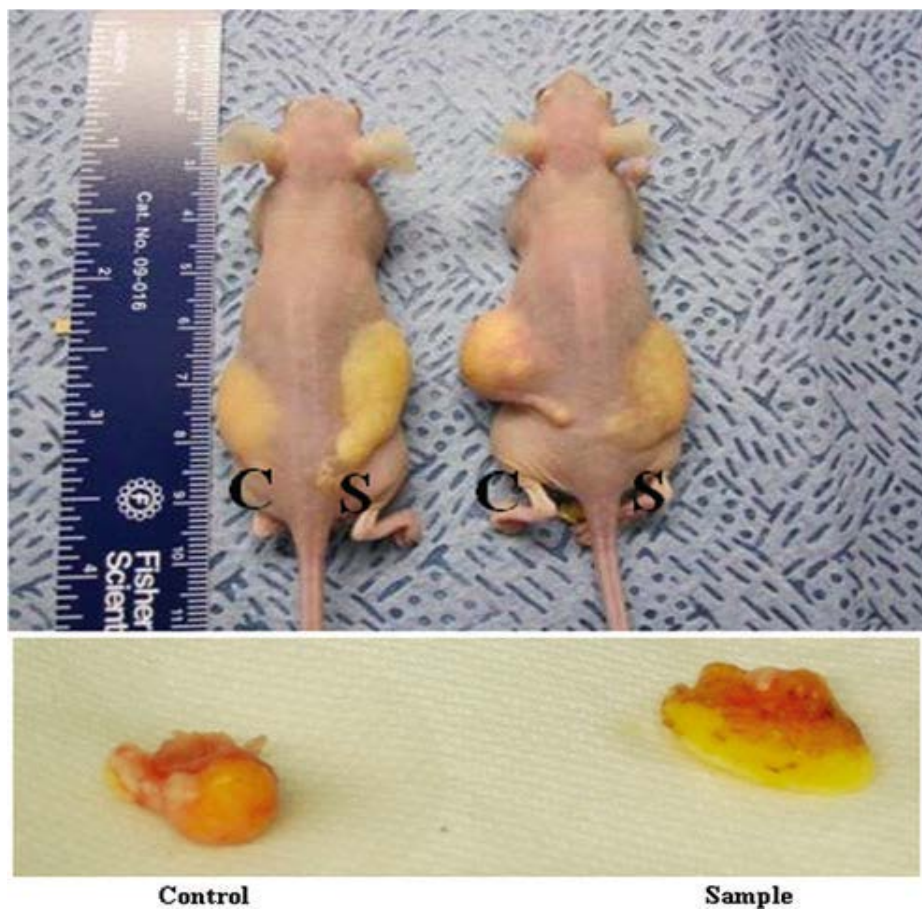


Figure 11. Dex MS effect in adipose tissue enhancement

Gross images were photographed at the 5-week time point of explantation. In the treatment group, 80mg of Dex MS was mixed with 1 mL of lipoaspirate, whereas the control group consisted of 1mL of only human lipoaspirate (C= control without Dex and S = sample with Dex MS).

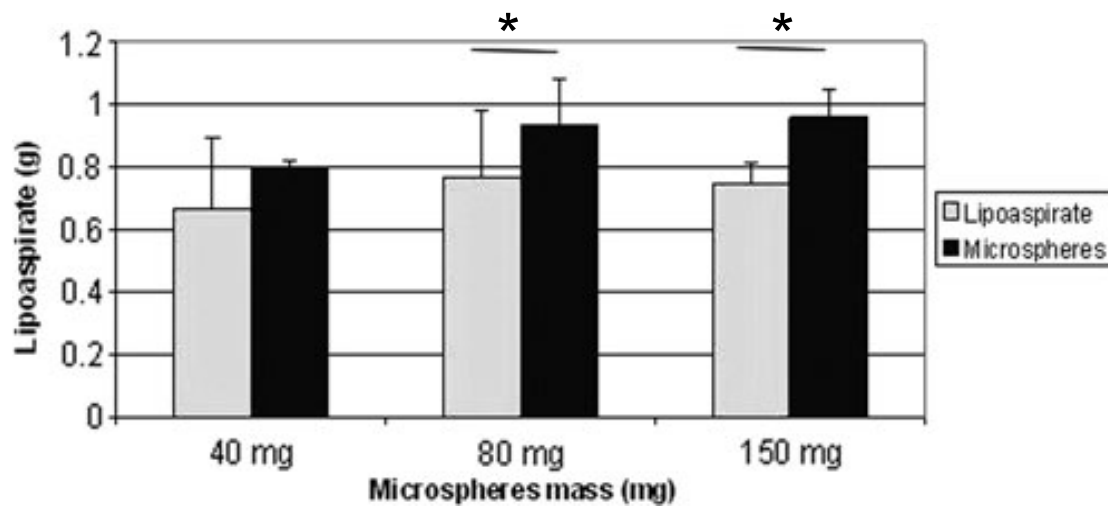


Figure 12. Results from adipose mass analysis of Dex MS treated animals. Mass of the explanted fat tissue at the end of 5 weeks was increased, as the dose of Dex MS was increased showing significant difference in the comparison with the control groups.

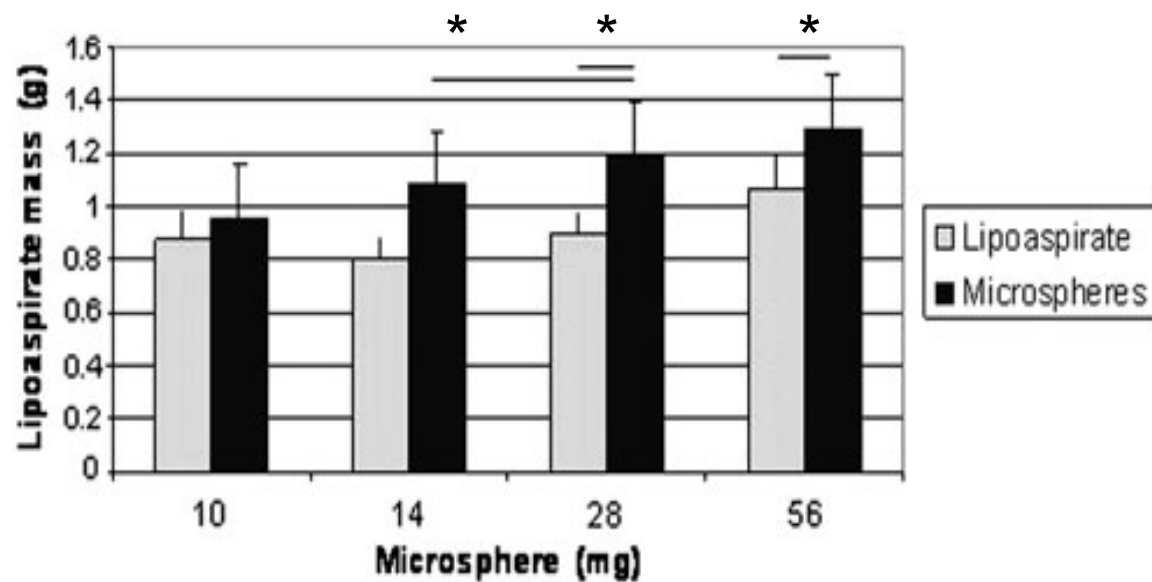


Figure 13. Results from adipose tissue treated with insulin MS animals. Mass of the explanted tissue after 5 weeks was increased, as the doses of insulin MS were increased, showing significant difference comparing with the control groups.

2.3.3 Combined drug *in vivo* studies

The animals from the Dex MS and insulin MS combined study were sacrificed after 5 weeks. Fat tissue was extracted, followed by measurements of volume and/or mass. The first numbers on the scale is the Dex part of MS and the second is the Insulin, 50 mg of Dex MS+ 90 mg of insulin MS mixed in 0.3mL of lipoaspirate. The single drug MS are the 27 mg of Dex MS and 10 mg of insulin MS mixed with 0.3mL of lipoaspirate and the controls are Empty MS and lipoaspirate only (L-left side and R- right side). Treatment groups are labeled with S (sample), whereas the control groups are the C (control).

The increase in mass after 5 weeks of MS treatment samples, as well as the decrease in volume for empty MS samples, was statistically significant depending on the dose (Dex MS Volume = 0.32 ± 0.043 mL, $p = 0.002$; Empty MS Volume = $- 0.14 \pm 0.051$ mL, $p = 0.646$).

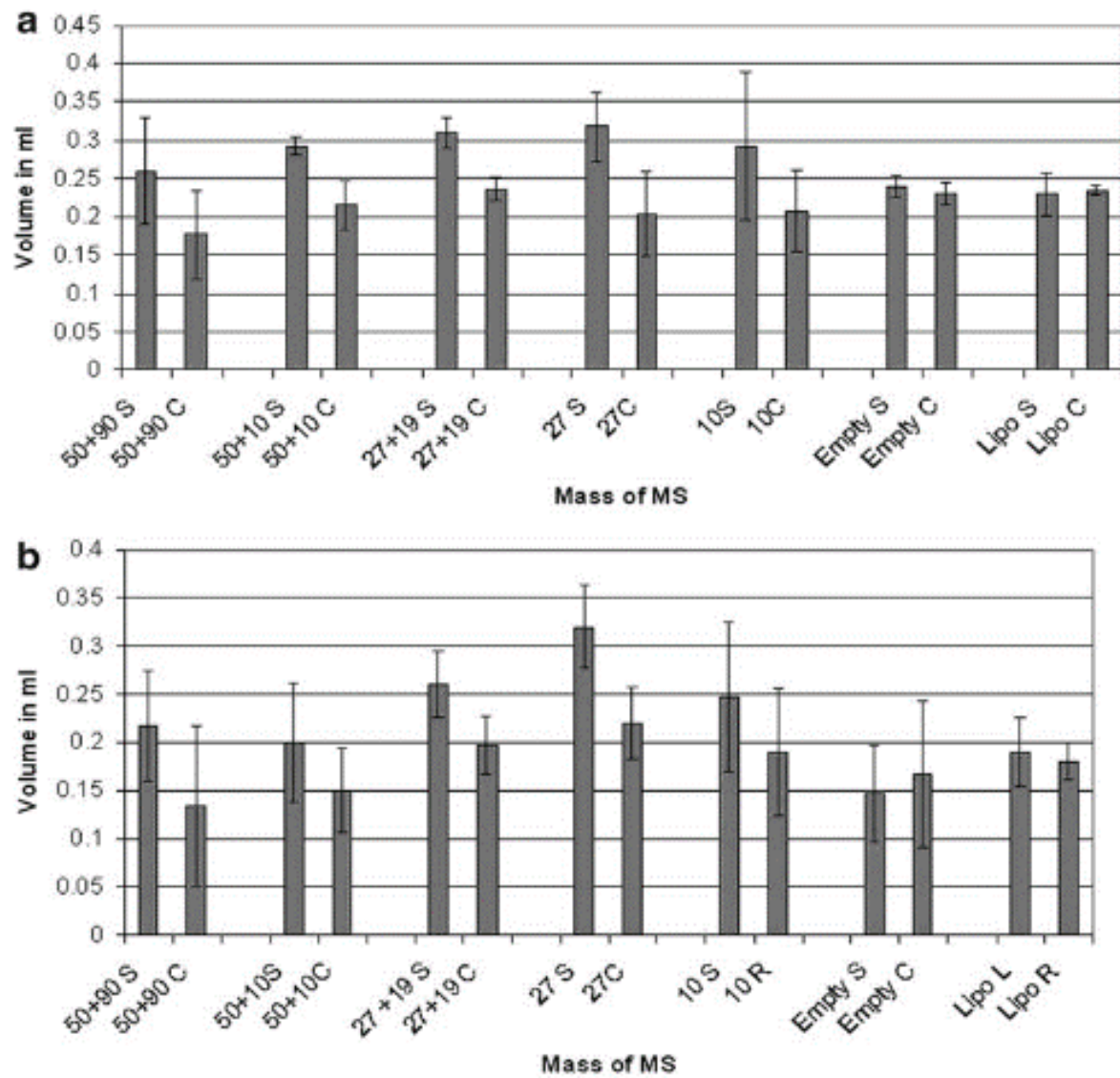


Figure 14. Results from a) mass and b) volume analysis of combined treated groups at 5 weeks.

In both graphs, the treatment side is labels with S and control side-lipoaspirate, with C

Differences were considered significant when $p < 0.05$. The combined *in vivo* study of both drugs did not result in a significant difference in volume compared with Dex or Insulin only (Figure 14). Although the results show a statistical difference compared with controls that were lipoaspirate only, there is a decrease in volume compared with samples that used individual drugs.

2.3.4 Long-term dexamethasone MS treatment groups

The animals from the Dex MS long-term study were sacrificed after monitoring 6 months. Fat tissue was extracted, followed by measurements of volume and/or mass. Significant difference was seen macroscopically between the Dex microspheres treatment groups and lipoaspirate only. (Figure 15)

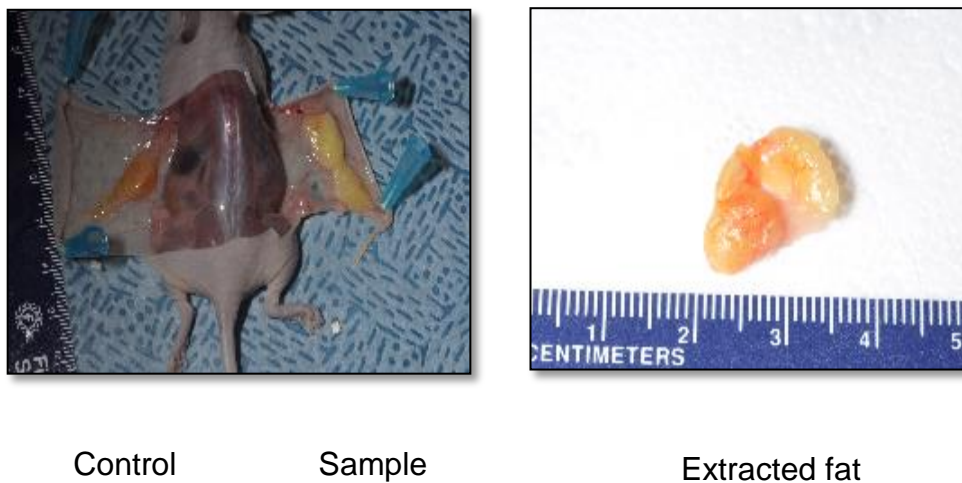


Figure 15. Long-term 27 mg Dex MS treatment results show a great difference between the treatment and control side. Extracted fat is highly vascularized

The volume displacement of the extracted tissue shown in the Figure 16, demonstrates a significant difference between the treatment and the control side. High dose Dex MS (50 mg) volume = 0.12 ± 0.040 mL, $p = 0.003$ low dose Dex MS (27 mg) volume = 0.10 ± 0.032 , $p = 0.004$; Empty MS Volume = 0.05 ± 0.058 mL, $p = 0.646$.

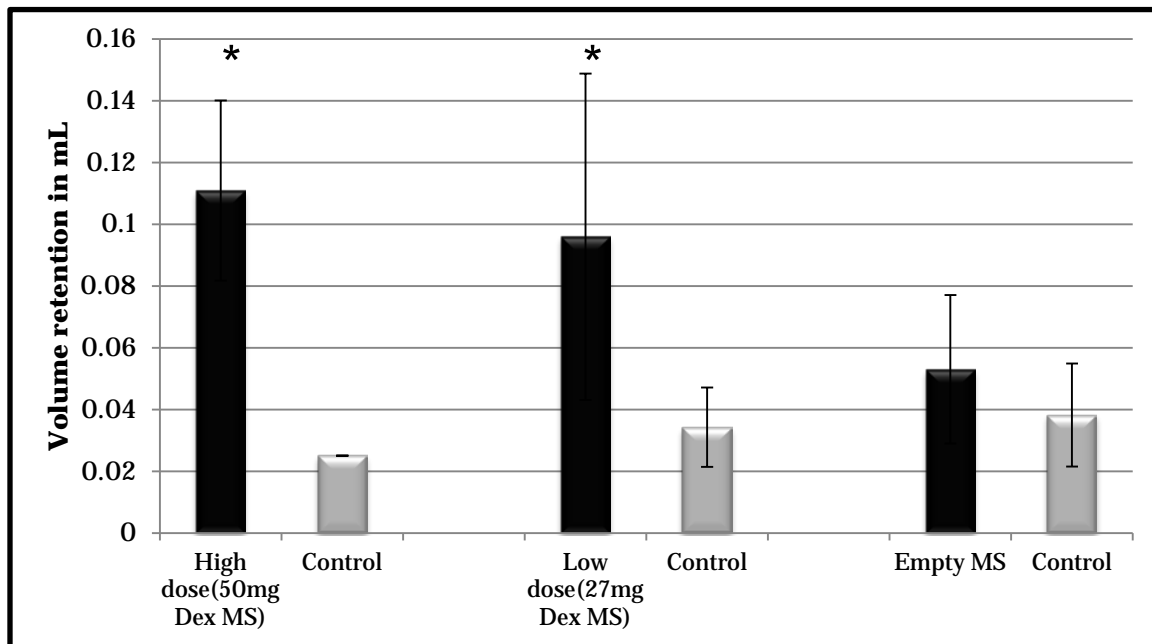


Figure 16. Volume measurements of long-term Dex MS animal study

2.3.5 Histological and Image J analysis

Ten tissue sections were stained with H&E, demonstrating gross architecture of the tissue. Blood vessels were quantified using ImageJ from the CD31 staining (Figure 17), resulting in a higher number of vessels in the treatment groups.

The results shown in Figure 18 elaborate the difference in vascularization between treatment and control samples in the single-dose study (Figure 18a) and the combined drug study (Figure 18b).

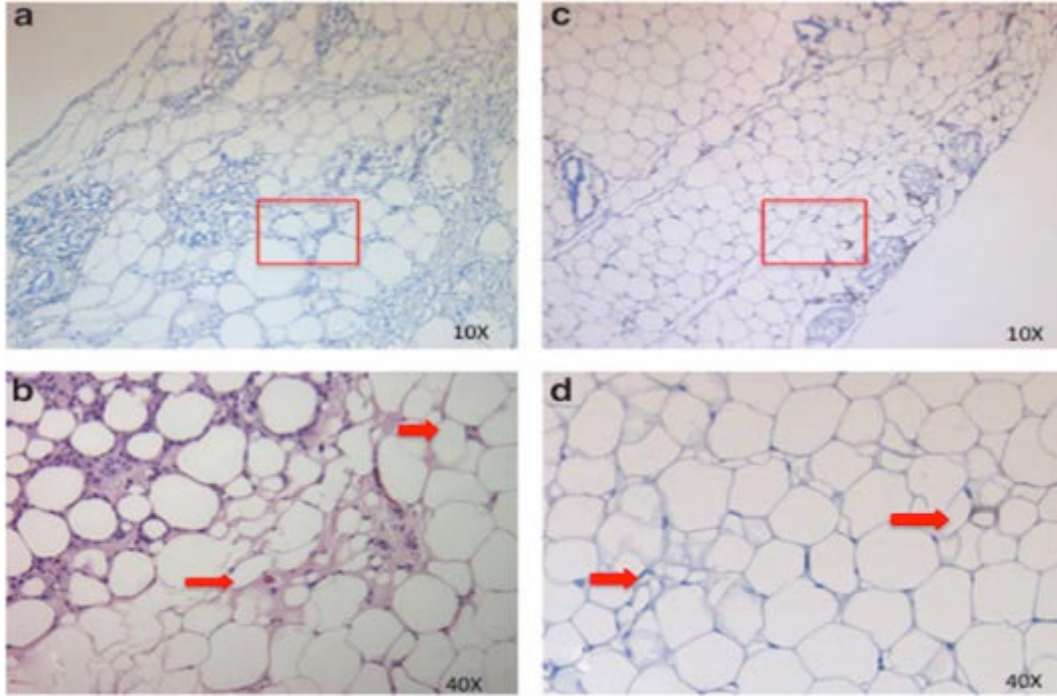


Figure 17. Human CD31 staining of Dex-loaded MS and Insulin-loaded MS, a) Dex-loaded microspheres group (80 mg Dex MS), b) magnified image of the Dex MS treated group (a), c) combined drug group CD31 staining and d) magnified figure of combined drug study (c)

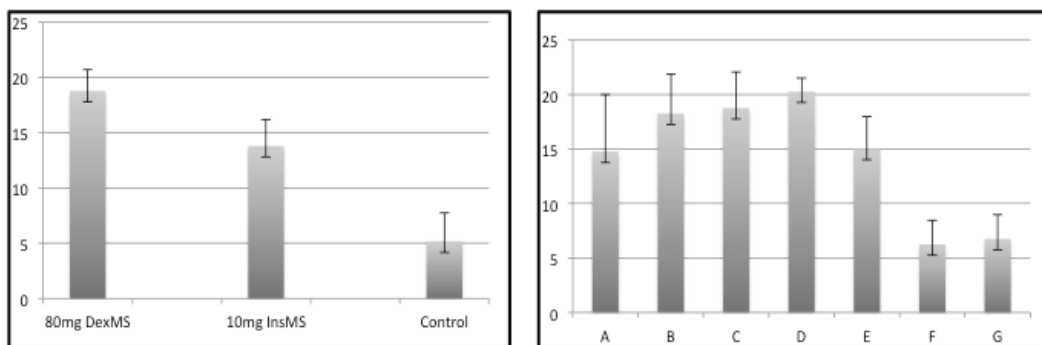


Figure 18. Blood vessels count, a) Dex MS treated groups and b) Combined drug MS treatment groups-Group A(50 mg Dex MS+90 mg Insulin MS), group B (50 mg Dex MS+10 mg Insulin MS), group C(27 mg Dex MS+19 mg Insulin MS), group D (27 mg Dex MS), group E(10 mg Insulin MS), group F(100 mg empty MS) and group G (lipoaspirate)

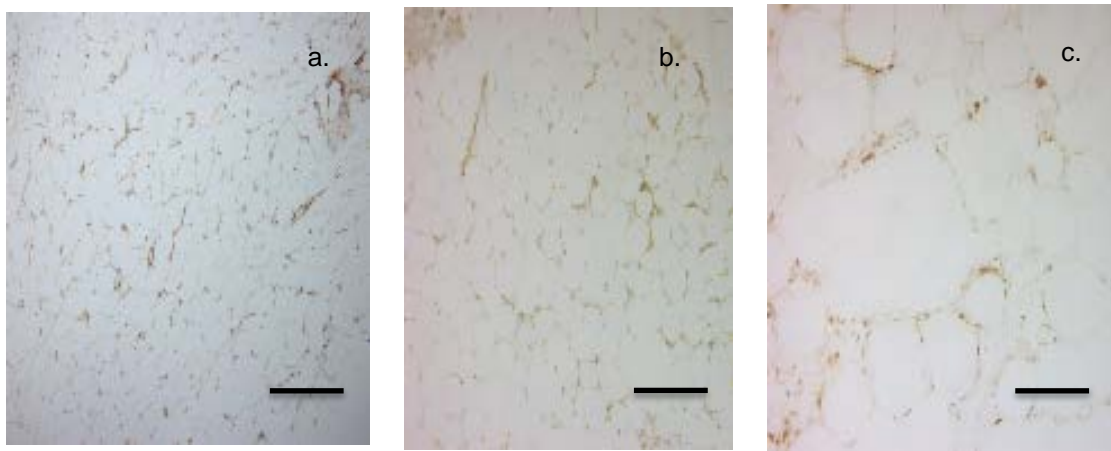


Figure 19. Long-term Dex MS treatment CD31 staining of the extracted fat, a) High dose (50 mg Dex MS), b) Low dose (27 mg Dex MS) and c) Lipoaspirate only

Long-term (6 months) Dexamethasone microsphere treatment groups (Figure 19a and Figure 19b) histology demonstrates highly vascularized tissue and healthy tissue morphology. Lipoaspirate group shows distracted tissue morphology with an evident lack of blood vessels (Figure 19c).

2.3.6 Statistical analysis

All results are presented as mean \pm standard deviation. The number of specimens in each group is presented in the above sections. Data was analyzed in Minitab 16 Statistical Software. Paired t-test, two sample t-test and/or one factor analysis of variance (ANOVA) tests were performed (where applicable) in order to determine if the differences between groups (control vs. experimental) were statistically different at $\alpha = 0.05$ significance level.

2.4 DISCUSSION

Resection of tumors in the head and neck, upper and lower extremities, as well as trauma and congenital abnormalities often resulted in contour defects due to the loss of soft tissue, largely composed of subcutaneous adipose tissue. Adipose tissue is a dynamic and multifunctional tissue that is ubiquitous throughout the human body.(35-37) Fat functions as a specialized organ that maintains the energy balance through controlled storage and release.

Adipocytes store energy in the form of triglycerides and accumulate or mobilize triacylglycerol in response to the body's energy requirements. (37) Adipose tissue is highly plastic and can adapt to facilitate greater storage through the hypertrophic expansion of terminally differentiated mature adipocytes, as well as the hyperplastic growth and differentiation of precursor cells present in the stroma. However, as mature adipose tissue does not transplant effectively, numerous natural, synthetic, and hybrid materials have been used to act as adipose surrogates.

This study outlines a new technology for fat retention during fat transfer using both Dex- and insulin-loaded PLGA MS. The effect of Dex in fat tissue enhancement has been studied before. As a synthetic glucocorticoid, Dex is more potent than the natural hormone cortisol, and its action in the enhancement of fat tissue formation by increasing the expression of C/EBP and PPAR- γ has been demonstrated. (38-40) Hence, there are a number of cases where Dex is used in medicine and in bioengineering research as an adipogenic catalyst. (40,41)

Insulin is a hormone that is known for regulation of carbohydrates and fat metabolism in the body. The presence of insulin in tissue stops the process of using fat as an energy resource. When insulin is absent, glucose is not taken up by body cells and the body begins to use fat as an energy source, or by transfer of lipids from adipose tissue to the liver for mobilization as an energy source.(42,43) The release of insulin to induce adipogenesis has been demonstrated, using *in vitro* and *in vivo* studies, resulting in tissue increased weight. (43,44)

Biodegradable drug delivery systems, such as PLGA-polyethylene glycol (PLGA-PEG) MS, have been studied as delivery vehicles for insulin, insulin-like growth factor-1 (IGF-1), and basic fibroblast growth factor. In a subcutaneous rat model, incorporating these growth factors improved autologous free fat graft weight and volume, with the best results observed for either insulin or IGF-1 alone or in combination. PLGA (75:25) foam was also assessed *in vivo* in combination with IGF-1 and insulin with fibroelastic tissue formation at the implantation site at 12 weeks. (46,47) In this study, we demonstrated that drug-loaded PLGA MS can have an impact on fat tissue enhancement through release of adipogenic drugs. The Dex-loaded MS successfully released the drug in a controlled fashion for duration of 3–5 weeks. Demonstrated by release studies, 70% of drug was released in the first 3 weeks with 100% release in 4–5 weeks.

Results demonstrated that after a 5-week period, there was a significant increase in mass and volume of the samples, which proved to be statistically significant. This observation was also seen with histological analysis. The results were notable when examined Dex only, which is known to be a synthetic steroid used as a strong anti-inflammatory drug, and also is a highly adipogenic factor.(48) increasing the expression of the C/EBP and PPAR γ genes.

An increase in mass, volume, and vascularization was also observed in the experiments with insulin-MS, which is a known lipogenic drug that enhances the lipid filling, as shown in previous *in vitro* and *in vivo* studies.(49) Insulin-loaded MS successfully resulted in a controlled release of insulin for a duration of 3–5 weeks, with 70% of insulin released in the first 3 weeks and 100% release in 4–5 weeks.

Results demonstrated that after a 5-week period, there was also a significant increase in mass and volume of the samples, but not as striking as Dex alone.

The animal groups treated with dexamethasone microspheres for a 6 month period, demonstrated adipose tissue retention compared to the group that was treated with empty microspheres and lipoaspirate only (Figure 16). Histology of the extracted adipose tissue from animals demonstrated a highly vascularized tissue in the dexamethasone microspheres treatment groups, with unobstructed adipose tissue structure.

2.5 CONCLUSION

The aim of this study was to improve the adipogenesis and angiogenesis of the adipose tissue after fat grafting via dexamethasone and insulin delivery by polymer based microspheres. In this study we elaborated the usage of encapsulated adipogenic drugs, such as insulin and dexamethasone, for stimulating fat tissue enhancement *in vivo*. A number of different injectable soft tissue replacement are used to reconstruct and regenerate the soft tissue after a defect, but most fail because of lack of vascularization before the scaffold gets integrated in the in site surrounding tissue. By preserving the adipogenesis and angiogenesis in the injected tissue with adipogenic drugs, we achieved enhanced retention of adipose tissue after grafting. Sustained delivery of the drugs through PLGA MS, induced enhanced adipose retention, showed by gross and histological differences in fat tissue vascularization compared with lipoaspirate alone.

Taking into consideration that all the components of the microspheres are FDA-approved and with a long clinical history including the health/biohazard details, this model shows a potentially relevant therapy for soft tissue reconstruction in clinical settings. The preliminary data supports that a 4-5 week delivery of the dexamethasone encapsulated in single-walled PLGA microspheres resulted in enhanced adipogenesis in an *in vitro* environment (8); therefore single-walled microspheres in *in vivo* settings were expected to work in same manner by increasing the adipogenesis in injected fat by maintaining a stable lipoaspirate volume at both time points. Doses of the drug released are undetectable by hormonal passage of the animals, and no adverse side effects were evident.

The mechanisms of dexamethasone and insulin in adipose retention are still unknown. There are studies that show that adipocyte going through the process of hypertrophy during the effect of dexamethasone or insulin. More research is needed to elucidate a clear mechanism. We have demonstrated that adipose tissue preserved volume after 5 weeks in an athymic mouse model compared to control lipoaspirate only treatments. These promising studies will support future longer-term studies as well as potential larger animal models.

3.0 DEXAMETHASONE-LOADED DOUBLE-WALLED MICROSPHERES

3.1 INTRODUCTION

The adipose tissue has become a focus area for soft tissue engineering based on a large number of reconstructive and correctional conditions that could be corrected with adipose tissue engineering strategies. Adipose tissue is a dynamic and multi-functional tissue that is ubiquitous throughout the human body.(50-51) Fat functions as a specialized organ that maintains the energy balance through controlled storage and release. Adipocytes store energy in the form of triglycerides, and accumulate or mobilize triacylglycerol in response to the body's energy requirements.(52) Adipose tissue is an active and dynamic tissue, which can adapt to facilitate greater storage through the hypertrophic expansion of terminally differentiated mature adipocytes, as well as the hyperplastic growth and differentiation of precursor cells present in the stroma. Mature adipose tissue does not transplant effectively; therefore, numerous natural, synthetic, and hybrid materials have been used to act as adipose surrogates.(53)

Due to issues of variability of retention of grafted fat, much research has been conducted to overcome and improve the survival of adipose tissue grafting. Processing of fat tissue prior to fat grafting is one method that has been manipulated for the purpose of maintaining improved adipose tissue retention. (54-55)

The effect of dexamethasone in fat tissue enhancement has been studied through the years. Being a synthetic glucocorticoid, dexamethasone is more potent than the natural hormone cortisol, the role and its action in enhancement in fat tissue formation by increasing the expression of C/EBP and PPAR- γ . (57) Hence, there are a number of cases where Dex is used in medicine and in bioengineering research as an adipogenic catalyst. In this objective, dexamethasone was encapsulated in a PLGA microsphere core surrounded by PLLA shell with the objective of a slower and better-controlled drug delivery. By encapsulation of dexamethasone in double-walled microspheres, the drug will be released in a sustained and controlled fashion for an average of 80-120 days. The release of the adipogenic drug in a slow and more controlled environment should favorably affect adipose tissue retention.

3.1.1 Specific Aim 2.

The specific aim of this chapter was to fabricate and optimize dexamethasone encapsulation in double wall microspheres, together with optimizing the doses of Dex DW MS in *in vivo* testing. The rationale of this aim was based on the fact that dexamethasone, protected by the PLGA and PLLA, will be released in a controlled manner for duration of 10-24 weeks, with a minimal burst release effect.

Slow and controlled release of dexamethasone within injected fat in a mouse model will enhance adipogenesis in a controlled fashion, therefore increasing adipose tissue retention.

To test this hypothesis, dexamethasone was encapsulated double wall microspheres and proven the core-shell architecture together with drug release profile. Once the parameters of the microspheres were established, they mixed with human lipoaspirate and injected in athymic mouse model, testing the adipogenesis in two time points. Volume and mass measurements of the extracted tissue were calculated followed by histology testing including tissue morphology and presence of blood vessels.

3.2 METHODS

3.2.1 Dexamethasone encapsulation within double-walled microspheres

In order to create double-walled microspheres, 400 mg of PLGA (75:25) was dissolved in 4.5 mL of dichloromethane (DCM). In a separate vial, a 10% solution of PLLA of equal polymer mass was prepared. After both polymers were dissolved, 20 mg of dexamethasone-sodium phosphate was added to the PLGA solution and vortexed until the solution was homogenous. The PLGA solution was then added to the PLLA solution and vortexed for additional 60 seconds.

This solution was then added via pipette to 400 mL of aqueous 1% poly(vinyl alcohol) solution and stirred at 500 rpm for 3 h. The microspheres were collected by means of centrifugation, followed by freeze-drying for a 24-hour period (LabConco Freezone 4.5). Empty double-walled microspheres (Empty DW MS) were created following the same protocol as dexamethasone double-walled microspheres (Dex DW MS), omitting the addition of dexamethasone.

3.2.2 Dexamethasone double-walled microsphere characterization

The morphology of both single and double-walled microspheres was determined using scanning electron microscopy (SEM). Initially, the microspheres were gold coated using a Cressington 108 Auto (Cressington), followed by the usage of a JSM-6335F SEM (JEOL) operated at an acceleration of 3.0 kV for morphology characterization. The size distribution of microspheres was determined by measuring the diameters of 30-50 microspheres using SEM images. Microsphere loading capacity was determined by using the equation $LC = \text{Amount of drug loaded (AD)} / \text{amount of polymer and amount of drug loaded (AP+AD)}$. Encapsulation efficiency (EC) was determined by amount of actual drug concentration (C_a) divided by theoretical concentration (C_{th}).

3.2.3 Dexamethasone double-walled microspheres ethyl acetate test

In order to confirm the polymer core-shell architecture in the double-walled microsphere, a solvent-specific ethyl acetate dissolution test was conducted. The polymer PLLA is not soluble in ethyl acetate, while PLGA is soluble. Therefore, dexamethasone loaded double-walled microspheres were sectioned and mounted on metal stubs using double-sided copper tape. After the microsphere were attached onto the metal stubs, they were exposing the ethyl acetate for 2 days. After this period the stubs were dehydrated and imaged with SEM. Because the PLGA component of the double-walled microsphere was dissolved, PLLA spherical shell was expected to remain.

3.2.4 Dexamethasone release from microspheres

The release kinetics of dexamethasone from both single and double-walled microspheres were tested by incubating 10 mg of microspheres in 1 mL of phosphate-buffered saline (PBS) at 37 °C. Supernatant was collected weekly for 45 weeks. Each week, the solution was replaced with fresh PBS after being vortexed for 15 sec. The amount of dexamethasone released was analyzed using spectrophotometry. The data collected was calculated using a standard curve.

3.2.5 Dexamethasone-loaded microspheres *in vivo* study design

Animals used in the study were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. A total number of 60 female athymic nude mice (5–10 weeks old; Harlan Laboratories) were used for the study. Animals were separated into 12 groups, each containing 5 mice per group (Table 5). The right side injection was the experimental (sample) group, and the left side consisted of the control group. Single and doubled walled dexamethasone microspheres were tested at two different time points, 6 weeks and 6 months. Two different combinations of dexamethasone microspheres were examined as shown in table 5.

Table 5. Doses of dexamethasone loaded single and double-walled microspheres

Treatment 0.3mL injections	Drug treatment dose	Number of animals	Time points
High dose Double-walled MS	50 mg Dex MS	5	6 weeks/ months
Low dose Double-walled MS	27 mg Dex MS	5	6 weeks/ months
High dose Single-walled MS	50 mg Dex MS	5	6 weeks/ months
Low dose Single-walled MS	27 mg Dex MS	5	6 weeks/ months
Empty MS A	50 mg MS	5	6 weeks/ months
Lipoaspirate B	NA	5	6 weeks/ months

The dosage of the drug is determined by the mass of the microspheres injected. The highest dose contained 50 mg of dexamethasone microspheres and the lowest dose consisted of 27 mg of dexamethasone microspheres, which correlated to ≈ 500 ng of Dex and ≈ 270 ng of Dex respectively. In addition to experimental groups, two control groups were tested. Control group 'A' contained empty microspheres, whereas control group 'B' consisted of only lipoaspirate. The doses were chosen based on our results from a previous study.(58)

The doses correspond to concentrations currently used in humans that are within the safe and therapeutic range. (60,61) Human lipoaspirate was processed as approved by the University of Pittsburgh Institutional Review Board, obtained by patients undergoing elective surgery using the Coleman method as described in previous studies.(58,59)

3.2.6 Animal surgeries

Animals were anesthetized with 3% isoflurane in oxygen at the time of the surgery. The lipoaspirate/MS injections were performed using a 16-gauge surgical cannula, in both sides of the dorsal area of the animal. The right side was injected with the experimental group and the left side contained lipoaspirate exclusively (Figure 20). Photographs were taken immediately following the surgery and weekly throughout the study.

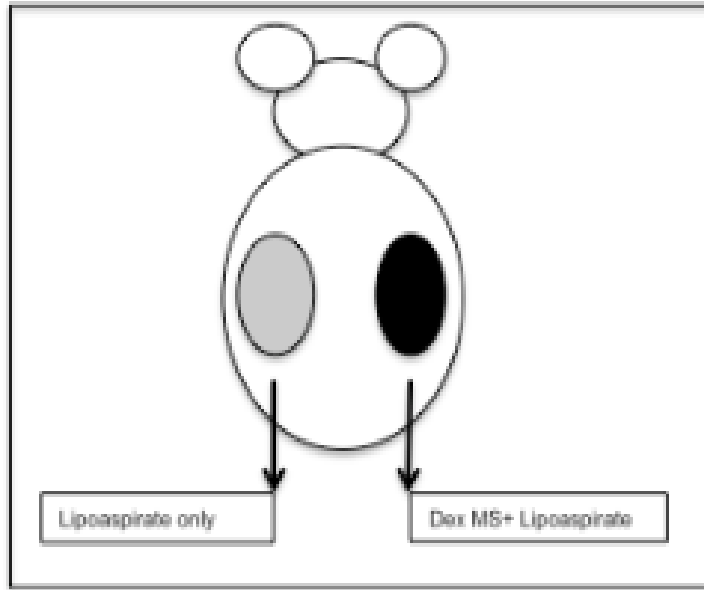


Figure 20. Animal surgery design

3.2.7 Histological analysis

Following extraction, the samples were fixed in 4% paraformaldehyde, incubated overnight in 30% sucrose (Sigma-Aldrich), and then embedded in paraffin, then cryosectioned at a thickness of 10 μm . Hematoxylin (Santa Cruz Biotechnology, Inc.) and eosin (Sigma-Aldrich) (H&E) staining was conducted using the same protocol as in our previous study.(58) Vascularization of the extracted samples was determined by assessing a pan peroxidase-based CD31 antibody staining to confirm the presence of blood vessels.

3.2.8 Image analysis

The Olympus Provis microscope (Olympus) was used to image extracted adipose tissue slides. Adipose tissue architecture was assessed by analyzing the tissue morphology of H&E stained slides. Blood vessels were counted using ImageJ (NIH). Blood vessel lumens were counted using CD31-stained slides, containing 5 slides per group. CD31-stained slides were used to evaluate 20 random cell surface areas. Beginning at the center of the slide and counting five cells in four directions, Image J was used to set the difference in cell areas between the control and treatment groups. Ten random tissue sections were quantified for each sample and means were determined for the aforementioned vessels and vessel lumens.

3.2.9 Dexamethasone-loaded microspheres systemic effects

Dexamethasone is able to effectively suppress levels of corticosterone as negative feedback from adrenocorticotrophic hormones.(62-64) The levels of dexamethasone in treatment groups were tested by drawing blood from animals at the 6 week timepoint. Before sacrifice, blood was collected and preserved in EDTA-coated tubes from four animals from each group under light anesthesia. The plasma was then separated and stored at -80 °C until used. Corticosterone levels in the plasma were quantified using an ELISA kit following the manufacturer's instructions (Enzo Life Sciences).(65-67)

3.2.10 Statistical analysis

Data was analyzed using SPSS Statistical Software. Paired t-tests, two-sample t-tests, and mixed way analysis of variance tests (ANOVA) were performed to determine if the differences between the experimental and control groups were statistically significant at the $p \leq 0.05$ significance level. All results are presented as mean \pm standard deviation.

3.3 RESULTS

3.3.1 Dexamethasone microspheres characterization

Single and double-walled microspheres were examined using SEM images. Single-walled microspheres both dexamethasone loaded and empty are an average of $100 \pm 10.5 \mu\text{m}$ in diameter (Figure 21a). Double-walled microspheres are an average of $250 \pm 20.5 \mu\text{m}$ of diameter (Figure 21b). Single-walled dexamethasone-loaded microspheres released the drug in 80 days, with an average of $30 \pm 5.5\%$ being released in the first week. After 85 days, around $95 \pm 3.5\%$ was released in total (Figure 22). Double-walled dexamethasone loaded microspheres released the drug in a period of ~ 300 days, with $\sim 10 \pm 5.5\%$ released in the first 40 days, resulting in a minimal burst release effect.

At day 300, $90 \pm 2.7\%$ of the drug was released. Double-walled dexamethasone loading capacity was found to be $\text{LC} = 42 \pm 3.6 \text{ mg Dex/g MS}$ and the encapsulation efficiency was calculated as $\text{EE} = 43 \pm 2.3 \%$.

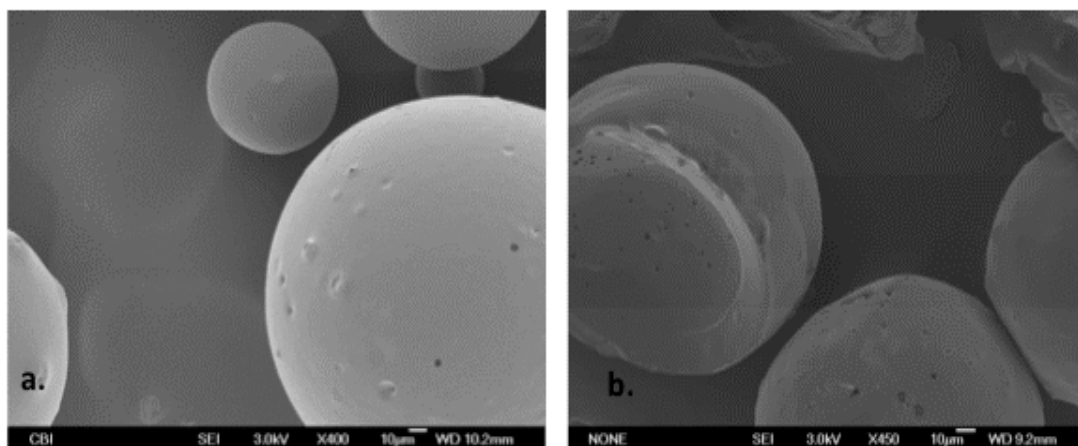


Figure 21. SEM images of dexamethasone loaded microspheres, a) Single-walled poly(lactic-co-glycolic acid) (PLGA) dexamethasone loaded microspheres, b) Double-walled poly(lactic-co-glycolic acid) (PLGA)- poly-L-lactide (PLLA) dexamethasone loaded microspheres

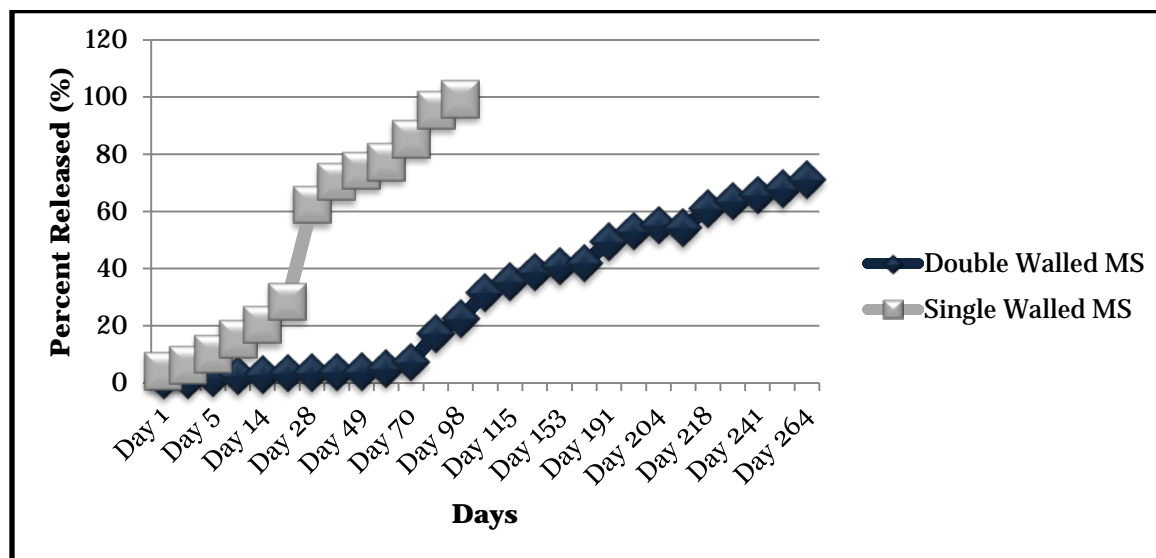


Figure 22. Drug release profile of Dex SW MS and Dex DW MS

3.3.2 Dexamethasone double-walled microsphere polymer orientation

Core-shell architecture of the microspheres was tested with a dissolution ethyl acetate test. Microspheres were first sectioned and treated with ethyl acetate, a solvent that dissolves PLGA and not PLLA. The SEM imaging of microspheres treated for 48h with ethyl acetate show mass loss of inner PLGA core. The PLLA shell was preserved to some extent in spheres treated with ethyl acetate. Figure 23 shows the results from ethyl acetate test, which shows the presence of the core-shell architecture of double-walled microspheres.

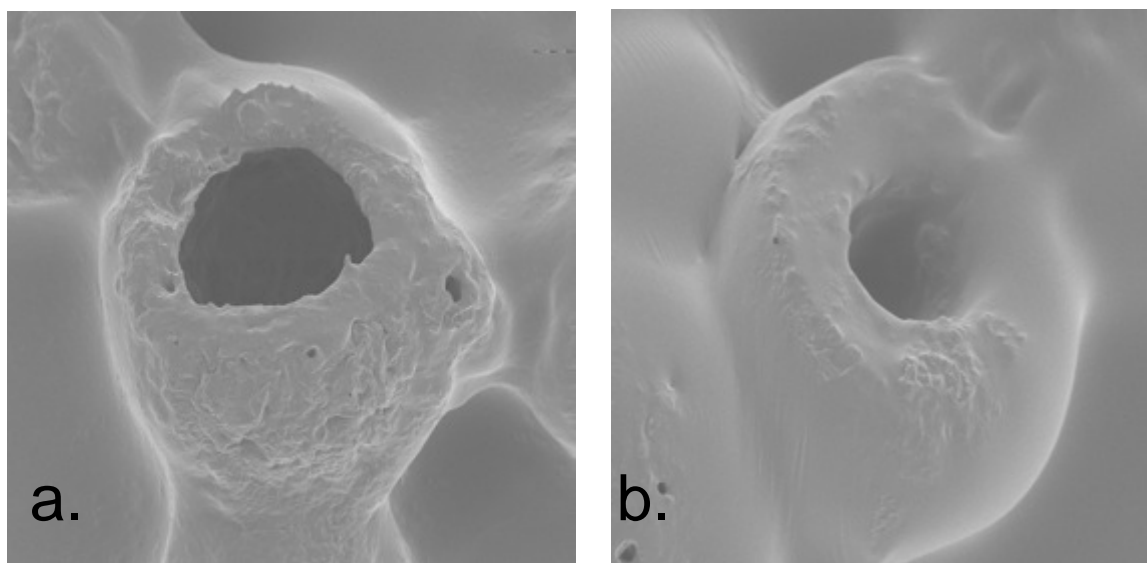


Figure 23. Core-shell orientation of polymers in double-walled microspheres tested with ethyl acetate test, a) Empty double-walled microsphere and b) Dexamethasone loaded double-walled microsphere

3.3.3 *In vivo* studies

Animals were sacrificed at two different time points: 6 weeks and 6 months. Dexamethasone-loaded microspheres were mixed with 0.3 mL of lipoaspirate, whereas the control consisted of 0.3 mL of only human lipoaspirate (C=Control without microspheres and S=sample with Dex MS). All animals were also compared with a control group that consisted of empty microspheres injected in one side and lipoaspirate in the other, as well as with a group that had lipoaspirate injected in both sites of the animal labeled a. and b. A significant difference can be seen between the control and the single-walled microsphere treatment groups in the photographs taken at the 6-week time point (Figure 24a) On the contrary, no noticeable difference can be seen between the double-walled microsphere treatment group and the control side at the earlier 6 week time point (Figure 24b).

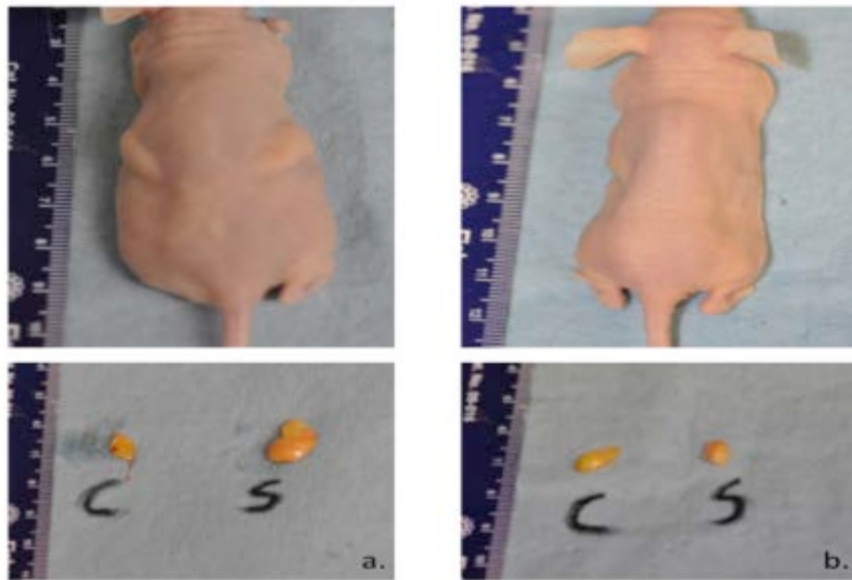


Figure 24. Gross images of extracted adipose tissue at week 6 a) Dexamethasone single-walled microspheres (27 mg Dex SW MS) treatment, and b) Dexamethasone double-walled microspheres (27 mg Dex DW MS) treatment

Photographs taken at the 6-month time point, however, show significant differences between the treatment group and the control group in both single-walled (Figure 25a) and double-walled dexamethasone microspheres (Figure 25b).

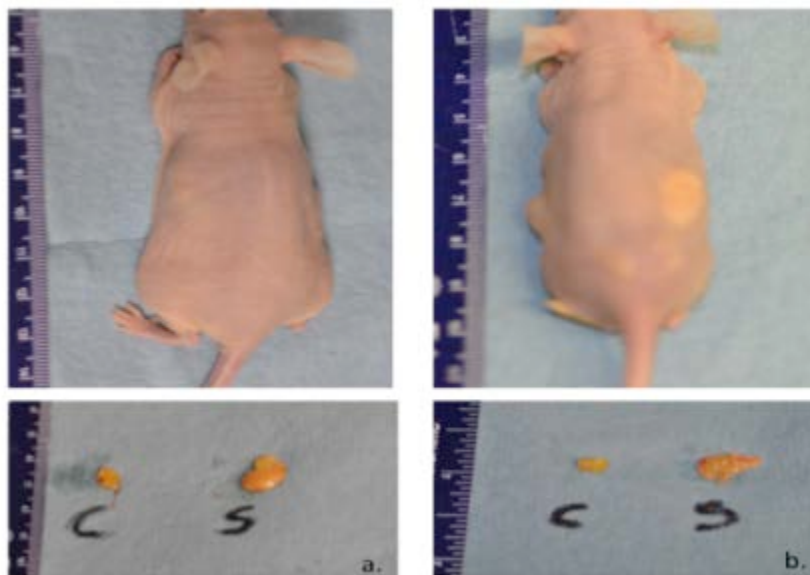


Figure 25. Extracted adipose tissue from animals at 6 months' time point, a) Dexamethasone single-walled microspheres (Dex SW MS) treatment and b) Dexamethasone double-walled microspheres (Dex DW MS) treatment

3.3.4 Adipose tissue mass and volume measurements

Macroscopic differences observed within the injections that contained dexamethasone microsphere treatments compared to tissue without Dex were confirmed by measuring mass and volume displacement of the extracted lipoaspirate in all groups. Tissue extracted from the earlier time point (6 weeks), show increase in mass and volume in single-walled dexamethasone microspheres treatment (Figure 26a and b), likely due to faster release of the drug from the single-walled microspheres.

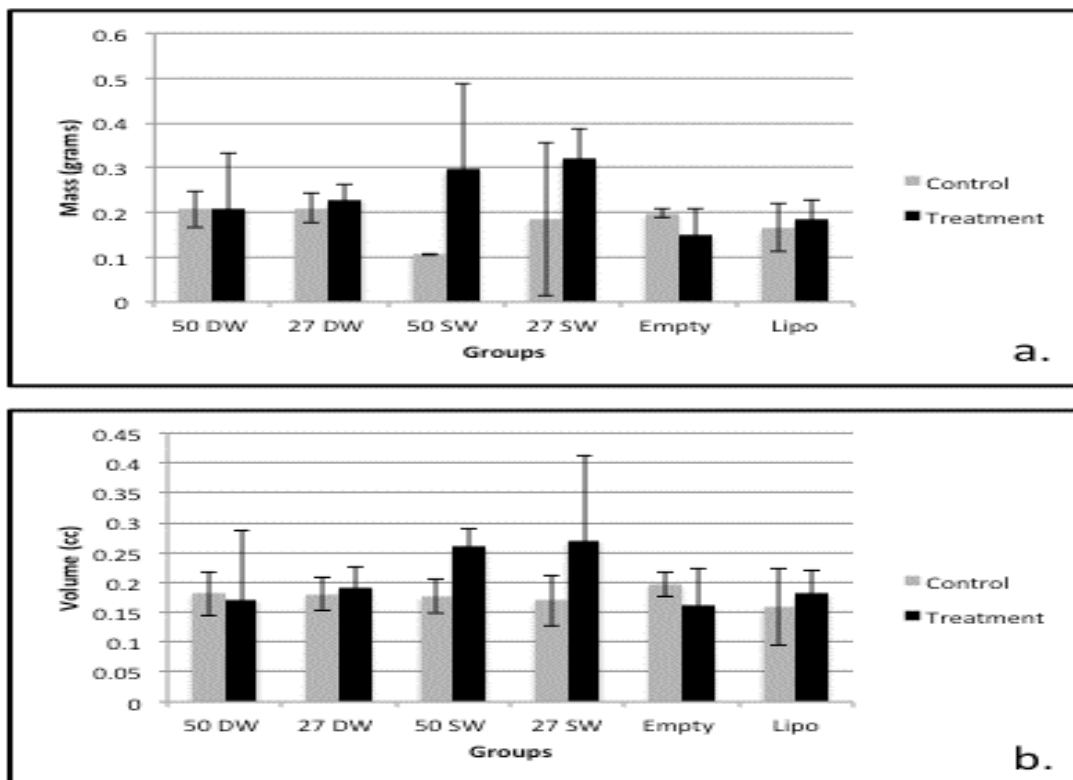


Figure 26. Adipose tissue extracted from animals at 6 week time point mass measurements (Fig. 21a) and extracted adipose tissue volume measurements (Fig. 21b)

Adipose tissue extracted from the second time point, 6 months respectfully, show a higher increase in mass and volume measurements in double-walled dexamethasone microspheres treatment groups compared to control groups of empty microspheres and lipoaspirate only (Figure 27a and b).

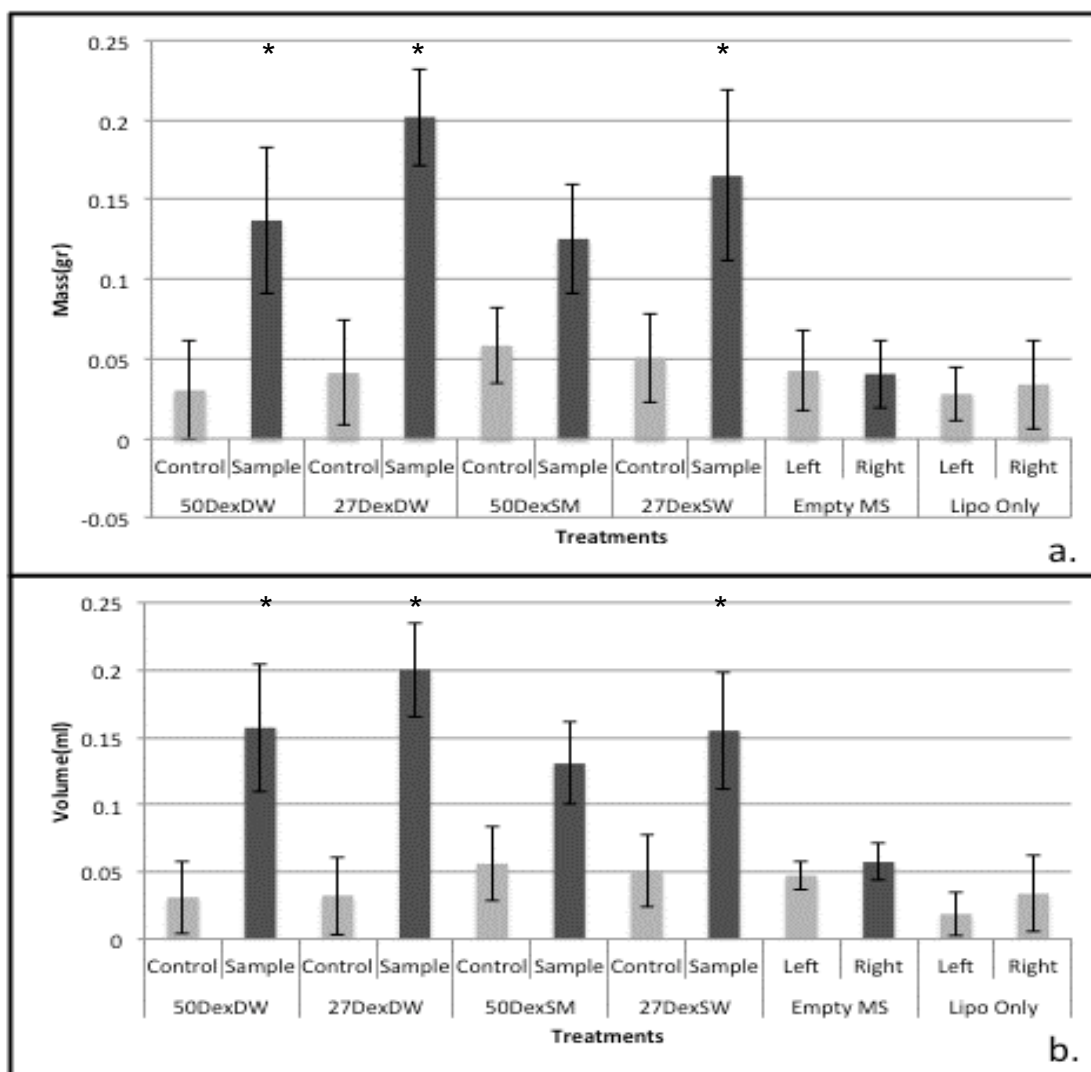


Figure 27. Adipose tissue extracted from animals at 6 months' time point mass measurements (Fig. 26a) and extracted adipose tissue volume displacement (Fig. 26b)

3.3.5 Dexamethasone loaded microspheres systemic effect

Plasma collected from four animals (n=4) per group of the 6-week time point was tested for the presence of dexamethasone in the systemic blood stream using the Corticosterone ELISA kit and by following the manufacturer's instructions (Enzo Life Sciences). Corticosterone levels were similar in all groups ($\approx 1,600$ pg/mL), including the treatment and control groups, showing no significant difference in corticosterone levels on a systemic scale in any of the groups (Figure 28).

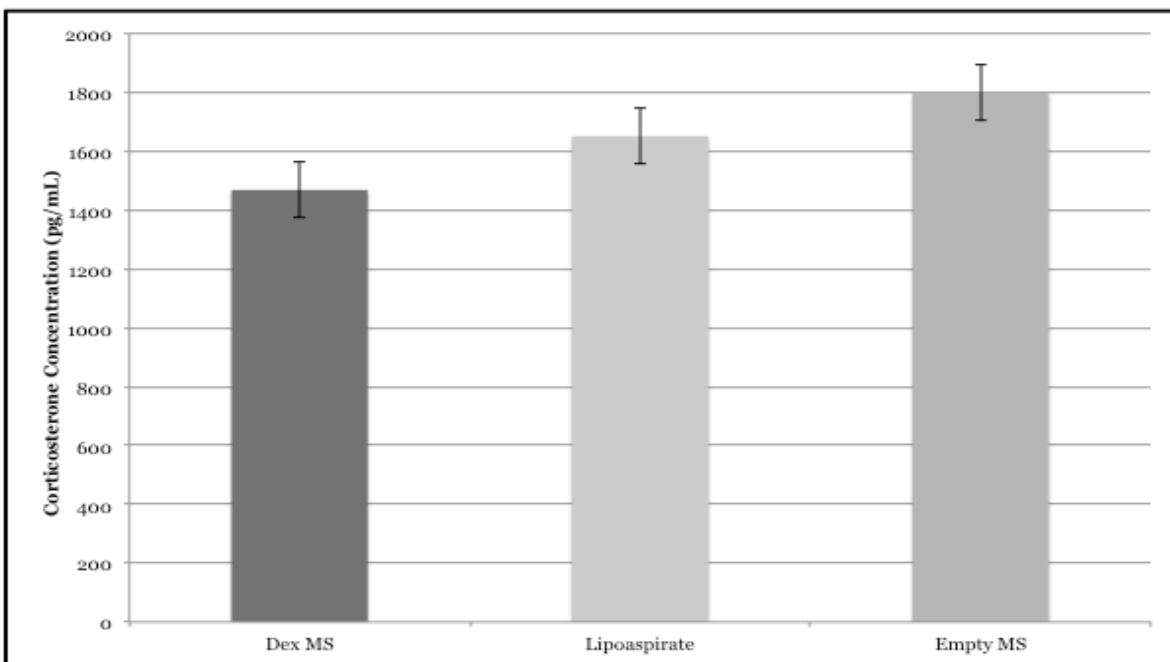
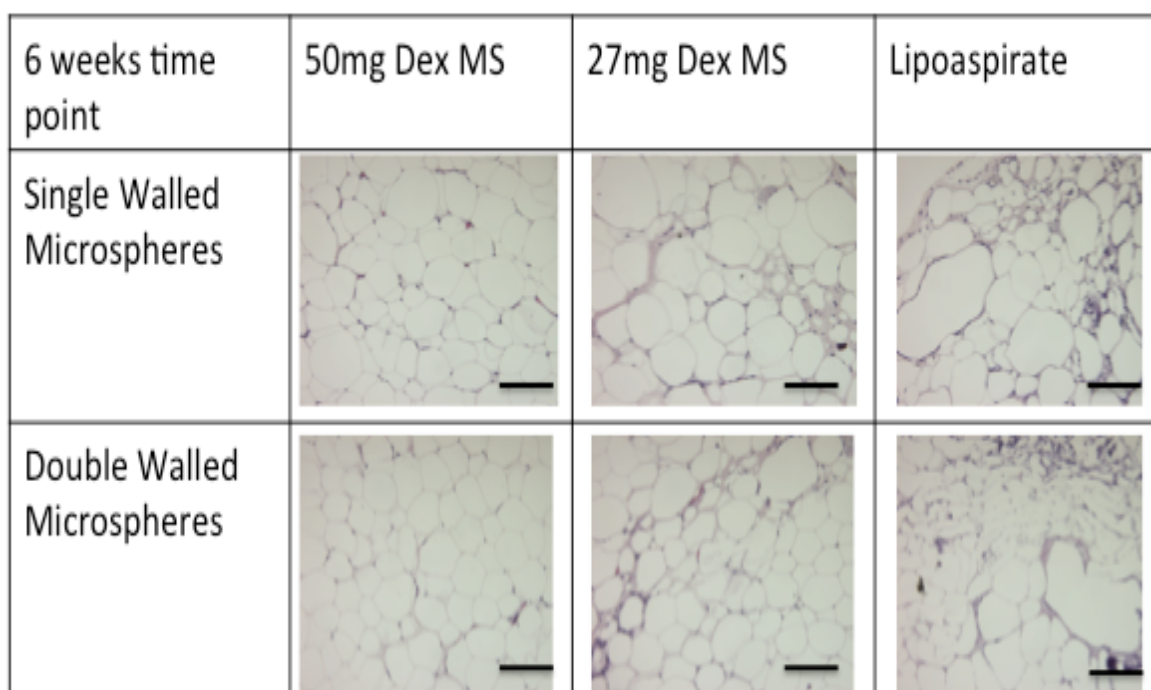


Figure 28. Corticosterone levels in all groups were average of 1,600 pg/mL, including the treatment and control groups

3.3.6 Histological analysis

Three tissue sections from each lipoaspirate extraction were stained with H&E in order to analyze gross architecture of the tissue (Figure 29). H&E images show healthy morphology of the adipose tissue in all treatment groups, without any interruption from the polymer microspheres. Adipose tissue without any microsphere treatment shows large adipocytes with surrounding disrupted tissue.



Scale bar 0.06mm

Figure 29. Histology of the extracted adipose tissue at 6 weeks' time point. H&E staining of the treatment groups and lipoaspirate control

Blood vessels were analyzed using Image J to view CD31-stained slides. The differences in vascularization between treatment and control samples are demonstrated in Figure 30. Presence of blood vessels were evident in all treatment groups, 50 mg Dex MS and 27 mg Dex MS, single and double-walled microspheres, with predominantly higher number of blood vessels in the double-walled microspheres treatment groups. Control group, lipoaspirate showed significantly fewer blood vessels compared to all the treatment groups. The difference in mass measurements between all groups was analyzed statistically with SPSS software, showing a statistically significant difference between the treatment group from the 6-month time point, which had results similar to the volume displacement measurements.

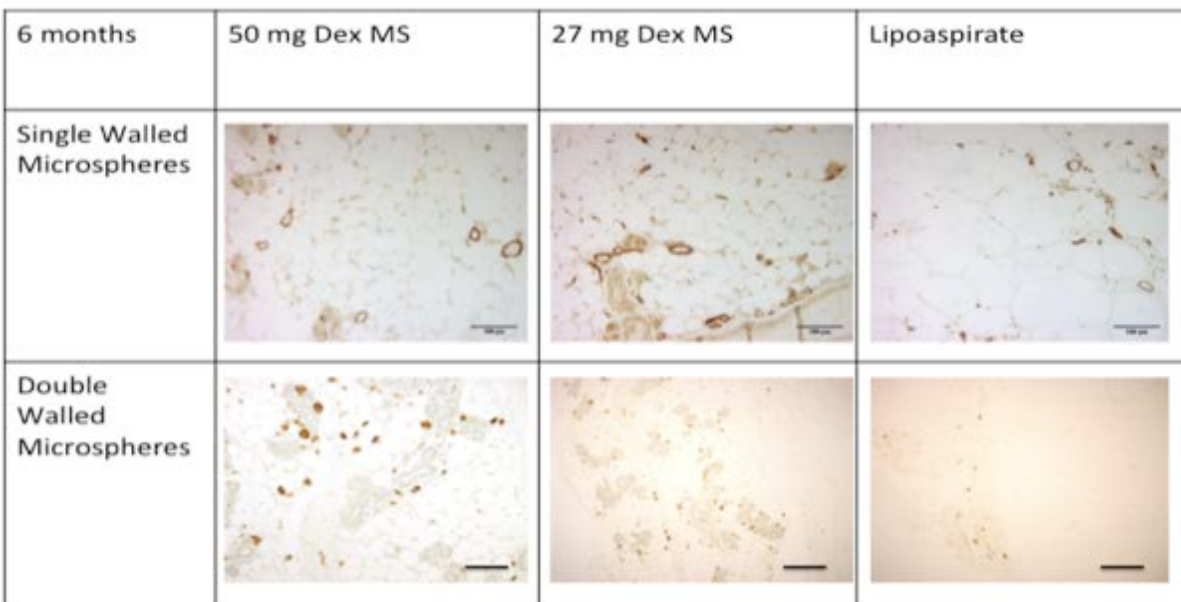


Figure 30. Histology of extracted fat at 6 month time point. CD31 staining shows a significant difference on blood vessel presence in treatment groups compared to control

All treatment groups had significant volume retention, with the 27 mg Dex DW MS group retaining up to 80% of the initially injected lipoaspirate (Figure 27b). Measurements from the 6-week time trial showed mass and volume retention of up to 60% for tissue treated with single-walled microspheres compared to the empty microsphere and lipoaspirate groups (Figure 26).

3.3.7 Dexamethasone microspheres treatment extracted fat cell size

Tissue explants were also analyzed to determine trends in cell size (hyperplasia v. hypertrophy) for various treatment groups and controls utilizing the microscope imaging software from NIH, ImageJ (Figure 31). Hyperplasia results from an increase in cell number which would indicate adipose tissue regeneration and adipose stem cell (ASC) differentiation. Contrastingly, hypertrophy results from an increase in cell size. Hypertrophy can indicate the initial stages of necrosis and apoptosis or increased lipid retention. Analysis involved randomly choosing five adipocytes from each explant sample and measuring their area in square μm . The average area for each group was calculated with percent standard of deviation. A paired, two-tailed t-test via SPSS software was utilized to determine if the size differences of adipocytes between control and experimental samples held any statistically significant differences with any comparison with a $p\text{-value} < 0.05$ resulting in statistical significance.

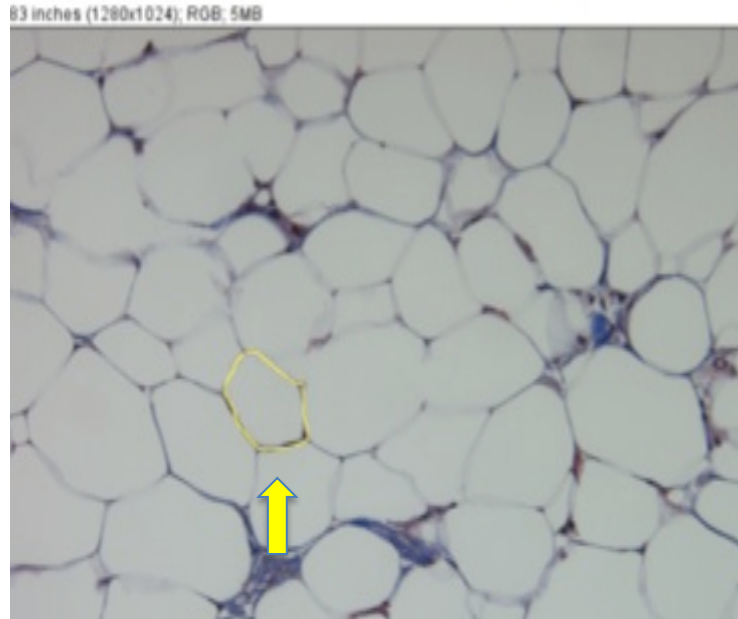


Figure 31. CD31-ImageJ NIH software image of Dex 27 mg SW MS treatment group. Arrow shows the process of labeling adipocyte area

The results from this data analysis is shown in Figure 32 From the data it was determined that adipocytes from the experimental groups were significantly smaller in size, and thus higher in number, compared to the control explant groups that did not receive Dex MS treatment. This indicates that the Dex MS impacted hyperplasia within the adipose tissue.

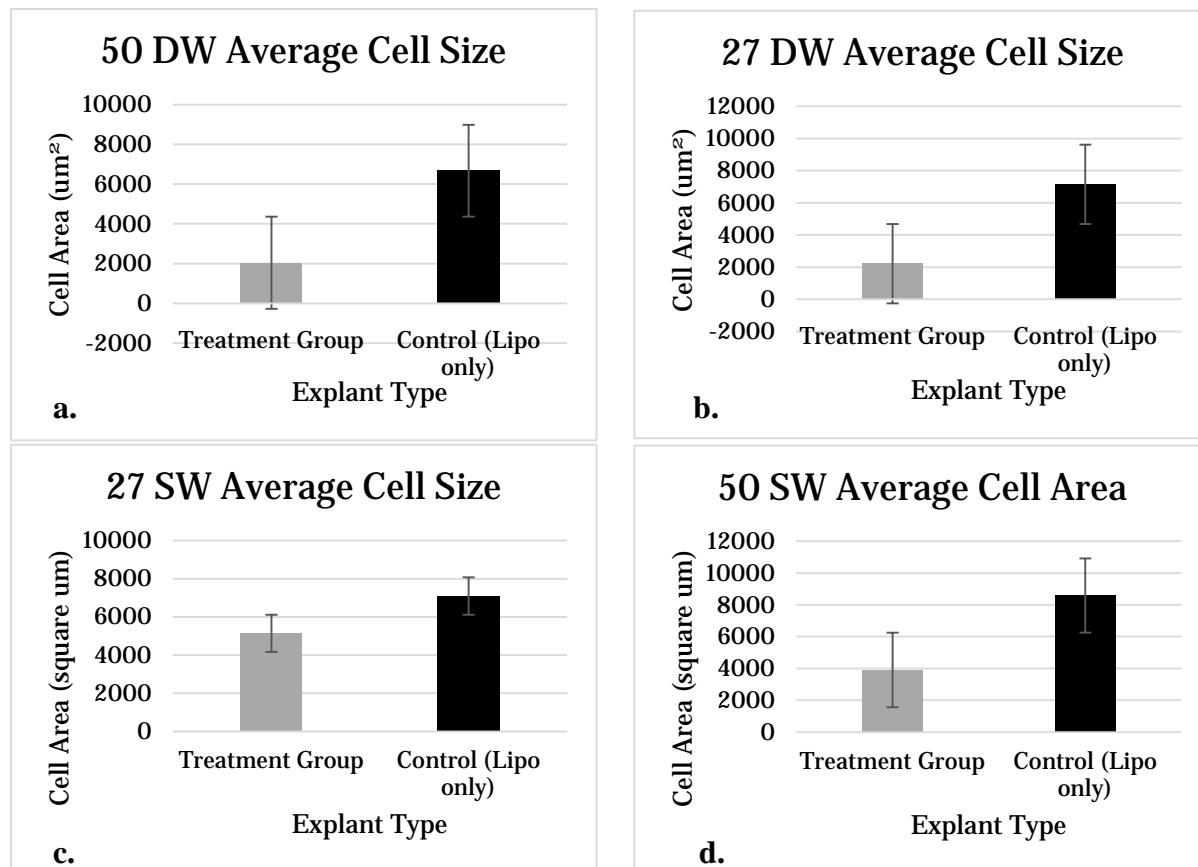


Figure 32. Adipocyte cell size in all the treatments, a) 50 mg Dex DW MS, b) 27 mg Dex SW MS, c) 50 mg Dex SW MS and d) 27 mg Dex SW MS

Additionally, there was a trend that 50 mg (higher dosage) treatment groups had a larger statistical difference than the 27 mg (lower dosage) treatment groups. Similarly, the SW treatment group exhibited less statistical significance compared to the DW treatment group. Thus, we assume that both increased Dex dosage and extended release period results in more blatant hyperplasia. However, because this experiment was not blinded it is suggested that this phenomenon is further investigated in future studies.

3.4 DISCUSSION

According to the American Society of Plastic Surgeons, there were a total of 5,814,998 reconstructive surgeries conducted in 2015 alone.(68) With such a large number of reconstructive surgeries occurring each year, it is critical that an optimal substance be used in tissue repair. Autologous fat, or mature adipose tissue, has been investigated as a key source of material for soft tissue reconstruction. Fat tissue is easily harvested and many patients possess excessive amounts of mature adipose tissue that can be collected without producing significant contour defects. Despite the incredible potential advantages of using adipose tissue in restorative tissue surgery, autologous fat transplantation has demonstrated poor results, with a 40 to 60% reduction in graft volume due to resorption.(69,70) Such high rates of adipose tissue resorption are theorized to be related to insufficient revascularization and mechanical damage.(71) Recent work has demonstrated that microvascular endothelial cells play a critical role in protecting adipose cells from hypoxia.(72) Moreover, adipose tissue mass is governed by the formation of healthy vasculature.(73)

Dexamethasone, a synthetic glucocorticoid, promotes adipogenesis by increasing the gene expression of CCAAT-enhancer-binding protein (C/EBP) and Peroxisome proliferator-activated receptor-gamma (PPAR-gamma).(8,58,71,72) The sustained delivery of adipogenic drugs, such as dexamethasone, to the healing tissue not only promotes cell differentiation and vascularization, but also acts to support long-term retention of the injected tissue. In this study, we demonstrated the ability to encapsulate and deliver dexamethasone within biodegradable double-walled microspheres to the injected adipose tissue for a period of over 6 months.

The long-term delivery of an adipogenic drug to the surgery site is critical for tissue repair and retention. According to the release studies, dexamethasone was released steadily from the Dex DW MS for the entirety of the 6-month trial. In both the 6-week and 6-month trials, the injected adipose tissue treated with Dex DW MS resulted in higher overall mass and volume retention when compared to the lipoaspirate and empty MS control groups. In addition, a greater number of blood vessels were observed in the treatment group samples following CD31 and H&E staining, suggesting improved vascularization of the treated tissue.

Scaffolds comprised of with synthetic materials are another means of supporting tissue regeneration by offering mechanical support. Yuksel et al., successfully described the delivery of adipogenic agents such as insulin and insulin-like growth factor-1 via PLGA/polyethylene glycol microspheres for the purpose of creating *de novo* fat tissue, which supported adipogenesis for a period of 4 weeks.(73,74) Adipocyte differentiation is multistep process requiring the sequential activation of several groups of transcription factors, including C/EBP gene family and PPAR- γ .(75-77)

Dexamethasone as a highly potent synthetic glucocorticoid works through activation of the glucocorticoid receptor, which is a nuclear hormone receptor in the same superfamily as PPAR- γ . Dexamethasone induces C/EBP- δ adipogenic activity and reduces the expression of pref-1, a negative regulator of adipogenesis.(78,79)

3.5 CONCLUSION

Although these findings are promising, to date, research has shown limited data on creating a proficient system for delivering adipogenic drugs over a prolonged period of time. By encapsulating an adipogenic drug, such as dexamethasone in doubled-walled PLGA/PLLA microspheres, we have shown that one can achieve slow and sustained drug delivery in a local environment, resulting in a long-term effect of dexamethasone in the implanted fat tissue for 6 months.

While the sustained and controlled release of the dexamethasone from double-walled microspheres has significantly increased the adipose tissue retention, it has also contributed on increasing blood supply in the added tissue. The immunohistochemistry staining shows more positive staining in the groups with double-walled microsphere treatment compared to single walled microspheres, empty microspheres and lipoaspirate only. Blood drawn from the animals show the absence of the drug in the blood stream, meaning that the systemic effects of the dexamethasone loaded double-walled microspheres is minimum to completely absent. This drug delivery system presents a clinical relevant form of successful drug delivery device with promising results.

Encapsulation of dexamethasone in double-walled PLGA/PLLA microspheres with the purpose of releasing the drug in a more sustained rate, presents an improved drug delivery system for prolonged adipose tissue retention. Sustained drug release, in this case dexamethasone, is successfully reached with local effect in grafted adipose tissue.

4.0 COMBINED SINGLE AND DOUBLE-WALLED MICROSPHERES DRUG DELIVERY SYSTEM EFFECT IN ADIPOSE TISSUE RETENTION

4.1 INTRODUCTION

4.1.1 Adipose tissue biology and function

Soft tissue engineering has been developed through the years, aiming to rebuild soft tissue loss. Adipose tissue is the key component needed for soft tissue reconstruction. Fat is an active and dynamic tissue, highly vascularized, distributed around the main organs and under the dermal part of the skin. Adipose tissue also gives shape and contour to the body. The minimal changes due to tumor resections or trauma can cause loss and deformity of the physiological shape of the body. Furthermore, adipose tissue is responsible for maintaining energy metabolism through storage of lipid, a task carried out by the mature adipocytes.

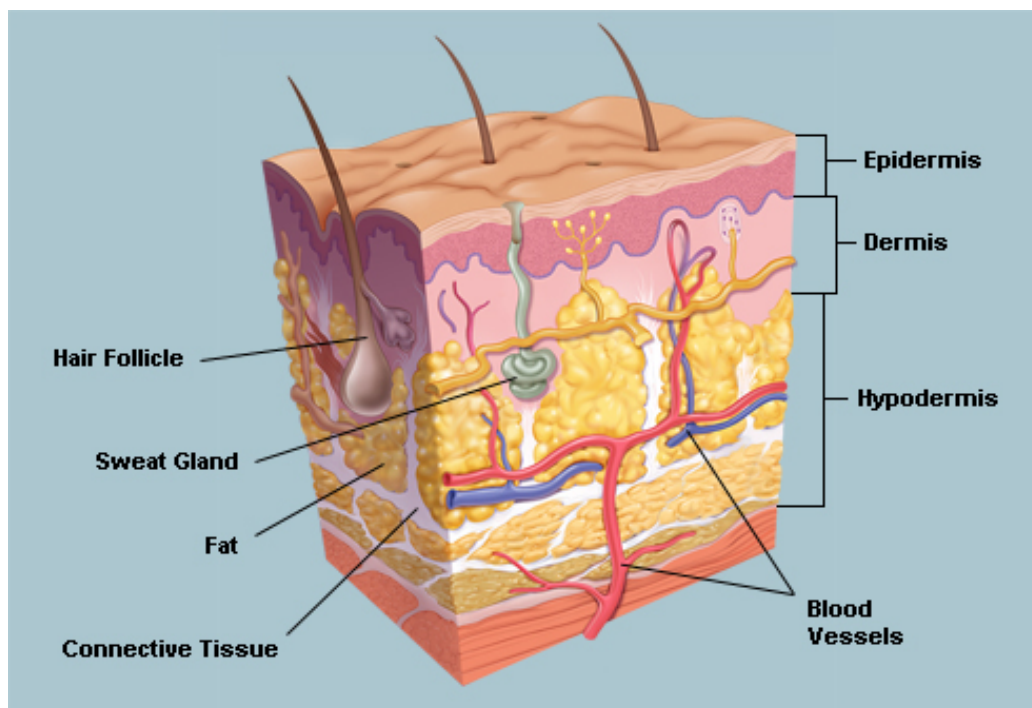


Figure 33. Adipose tissue (www.am-medicine.com)

The adipose tissue functional mechanisms overlap and interact with surrounding tissues and capillaries in addition to influencing energy homeostasis throughout the entire organism. Understanding of adipocyte biology and function helps establishing the different methods of handling adipose tissue prior to grafting. Different types of fat that have different functions in physiology and metabolic diseases. Relative to white adipose tissue, which is the body's main energy storage, brown adipose tissue can dissipate energy as a defense against cold temperatures and maintains energy balance in the body. The transition from an immature to a mature adipocyte starts initially by the determination and differentiation of a multipotent stem cell into an adipo-blast and pre-adipocyte, followed by differentiation into a mature adipocyte.(85)

4.1.2 Long-term viability of grafted adipose tissue

As discussed throughout this thesis, adipose tissue transplantation for soft tissue augmentation is a commonly used technique in plastic surgery. The outcomes include a variety of reports showing degrees of success or failure. The different methods of processing adipose tissue prior to injection can affect the survival of adipose tissue.(80) Autologous adipose tissue retention after fat grafting has been a hot topic for some time now. The literature reports a large number of autologous fat tissue grafting techniques; however, there is no optimized standard test for determining fat viability or volume augmentation after grafting.(81) Average retention of adipose after fat grafting by even experienced surgeons ranges from about 50-90%.(82) Additional procedures are usually necessary to achieve an optimal result. However, there is very limited data, which has addressed the timing of subsequent fat grafting. Typically, fat survival is adequate for 6-8 months before a repeat procedure is required.(83)

Autologous fat grafting is considered a challenging procedure, due to the un-optimized characteristic of adipose tissue. Viability of transplanted adipose tissue is a main consideration when fat grafting is performed, and significant research has been done to understand and predict the fate of grafted fat. Poor fat viability that produces inadequate results can be considered as a complication of this procedure. Many studies have demonstrated that fat longevity depends donor site selection, methods of harvesting, processing and handling of fat.(84)

Lack of vascularization of the transplanted tissue can create fat pseudo-cysts that, over time, are absorbed, leading to volume loss of the tissue. (85)

4.1.3 Specific Aim 3

In this study we aimed to optimize and evaluate the *in vivo* effect of combining single and double-walled microspheres for the purpose of prolonged and efficient fat grafting retention. Dexamethasone-loaded microspheres, both single and double-walled, will release the drug in a controlled fashion with doses being tailored to release by utilizing both types of microspheres to achieve enhanced fat grafting for shorter and longer periods of time. By combining both single-walled PLGA microspheres and double-walled PLGA-PLLA microspheres, it was hypothesized that the adipose tissue will have the acute effect of the single-walled microspheres (due to the burst release by the time that its crucial for fat tissue survival after being grafted) and followed by slow and steady release of the drug from the double-walled microspheres. Retention of the adipose tissue will be significantly maintained with using the combination of both types of microspheres.

4.2 METHODS

4.2.1 Fabrication of single and double-walled microspheres

Fabrication of single and double-walled microspheres has been described in the previous chapters. Dexamethasone loaded single-walled microspheres were prepared using single emulsion, protocol established previously in the lab.(8)

Double-walled dexamethasone loaded microspheres were prepared using a double emulsion protocol, creating the core-shell architecture of the microspheres. Empty single and double-walled microspheres were prepared using the same conditions as dexamethasone-loaded microspheres but omitting the drug.

4.2.2 Human lipoaspirate processing process

Adipose tissue was harvest from abdominal area of the University of Pittsburgh IRB approved patients. Harvested lipoaspirate was processed with Coleman technique as described in the previous two chapters. The processed human lipoaspirate was then mixed manually with microspheres prior to injecting into the mouse model.

4.2.3 Combined microspheres dosage

The doses for combined microspheres study were based on the hypothesis that dexamethasone loaded microspheres both single and double-walled will release the drug in a controlled fashion with doses being tailored to release dex for shorter and longer period of time. The combination of both single-walled PLGA microspheres and double-walled PLGA-PLLA microspheres will result in the beneficial effects of the short term dex release from single-walled microspheres along with the long-term release of dex from the double-walled microspheres.

4.2.4 Combined single and double-walled microspheres In vivo study design

For testing statistical significance for aim 3, total number of 70 animals was used. There were 7 groups of mice, each containing 5 mice per group, tested at 2 time points, 6 weeks and 6 months respectfully (Table 6). One site of injection was the experimental (sample) group, with the other site consisting of the control group. The ‘A’ control group consisted of empty microspheres, whereas ‘B’ control group consisted of lipoaspirate alone.

Table 6. Combined single and double-walled microsphere *in vivo* study design

Groups	Treatment Doses	Single-double walled microspheres ratio (SW:DW)	Dose of the drug	Number of animals	Time points
Group A (High dose of SW MS)	36 mg SW / 15 mg DW MS	2:1	402 µg	5	6 weeks/months
Group B (Equal dose of SW/DW MS)	24 mg SW/ 24 mg DW MS	1:1	408 µg	5	6 weeks/months
Group C (High dose of DW MS)	15 mg SW/ 30 mg DW MS	1:2	405 µg	5	6 weeks/months
Group D (SW MS)	57 mg SW MS	SW MS only	400 µg	5	6 weeks/months
Group E (DW MS)	40 mg DW MS	DW MS only	400 µg	5	6 weeks/months
Group F (Empty MS)	25 mg SWe/ 25 mg DW e	1:1	0 µg	5	6 weeks/months
Lipoaspirate	NA	NA	0 µg	5	6 weeks/months

Three different dose ratios of single and double-walled microspheres are described in Table 6. These dosages were chosen based on our results in the previous studies. Also, these doses correspond to doses in humans that are in the safe, therapeutic range. Furthermore, the dosage of drug is determined by the mass of microspheres injected. In addition to experimental groups, two control groups were tested. One group contained lipoaspirate alone and the second group consisted of empty double-walled microspheres.

4.2.5 Animal Surgeries

Animals in Aim 3 went through the same surgeries as described in Aims 1 and 2. Animals used in the study were athymic mice (5–10 weeks old; Harlan Laboratories). Animals were anesthetized with 3% isoflurane in oxygen at the time of the surgery. The lipoaspirate/MS injections were performed using a 16-gauge surgical cannula, in both sides of the dorsal area of the animal. The right side was injected with the experimental group and the left side contained lipoaspirate exclusively. Photographs were taken immediately following the surgery and weekly throughout the study.

4.2.6 Dexamethasone-loaded microspheres systemic effects

The levels of dexamethasone in treatment groups were tested by drawing blood from animals of the 6 week treatment groups. Blood was collected before sacrifice the animals and preserved in EDTA coated tubes from four animals from each group under light anesthesia. The plasma was then separated and stored at -80°C until used. Corticosterone levels in the plasma were quantified using an ELISA kit following the manufacturer's instructions (Enzo Life Sciences).(65-67)

4.2.7 Histological analysis

The samples were fixed in 4% paraformaldehyde, incubated overnight in 30% sucrose (Sigma-Aldrich), and then embedded in paraffin, then cryosectioned at a thickness of 10 μm . Hematoxylin (Santa Cruz Biotechnology, Inc.) and eosin (Sigma-Aldrich) (H&E) staining was conducted using the same protocol as in previous study. Vascularization of the extracted samples was determined by assessing a pan peroxidase-based CD31 antibody staining to confirm the presence of blood vessels.

4.2.8 Image analysis

As in the previous studies, Olympus Provis microscope was used to image extracted adipose tissue slides. Adipose tissue architecture was assessed by analyzing the tissue morphology of H&E stained slides. Blood vessel were counted using CD31-stained slides, containing 5 slides per group. Ten random tissue sections were quantified for each sample and means were determined for the aforementioned vessels and vessel lumens.

4.2.9 Statistical analyses

All the data was analyzed using SPSS Statistical Software. Paired t-tests, two-sample t-tests, and mixed way analysis of variance tests (ANOVA) were performed to determine if the differences between the experimental and control groups were statistically significant at the $p \leq 0.05$ significance level. All results are presented as mean \pm standard deviation.

4.3 RESULTS

4.3.1 *In vivo* studies

4.3.1.1 *In vivo* results from 6 weeks' time point

Animals from the combined dexamethasone loaded single and double-walled microspheres were sacrificed at two different time points: 6 weeks and 6 months. All the groups with combined dexamethasone loaded microspheres were mixed with 0.3 mL of lipoaspirate, whereas the control consisted of 0.3 mL of only human lipoaspirate (C=Control without microspheres and S=sample with microspheres). The animals were also compared with a control group that consisted of combined single and double-walled empty microspheres injected in one side and lipoaspirate in the other, as well as with a group that had lipoaspirate injected in both sites of the animal labeled a. and b.

At the 6 weeks' time point, no evident differences were seen in any of the groups that had the treatment with the combined single and double-walled microspheres. However, a difference was observed between the control and sample side on the group that had only single-walled microsphere treatment (group D).

The photographs show differences in adipose retention in the photographs taken at the 6-week time point (Figure 34) Control side, labeled with C appears smaller than sample or treatment side, labeled as S in Figure 34.

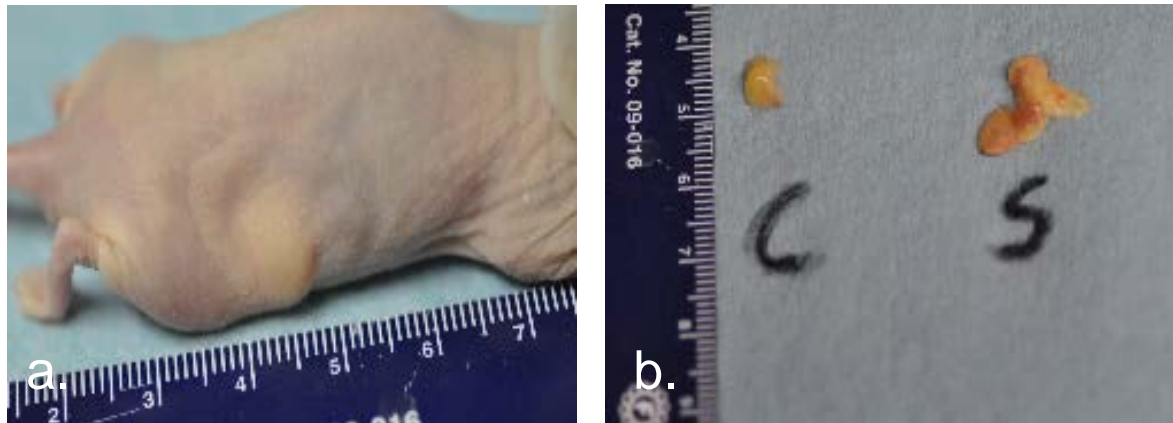


Figure 34. Animal with group D treatment and lipoaspirate (a) and adipose tissue extracted from group D, dexamethasone loaded single-walled microspheres (b)

No noticeable difference was observed between the treatment group and the control side at any of the combined single and double-walled microspheres treatment, including the group with double-walled microspheres at the 6 weeks' time point (Figure 35); however, differences of treatment groups compared to the controls were observed.

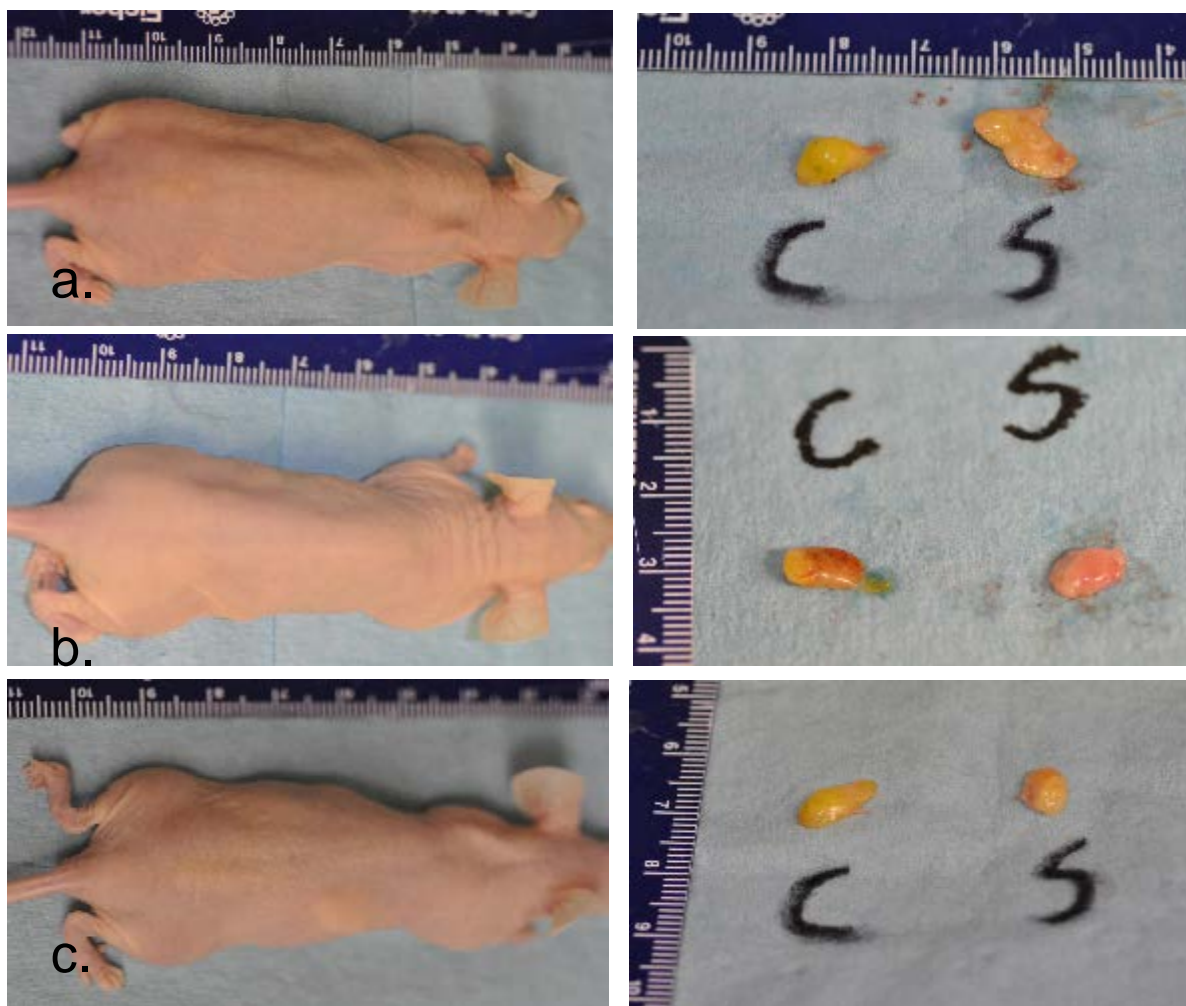


Figure 35. Adipose tissue extracted from 6 weeks animals with the following treatments: a) Group A-2:1 SW:DW ratio, b) Group C-1:2 SW:DW and c) Group F-Empty MS 1:1 SW:DW ratio

The extracted fat from the combined single and double-walled microspheres groups was grossly not different from combined single and double-walled empty microspheres or lipoaspirate group at 6 weeks' time point. It is anticipated that there's no difference seen because of the slow release of dexamethasone from double walled microspheres. Figure 35 shows the extracted adipose tissue from 3 groups of treatment.

Group A, the groups with the higher ratio of dexamethasone single-walled microspheres (36 mg Dex SW MS: 15 mg Dex DW MS) grossly don't show any evidence of the treatment side being different from the control side. The similar case follows the other combination of single and double-walled microspheres treatment (Group B- 25 mg Dex SW MS/ 25 mg Dex DW MS and group C- 15 mg Dex SW MS/ 30 mg Dex DW MS). Group F, combined single and double-walled empty microspheres shows no adipose tissue macroscopic difference between the treatment and control side.

4.3.1.2 *In vivo* results from 6 months' time point

The combined dexamethasone loaded single and double-walled microspheres study was analyzed at two time points, short-time 6 weeks and longer-time point 6 months respectfully. All the groups with combined dexamethasone loaded microspheres were mixed with 0.3 mL of lipoaspirate, whereas the control consisted of 0.3 mL of only human lipoaspirate (C=Control without microspheres and S=sample with microspheres). The animals were also compared with a control group that consisted of combined single and double-walled empty microspheres injected in one side, and lipoaspirate in the other, as well as with a group that had lipoaspirate injected in both sites of the animal labeled a. and b. Whereas the gross results from the first (short) time point show that only in the group with the single-walled microspheres there was an evident difference between the microspheres treatment and control side, none of the combined microspheres treatments groups showed differences in gross morphology of the extracted fat.

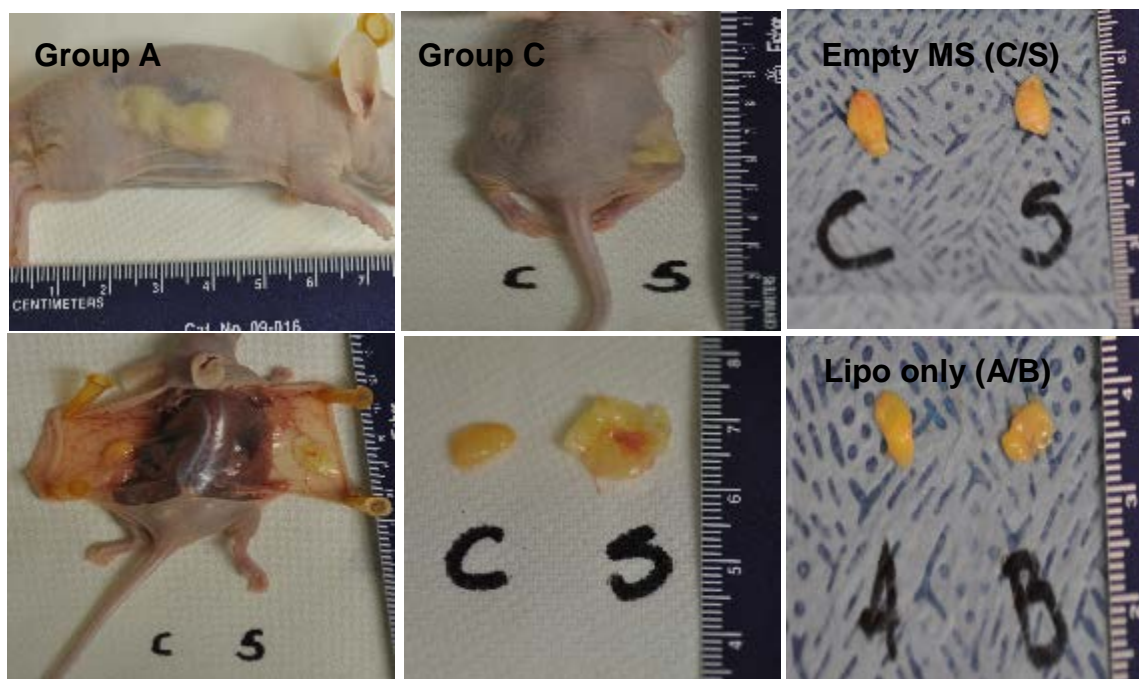


Figure 36. Adipose tissue extracted from animals treated for 6 months, group A (2:1-SW/DW MS), group C (1:2-SW/DW MS), empty MS (1:1-SW/DW empty MS) and lipoaspirate only (A/B)

The treatment group A, combined single-walled (36 mg SW MS) and double-walled (15 mg DW MS) shows a difference between the lipoaspirate in the treatment side and the control side of the injections (Fig.31). Adipose tissue extracted from the group C (15 mg SW MS / 30 mg DW MS) was macroscopically larger than the control side or any of the control groups. Empty microspheres group showed the lipoaspirate with no evident changes on the side that had the treatment and the side with lipoaspirate (Figure 36).

Understanding the morphology and properties of the double-walled dexamethasone loaded microspheres, the sustained and slow of the drug, explains the difference seen in the explanted adipose tissue at the 6 month time point compared to results of 6 week time point.

4.3.3. Mass and volume measurements analysis

The differences observed within the treatment groups that contained combined dexamethasone microsphere treatments compared to tissue without the drug were confirmed by measuring mass and volume displacement of the extracted lipoaspirate in all groups. Tissue extracted from the first time point (6 weeks), show a significant increase in volume only with the single-walled dexamethasone microspheres treatment (Figure 37), which may be due to faster release of the drug from the single-walled microspheres.

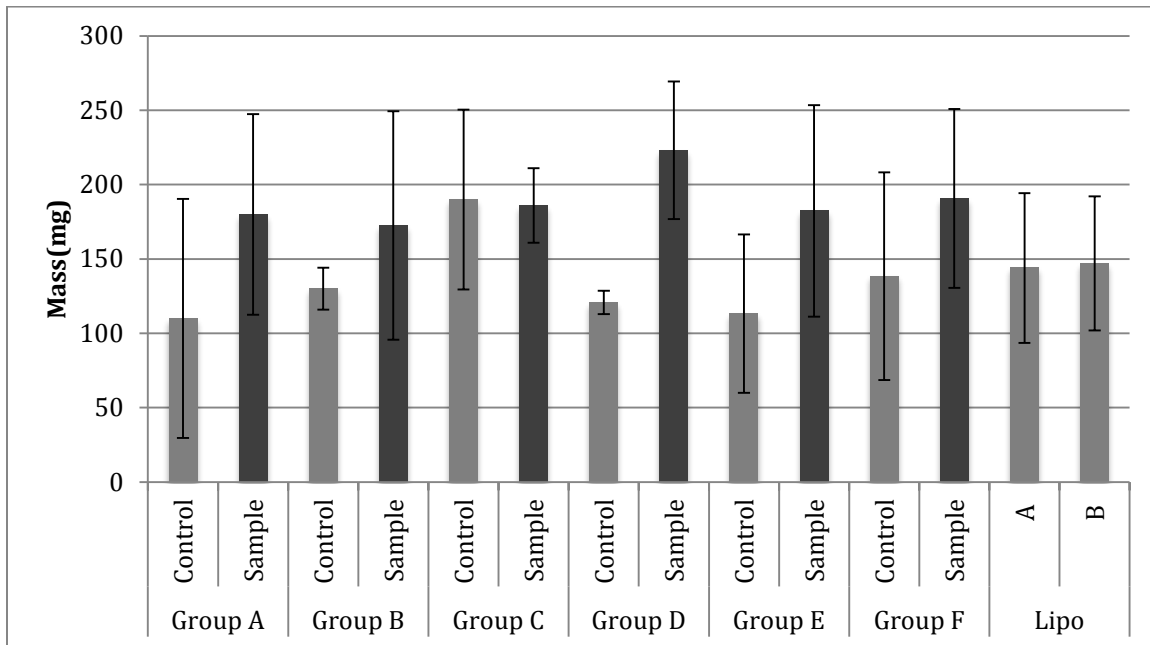


Figure 37. Adipose tissue volume measurements at 6 weeks' time point

The extracted lipoaspirate group showed a change between the treatment and control side at the longer time point of 6 months. Combined single and double-walled microspheres demonstrated higher volume retention compared to control groups. Animals were initially injected with 0.3mL of lipoaspirate combined with microspheres in the treatment side and lipoaspirate only on the control side. Figure 38 depicts the volume retention with group A of ~95 % compare to group F (empty SW/DW microspheres) of ~15% and lipoaspirate only ~12% volume retention. The statistically significant difference between the treatment and control sides has been determined One-Way ANOVA comparison, SPSS software, p-value <0.005.

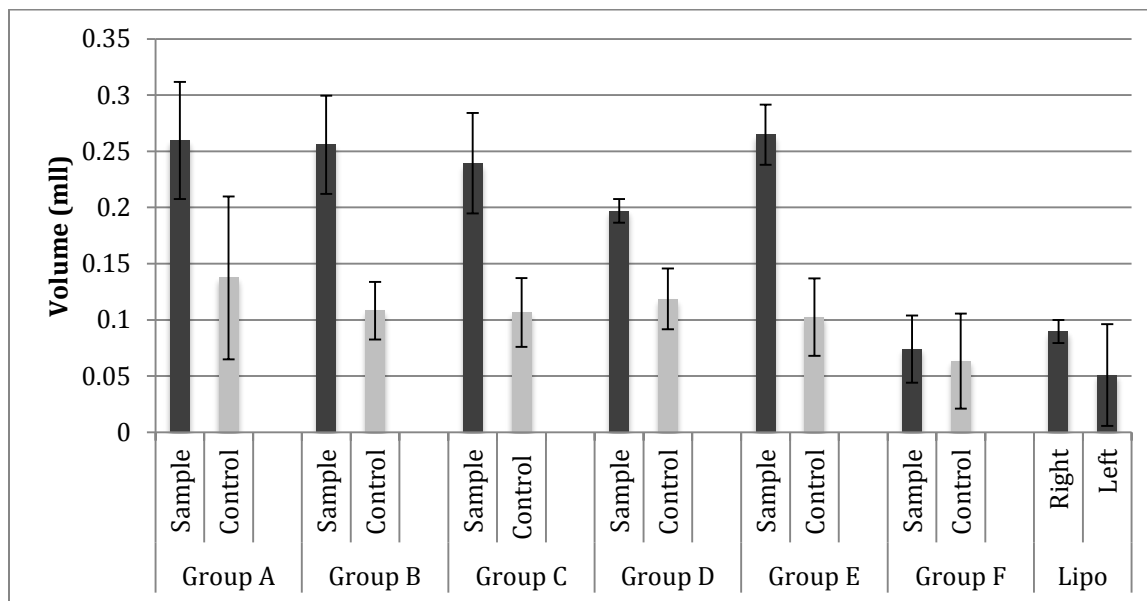


Figure 38. Adipose tissue volume retention at 6 months' time-point

4.3.4. Sample histology

Three tissue sections from each lipoaspirate extraction were stained with H&E in order to analyze gross architecture of the tissue. H&E images show indestructible architecture of the adipose tissue in all treatment groups, without any interruption from the combined single and double-walled microspheres (Figure 39). Adipose tissue without any microsphere treatment shows large adipocytes with surrounding disrupted tissue.

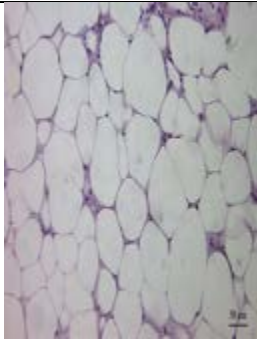
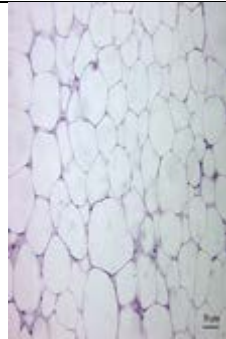
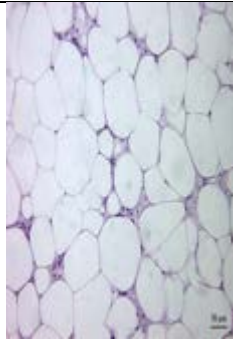
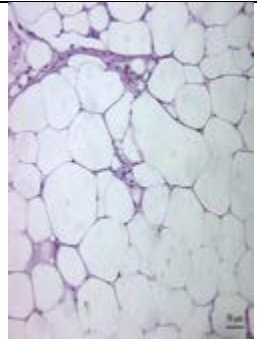
Treatment groups 6 weeks time point	Group A (High SW MS dose)	Group B (Equal SW/DW MS)	Group C (High DW MS dose)	Group F (Equal SW/DW empty MS)
H&E staining				

Figure 39. Histology of extracted tissue at 6-weeks time point. H&E staining of group A (High SW MS dose), group B (Equal SW/DW MS dose), group C (High DW MS dose) and group F (Equal SW/DW empty MS) treatment

Blood vessels were analyzed using Image J to view CD31-stained slides as described in the previous studies. The differences in vascularization between treatment and control samples are demonstrated in Figure 40.

The difference in mass measurements between all groups was analyzed statistically with SPSS software, showing a statistically significant difference between the treatment group at the 6-month time point, which had results similar to the volume displacement measurements.

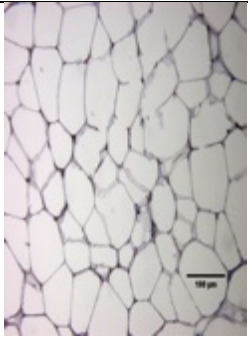
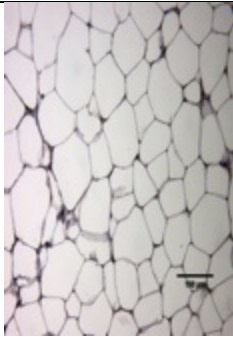
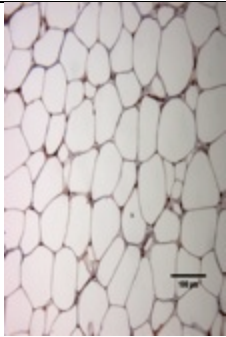
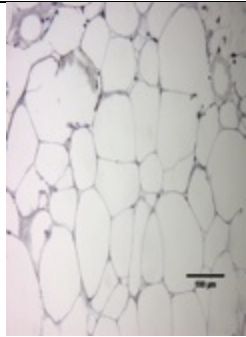
Treatment groups 6 weeks time point	Group A (High SW MS dose)	Group B (Equal SW/DW MS)	Group C (High DW MS dose)	Group F (Equal SW/DW empty MS)
CD31 staining				

Figure 40. Histology of the extracted fat at 6 months time point. . CD31 staining shows a significant difference on blood vessel presence in treatment groups compared to control

4.3.2 Dexamethasone combined microspheres study systemic effect

Dexamethasone/corticosterone levels in animals in the combined microspheres treatment group were tested at 6 weeks. Plasma was collected from four animals (n=4) per group in the 6-week time point and tested for the presence of dexamethasone in the systemic blood stream using the Corticosterone ELISA kit by following the manufacturer's instructions (Enzo Life Sciences).

Corticosterone levels didn't show any significant difference between the group A (High dose of SW MS), group B (Equal dose of SW/DW MS) and group C (High dose of DW MS) groups and group F (Empty microspheres) and lipoaspirate only. All the treatment groups showed an average of $\approx 1,600$ pg/ml corticosterone, showing no significant difference in corticosterone levels on a systemic scale in any of the groups (Figure 41)

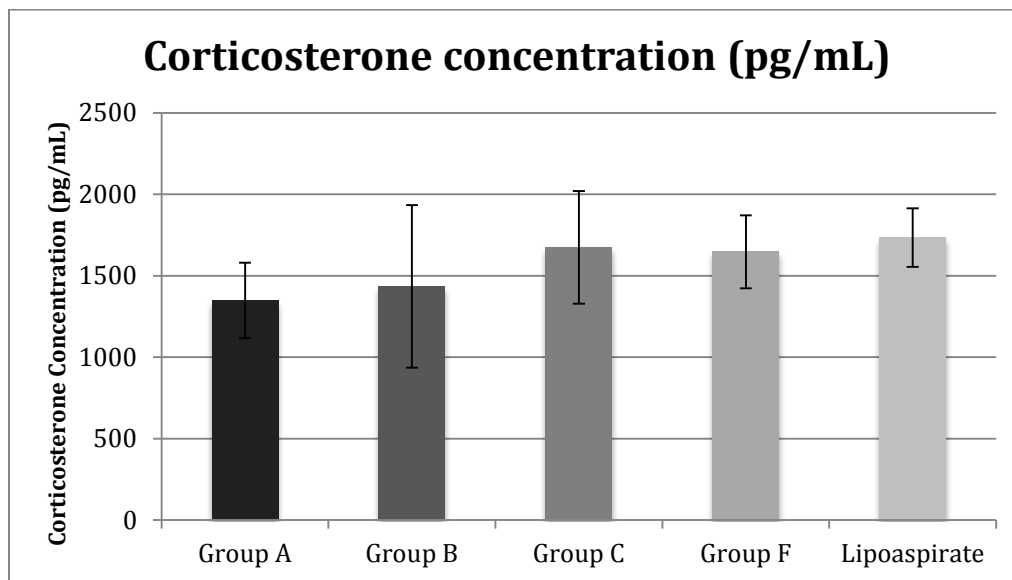


Figure 41. Corticosterone levels in combined microspheres study animals at 6 weeks.

4.4 DISCUSSION

Fat grafting is a very commonly used procedure in which fat is harvested by liposuction from a part of the body and transferred by injecting it into an area where it can be used to add volume, or correct a contour deformity. In late 1800, Neuber described the use of transplanted fat for reconstruction of facial scars.(88) The importance of fat grafting was described even in the earliest cases, when the surgeons recognized the importance of the transformation the fat into the scarred tissue.(89)

Fat grafting comes with a number of advantages, most importantly using patients' own tissues.. Also, different studies demonstrate that fat grafting will last longer than artificial fillers, which usually last a couple of months before reinjection is needed. Fat grafting, however, remains a challenging issue within plastic surgery with no solid protocol as to how to optimally perform the procedure. Various methods of harvesting and transfer have been tested and researched through the years. Despite rapidly growing work done to this field, it still remains one of the most influential directions of plastic surgery research.(90-92)

As a very promising therapeutic approach, fat grafting has numerous challenges that are still being investigated today. The loss of volume or even complete resorption of the graft has been evident due to either insufficient tissue quality by lack of cell differentiation or lack of vasculature.(93) This emphasizes that angiogenesis and adipogenesis are crucial for maintaining a stable volume of adipose tissue. (93) Numerous studies have demonstrated the important effects of factors and drugs on adipogenesis.(8,58,71,72), also including the vascularization for quality, volume and long-term survival of transplanted adipose tissue constructs.

In this chapter, both dexamethasone single and double-walled microspheres have been tested with the purpose of achieving a longer and more sustained volume of grafted adipose tissue. Three combinations of microspheres have been tested, group A with higher dose of single-walled microspheres (36 mg SW MS/ 15 mg DW MS), group B with equal doses of single and double-walled microspheres (25 mg SW MS/ 25 mg DW MS), group C with higher dose of double-walled microspheres (15 mg SW MS/ 30 mg DW MS) and were compared with a group with single walled only (group D), group with double-walled microspheres only (group E). Two control groups were group F with combined single and double-walled empty microspheres and a group with lipoaspirate only. All the treatments were tested at two time points, six weeks as a short time point and six months as a longer time point. In the first period, 6 weeks respectfully, the only group that showed a significant difference in mass and volume measurements was group D (57 mg SW MS), which was most likely due to the fast release of the drug from the single-walled microspheres (Figure 34). The histology showed healthy tissue morphology in all treatment groups (Figure 39). Extracted fat from animals of the 6 months' time point showed significant difference in mass and volume measurements in all the treatment groups compared to single and double-walled empty microspheres and lipoaspirate only shown in the Figure 36. The combination of both single-walled PLGA microspheres and double-walled PLGA-PLLA microspheres demonstrated that thefat has been effected by the acute effect of the single-walled microspheres and followed by a slow and steady release of the drug from the double-walled microspheres, resulting in a stable volume of grafted fat.

4.5 CONCLUSION

In this study, the specific aim was to achieve a prolonged yet still healthy adipose tissue volume after lipoaspirate grafting with microsphere drug delivery system. Single and double-walled microspheres loaded with dexamethasone were combined, with doses tailored to have the fast effect of the single-walled microspheres and followed by the slow and steady release of the drug from the double-walled microspheres. Adipose tissue mass and volume was preserved in the 6 months' time point ($\approx 90 \pm 5.2\%$ in the group A (High SW MS dose)) (Figure 38). While the sustained and controlled release of the dexamethasone from combined microspheres has affected adipose tissue retention, dex did not show any significant signs of being present systemically in animals' blood stream (Figure 41). The combined single and double-walled PLGA-PLLA microspheres has been demonstrated not only to be effective for delivering adipogenic drugs such as dexamethasone but also effecting adipose tissue in a local and controlled environment.

5.0 CONCLUSION AND FUTURE DIRECTIONS

The main goal of the project was to develop and test a controlled delivery system of adipogenic factors, and generate stable adipose tissue retention. Three specific aims are proposed to achieve the goals of this study. Optimizing single-walled microspheres loaded with two adipogenic drugs was described in Specific Aim 1. Local delivery of dexamethasone from PLGA microspheres had a significant role on retaining adipose tissue mass in two different time points, 5 weeks as a short-time point and 6 months as a longer time point. Insulin was also tested as the second drug delivery encapsulated in PLGA microspheres mixed with lipoaspirate and injected in athymic mice. Additionally, the combination of both dexamethasone and insulin loaded PLGA MS, was studied. The volume of extracted tissue with the combined drugs study design didn't show any significant effect compared to the solo treatment with Dex MS or Insulin MS.

Specific Aim 2 was designed to address the fabrication and optimization of dexamethasone loaded double-walled PLGA/PLLA microspheres and also examine the effect in adipose tissue *in vivo*. Dexamethasone, a highly adipogenic drug, was encapsulated in a PLGA core surrounded by PLLA shell with the purpose of a slower and controlled drug delivery. By encapsulation of dexamethasone in double-walled microspheres, the drug was released in a sustained and controlled fashion for an average of 80-120 days.

The release of an adipogenic drug in a slow and more controlled environment affected adipose tissue for a prolonged period. With no previous studies reported with the encapsulation of steroids in double-walled PLGA/PLLA microspheres, quantitative prediction of release profiles is challenging to interpret. Therefore, optimization of dexamethasone release upon trials was required. Microspheres were mixed with human lipoaspirate and injected into dorsal aspect of athymic mouse model with the purpose of testing the bioactivity of dexamethasone-loaded microspheres (Figure 20).

Figure 22 shows the difference in the drug release mode from the single and double-walled dexamethasone loaded microspheres. Injected adipose tissue when extracted from the animals at both 6 weeks and 6 months' time line showed a significant difference in mass and volume between the treatment and control groups. Figure 27 shows volume retention of adipose tissue averaged $\approx 80\%$ in 27 mg DW MS. Encapsulation of dexamethasone in double-walled PLGA/PLLA microspheres did affect the adipose tissue by releasing the drug in a decreased and more sustained rate developing an improved drug delivery system for prolonged adipose tissue retention.

Specific Aim 3 described the combination of single and double-walled dexamethasone loaded microspheres for the purpose of achieving higher sufficient adipose volume retention. The experimental design was based on effects of Dex MS *in vitro* was established as following: Dexamethasone was encapsulated in PLGA single-walled microspheres (9), tailored to be released in a controlled sustained fashion in matter of 4-6 weeks. Dexamethasone, a synthetic glucocorticoid, promotes adipogenesis by increasing the gene expression of CCAAT-enhancer-binding protein (C/EBP) and Peroxisome proliferator-activated receptor-gamma (PPAR-gamma).(8,58,71,72).

Also, by encapsulating an adipogenic drug such as dexamethasone in PLGA/PLLA microspheres, a slower and sustained drug delivery in the local environment was achieved and therefore created a prolonged effect of dexamethasone in implanted fat tissue for over 6 months. In this study, single and double-walled microspheres were combined with a purpose of tailoring the doses to have an acute effect in the first weeks from single-walled microspheres and followed by sustained release from double-walled microspheres. Three different ratios of single and double-walled were tested: a higher dose of single-walled microspheres, a higher dose of double-walled microspheres and an equal dose of single and double-walled microspheres. The combined treatments were compared with the group of only single-walled microspheres and a group of a double-walled microspheres that had the same corresponding dose of dexamethasone as the combined microspheres groups. The control groups were combined single and double-walled empty microspheres and lipoaspirate only group. Higher adipose tissue retention was demonstrated in 6 months' time point. Group A, high dose of single walled microspheres (2:1 SW/DW MS) did show retention in a range of 90% compared to the control lipoaspirate group and empty microspheres group with the retention average of 15%. Blood drawn from the animals at 6 weeks' time point, was tested for the levels of glucocorticoids (corticosterone) levels, and showed little to no difference between the treatment and control groups.

This project emphasizes the use of all FDA-approved components for the purpose of enhanced adipose tissue survival after fat grafting. Traditional methods for preserving adipose tissue after transfer have many inconsistent results.(94-101) The loss of volume results from a reduction in the subcutaneous fat, tissue atrophy, and leads to changes in shape.

Such loss of volume compounded with tissue, leads to the aged appearance of the periorbital, perioral, cheek, and mandibular areas.(120-126) A number of surgeons choose to replace this volume with various injectable agents, both synthetic and autologous, in search for the ideal soft tissue filler.

While many devices have been evaluated for controlled release drug delivery, biodegradable polymer microspheres are one of the most common types. Microspheres can be used to encapsulate many types of drugs including vaccine components.(102-105). Clinically used and commercial products that are based on polymer microspheres including Lupron Depot and Nutropin Depot. The disadvantages of microspheres start with difficulty of large-scale manufacturing, inactivation of drug during fabrication, and not easy controllable drug release rates(105-110). Nutropin Depot, which consist of Genentech's recombinant human growth hormone (rhGH) encapsulated within poly(D,L-lactide-co-glycolide) microspheres using Alkermes' proprietary ProLease_encapsulation technology, was recently withdrawn from the market because of high cost of manufacturing. However, polymer-based drug delivery systems such as biodegradable microspheres are simple to produce and can be administrated through various routes including oral, pulmonary, and parenteral injection and don't require surgical removal after release is completed. Unfortunately, with all this forms of administration, control of drug delivery rates remains limited.(105-110).

Single and double-walled microspheres were successfully fabricated and optimized in this study. The bioactivity effect was tested *in vivo*, in an ahtymic mouse model, injected with lipoaspirate as a scaffold of dexamethasone loaded microspheres.

Dexamethasone has been shown to affect adipose tissue by up-regulating the glucocorticoid receptors (118), however more details are needed to examine the effects of dexamethasone on different cell types within lipoaspirate.

Finally, the athymic mouse model was chosen as a primary testing animal model that enabled the examination of human adipose tissue. However, future studies that must be performed to demonstrate their potential for soft tissue reconstruction will include an immunocompetent animal model.

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