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Equine Model for Soft Tissue Regeneration

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Abstract

Soft tissue regeneration methods currently yield suboptimal clinical outcomes due to loss of tissue volume and a lack of functional tissue regeneration. Grafted tissues and natural biomaterials often degrade or resorb too quickly, while most synthetic materials do not degrade. In previous research we demonstrated that soft tissue regeneration can be supported using silk porous biomaterials for at least 18 months in vivo in a rodent model. In the present study, we scaled the system to a survival study using a large animal model and demonstrated the feasibility of these biomaterials for soft tissue regeneration in adult horses. Both slow and rapidly degrading silk matrices were evaluated in subcutaneous pocket and intramuscular defect depots. We showed that we can effectively employ an equine model over six months to simultaneously evaluate many different implants, reducing the number of animals needed. Furthermore, we were able to tailor matrix degradation by varying the initial format of the implanted silk. Finally, we demonstrate ultrasound imaging of implants to be an effective means for tracking tissue regeneration and implant degradation.

Keywords

Animal model; In vivo test; Mesenchymal stem cell; Scaffold; Silk

Introduction

Large soft tissue defects in humans are the result of traumatic injuries, tumor resections or “wasting” diseases such as lipodystrophy and muscular dystrophy (1–4). With both adipose and muscle tissue defects, there frequently remain functional, volumetric and esthetic insufficiencies and psychological disorders can accompany the resultant deformities (5, 6).

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Common treatment for volumetric loss of both tissue types is autologous tissue grafting, however, this frequently leads to donor site morbidity and less than ideal engraftment(4, 7).

In tissues where extracellular matrix dictates structure and organization, such as bone, autologous grafts are most likely to heal when firmly secured in direct contact with host bone(8). Under these circumstances, even if the grafted cells do not survive, healing occurs as long as there is sufficient infiltration of host cells which go on to resorb graft matrix and replace it with new bone (8). This is in contrast to tissues such as adipose and muscle whose cellular components dictate tissue organization. For these highly cellular tissues, it is more imperative that the grafted material will provide the initial biological cues to support the long-term regeneration of the tissue(8).

To help address these considerations, some investigators have assumed a tissue engineering approach for filling soft tissue defects. Under these circumstances a biomaterial scaffold, designed to fit the dimensions and needs of the tissue, can be implanted alone or with cells. Biomaterials for adipose tissue engineering vary from synthetic to natural. Poly-lactic-co-glycolic acid (PLGA) and poly-ethylene glycol (PEG) are the most common synthetic materials evaluated for adipogenesis (9–13). In all PLGA studies, adipogenesis was well supported both *in vivo* and *in vitro*, however, only for short-term time frames (9–13). An *in vivo* study demonstrated a loss in adipogenic outcomes by 3 months post-implantation, which occurred in concert with degradation of the PLGA scaffold (13). PEG, also supports adipogenesis, however, inherently does not contain any cell-binding motifs, leading to a lack of biodegradation which requires that it be modified to enhance attachment (14). The inability of cells to remodel their environment in PEG biomaterials, do not make these matrices an appropriate choice for soft tissue regeneration where host infiltration is important for remodeling and angiogenesis.

Decellularized extracellular matrix (ECM), with or without stem or progenitor cells, is the most frequently employed biomaterial for skeletal muscle regeneration. The ECM undergoes degeneration in the presence of a targeted immune response, releasing biological factors and structures (e.g., growth factors, basement membrane fragments) that interact with the host to create a pro-regenerative environment. A prolonged post-operative period is required to realize these mechanisms' regenerative potential (15). Muscle regeneration therapies involving synthetic biomaterials have undergone substantially less evaluation.

Various other natural biomaterials contain cell binding motifs; and they are able to be remodeled by the body, making them attractive options for soft tissue regeneration. Collagen, alginate, gelatin, hyaluronic acid (HA), and extracellular matrix proteins (ECM) have all been explored for adipose and muscle tissue engineering, yet they often need to be chemically crosslinked to optimize degradation profiles which then alters biological responses (16–32). Silk, a protein biomaterial, was recently FDA approved as a surgical mesh and has been used extensively for sutures (33,34). Moreover, it can be processed into different formats with controllable degradation rates (days to years) by modifying the level of protein crystallinity during processing (35). The crystallinity is associated with the physical beta-sheet crosslinks and therefore no chemical crosslinking is needed, which can

otherwise confound biological responses. Silk matrices can also support adipogenesis both *in vitro* and *in vivo* (36–39).

Recently, we have shown that the use of a silk sponge matrix seeded with human adipose derived stem cells (ASCs), *ex vivo* differentiated adipocytes, or seeded with human lipoaspirate, can maintain volume and actually regenerate adipose tissue over an 18 month period in a dorsal subcutaneous pocket model in male athymic rats (38). Interestingly, we did not detect regenerated adipose tissue until 12 months post-implantation (38). Conversely, if we implanted an unseeded silk sponge alone, only connective tissue was seen with no adipose tissue regeneration (38), implying the importance of the cellular component of the implant.

While these rodent model results are promising, we need to demonstrate the feasibility of the system in a larger animal model with an intact immune system to show clinical relevance. Many larger animal models for filling soft tissue defects have been unsatisfactory. Aside from rapid implant material resorption, insufficient volumes of subcutaneous adipose tissue for grafting are an additional drawback (13, 40). Under the latter circumstances, visceral adipose tissue has been an alternative source. However, differences in regeneration potential exist between the adipose sources, and collecting visceral tissue requires a laparotomy, an invasive procedure (13, 40). An equine model is at an advantage: comparatively large volumes of adipose donor tissue are readily harvestable from the subcutaneous space around the tail head or dorsal to the gluteal muscles. Indeed, these donor sites are already used clinically in horses as sources of adipose-derived stem/stromal cells and their products for tissue regeneration purposes (41, 42). The equine's large size also makes it possible for investigators to simultaneously implant and then screen large and multiple scaffolds at subcutaneous and intramuscular sites in a single animal.

Accordingly, for this proof-of-concept study, we chose an equine model to evaluate 4 different silk matrices that spanned a spectrum of degradation rates for soft tissue regeneration over 1, 3, or 6 months. For subcutaneous depots we tested silk gels, silk foams, and silk porous sponges with short (weeks to months) and long (years) degradation rates. For intramuscular defects, we tested silk porous sponges with either short or long degradation time frames. We hypothesized that the horse is a viable species in which to test, using a survival model, multiple soft tissue regenerative implants simultaneously at both intramuscular and subcutaneous sites; that silk matrix degradation can be tailored by manipulating the matrix preparation technique; that silk matrix-mediated tissue regeneration can be affected by not-loading/loading the matrix with progenitor cells; and that ultrasound imaging of implants is an effective means for tracking tissue regeneration and implant degradation.

Materials and Methods

Equine model

All animal related activities took place in their entirety in AAALAC-accredited facilities that are registered with the USDA. These facilities met or exceeded all applicable animal care and use federal guidelines including the Guide for the Care and Use of Animals. The

facilities incorporate dedicated animal housing, surgical intervention and procedures amenities. The research protocol was approved and in compliance with Tufts University's Institutional Animal Care and Use Committee (IACUC, protocol # G2010-140) in accordance with the Office of Laboratory Animal Welfare (OLAW) at the National Institutes of Health (NIH).

Both subjects were geldings (male). Subject 1 was an Appaloosa breed weighing 1276 lbs., and estimated to be 9 years old (equivalent to a 35 year old human). Subject 2 was a Standardbred breed, weighing 1166 lbs., estimated to be 15 years old (equivalent to a 45 year old human). Subjects were purchased at auction and therefore exact ages were unknown. Both subjects were deemed in good health prior to and during the study on the basis of daily physical evaluations through the duration of the study. Pre-operative complete blood count and blood chemistry data confirmed this finding. Subjects received annual vaccination (tetanus, encephalitis, flu, rhinopneumonitis, rabies, west Nile virus) and quarterly anthelmintic treatments (ivermectin/praziquantel or pyrantel pamoate rotated).

For the duration of the immediate peri-operative period (~ 4 weeks), animals were confined to their stalls (16 × 16 feet, wood shavings for bedding). Thereafter, in hand walking exercise progressed to small paddock turnout subject to weather conditions. Diet included ad libitum hay and concentrate feed supplementation on an as needed basis.

Surgical Implantation

Pre-operatively, an intravenous jugular catheter was aseptically placed in the jugular vein to facilitate administration of analgesics, sedatives, antibiotics and anti-inflammatories. Sedation was provided for all implant and harvest procedures using intravenous detomidine hydrochloride (20 or 40 mg/kg) and butorphanol (0.01-0.05 mg/kg) administered to effect. For all implant and harvest sites, carbocaine 2% (20 mg/mL) administered locally via a 22 gauge 1.5 inch needle provided local analgesia. Subjects were administered phenylbutazone (4.4 mg/kg loading dose and then 2.2 mg/kg twice daily) immediately prior to surgery orally and then once to twice daily while significant local inflammation persisted. Subjects were also administered ceftiofur (2.2 mg/kg, twice per day, intravenously) and gentamicin (6.0 mg/kg, daily, intravenously) for up to three days post-operatively. Upon completion of the study, the subjects were put up for adoption and sent to approved owners and homes, ensuring long-term wellbeing of the horses. Implant types and locations are outlined in Table 1.

Implant Surgery

Following onset of sedation, the implant sites overlying the neck, epaxial muscles, triceps, gluteal muscles, and semimembranosus/semitendinosus, were clipped and aseptically prepared for surgery. Each site was locally infiltrated with a ring block of 2% carbocaine (20 mg/mL) and then aseptically draped.

At intramuscular implant sites (triceps, gluteal muscles, and semimembranosus/semitendinosus) an approximately 5 cm long incision was completed through the skin, subcutaneous tissue and muscle fascia, and an approximately 25 mL volume of striated muscle was surgically excised from the exposed muscle belly using routine sharp dissection

and hemostasis (applied pressure and gauze tampon), as performed clinically for standing muscle biopsies in horses. One treatment group had the defect left empty as a control, but all other defects, except that of UMSCs only, were filled with a 25 mL volume implant. The implant was placed such that it was completely surrounded by muscle. The overlying muscle fascia and subcutaneous tissue were both re-apposed separately using 0-0 polydioxanone suture. The skin was re-apposed using a combination of 2-0 polypropylene and stainless steel staples.

For subcutaneous implants (neck and thoraco-lumbar paraspinal sites), an approximately 2-cm long incision was completed through the skin. After creating a subcutaneous pocket by bluntly undermining the adjacent skin, each pocket was filled with a 1-ml volume silk implant. The implant was placed such that it was completely surrounded by adipose tissue. The skin was then re-apposed with 0-0 polydioxanone suture and/or stainless steel staples. Sutures were removed 10 days post-operatively.

Silk Biomaterial Preparation

Bombyx mori silkworm cocoons were supplied by Tajima Shoji Co. (Yokohama, Japan).

Aqueous silk solution was prepared as published (47). To form the silk gels, aqueous silk solution was sterile filtered through 0.2µm syringe filter and concentrated to 8% w/v silk solution in sterile centrifugal filter units with a 3500 MW cutoff (Millipore Corp, Billerica, MA). The silk solution was vortexed (Vortex Genie, Fisher Scientific, Pittsburgh, PA) at a setting of 7 for 20 minutes at a volume of 10mL in a 15mL conical tube. The vortexed silk solution was cast in 10 cm diameter Petri dishes and allowed to gel for 30 mins at 37°C. After gelation, the gels were cut to the desired size (1cm³).

To form silk foams, aqueous silk solution was poured into a plastic Petri dish. The solution was then stored in an EdgeStar Model FP430 thermoelectric cooler maintained at -7°C for 3 days. It was then transferred to a VirTis Genesis (Model 25L Genesis SQ Super XL-70) lyophilizer for 3 days, resulting in a very consistent interconnected fine-pore structure. The foam was soaked in 70% methanol for 1 day to induce β-sheet formation, then dried, cut to the desired size (1 cm³), autoclaved and stored at 4°C until use.

For aqueous based sponges, the aqueous silk solution was diluted in water to 2% w/v. For solvent based sponges, the aqueous silk solution was lyophilized until dry and re-solubilized over 2 days in hexafluoro-2-propanol (HFIP) at 17% w/v. Salt crystals were sieved to the desired range of 500-600 microns, poured into Teflon molds and either aqueous silk or HFIP-silk solution was added. The molds were covered and left in a fume hood for 2 days, then were immersed in methanol overnight, left in the fume hood for 1 day for the methanol to evaporate and then placed in water to leach out the salt particles. The water was changed 3 times a day for 2 days. The sponges were removed from the molds, cut to the desired dimension (2.5 cm diameter × 5 cm height for intramuscular implants, 1 cm³ for subcutaneous implants). The scaffolds were left to dry, autoclaved, and then kept at 4°C until use.

Equine umbilical stem cell isolation and culture

Umbilical cord tissue mesenchymal stromal cells (UMSCs) were obtained from a fresh (<12 hr old) equine umbilical cord tissue that was preserved at 4°C. After extensive washing of a sub-segment of the equine cord in PBS, intervacular connective tissue (Wharton's Jelly) was dissected free and minced to fragments (~2 mm³) and plated on plastic, 4 fragments per well (6 well format) in shallow media (700 µL alpha MEM, 10% FBS, 10% Equine donor serum, 2 mM L-glutamine, and 1X penicillin/streptomycin /nystatin) for explanting of cells. After 4-7 days, outgrowth cells were passaged using trypsin (0.25%)/EDTA (0.1%) and plated at clonal density (2*10³/cm²) on large dishes (150 cm²) for expansion of colony forming cells. When dishes reached ~70% confluence, cells were harvested for labeling and seeding silk scaffolds. Viability of cells exceeded 98% using trypan blue exclusion. Surface expression of CD29 and CD90, and lack of CD14, CD45, and CD79 expression were confirmed by flow cytometry (10,000 events/sample, Accuri C6).

Pre-implantation preparation

The day prior to implantation, blood was collected from the jugular vein of each subject. The serum portion was collected, heat inactivated, and added to Dulbecco's Modified Eagles Medium (DMEM) so that the final concentration of autologous serum was 10% v/v. All silk biomaterials were soaked in 10% autologous serum in DMEM for at least 1 hour prior to implantation. In groups with equine umbilical stem cells seeded, implants were prepared as follows. Cells were labeled (3 min) with PKH26 (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions, and stained cells resuspended (20*10⁶/mL) in media containing autologous serum (10%) from recipient. Finally silk sponges were seeded by depositing 25µL of cell suspension onto surface at 40 equidistant locations followed by incubation at 37°C, 5% CO₂ for 1 hr prior to harvesting seeded implants for surgical (allogeneic) implantation into recipient. For cells that were injected without a biomaterial carrier, the cells were injected as is from a tuberculin syringe.

Adipose Harvest and Lipo-Soaked Implant Preparation

Following onset of sedation, the adipose harvest site adjacent to the tailhead was aseptically prepared for surgery. Local anesthetic was placed subcutaneously as a line block and an approximately 5cm long incision was made through the skin and subcutaneous tissue to access the para-coccygeal fat. Approximately 25 mL of adipose tissue was excised using sharp dissection and hemostasis (applied pressure and gauze tampon). A two layer closure was routinely completed using 0-0 absorbable suture for the subcutaneous tissues and surgical staples for the skin. Staples were removed 10 days post-operatively.

Approximately 25mL of adipose tissue per intramuscular implant or per 4 subcutaneous implants was taken at time of surgery and placed into a 50mL sterile conical tube and washed with an equal volume of saline until clear of any residual blood. The adipose tissue was finely minced with scissors, such that when the scissors were lifted from the tube, no chunks of tissue would remain adhered to the scissors. This tissue, referred to as 'lipo' in results and discussion, had the same appearance as human lipoaspirate. The implants were placed in the tubes (1 intramuscular implant or 4 subcutaneous implants per 25 mL adipose tissue per 50 mL tube) and allowed to adsorb the lipo for 15 minutes prior to implantation.

Pre- and post-operative care and monitoring

Physical examination assessing implant sites, disposition, heart rate, respiratory rate, rectal temperature, mucous membrane color, capillary refill time, gut sounds, appetite, thirst and musculoskeletal soundness were continued twice daily for the first three post-operative days, and then once daily for the first two post-operative weeks. Weights were taken pre-operatively and monthly.

Ultrasound

All implant sites were ultrasonographically evaluated by a single operator prior to implant surgery to provide control images, immediately post-implantation and then at weekly intervals for the first month post-implantation followed by monthly evaluations depending on the time interval of interest (1 month, 3 months or 6 months). In an effort to semi-quantitatively evaluate the implant integration with its surrounding tissue we used a subjective rating system on a scale of 0 to 5 (Table 2). A score of '0' indicates there was little to no difference between the implant and surrounding tissue. A score of '5' indicates a very clear demarcation between the implant and surrounding tissue. Assessed parameters included implant surface area, echogenicity, and fluid accumulation. An Esaote MyLab40 model ultrasound instrument was used with a 5 MHz linear probe to evaluate subcutaneous implants and a 12 MHz linear probe to record intramuscular structures. Subcutaneous implants were evaluated in a single plane, transversely, while intramuscular implants were evaluated orthogonally in both transverse and longitudinal sections. Focal zone, gain, and dynamic range were standardized for all ultrasound scans, and transducer positioning was standardized based on external landmarks. Ultrasound images were evaluated by tracking two different variables – size and a blinded qualitative ranking of tissue integration using the values in table 2.

Explantation

Following onset of sedation, aseptic preparation and draping of the implant site, local anesthetic was infused in a line block. An approximately 3.5cm long skin incision was made, and an approximately 10mL biopsy of implant remnant, replacement and adjacent tissue was sharply dissected. The overlying tissues were routinely re-apposed with 0-0 polydioxanone suture and stainless steel staples (skin).

All implants were measured with calipers, photographed and placed into formalin or frozen for histological analysis. Volumetric measurements were made by carefully dissecting the surrounding tissue from the implant and measuring the implant with calipers the height, width, length (for cube implants). Each dimension was measured 3 times and the average values were multiplied to get a volume. For cylindrical implants, the dimensions measured were height and diameter. Average values were used in the equation $volume = height * \pi * (diameter/2)^2$.

Histology

Histological solvents were purchased from Fisher Scientific (Pittsburgh, PA) and histological reagents were purchased from Sigma-Aldrich. Constructs were processed according to standard histology protocols. Formalin fixed samples were put through a series of

dehydration solvents and finally paraffin using an automated tissue processor. Samples were embedded in paraffin, cut in 10 micron sections, and let to adhere on glass slides. The sections were rehydrated and stained with Hematoxylin and Eosin (H&E) for general morphology and organization.

Statistics

As this was a proof-of-concept study we only studied 2 subjects. Subcutaneous implants were implanted as n=4 per group, while intramuscular were n=1. For subcutaneous implants volume measurements are presented as an average $\pm$ SD of n=4 implants. Histological sections are representative images of 3-5 sections taken through the center region of the implant.

Results

Implantation

Implantations of various formats and anatomical sites are listed in Table 1. The subjects sustained the implantation and tissue harvesting surgeries without significant complications or changes in weight. In subject 2, one intramuscular implantation site became locally infected at day 5 post implantation. Several skin sutures and staples were removed to allow local drainage and the infections resolved spontaneously over a 3-5 day period. Both subjects were functionally sound and adopted on completion of the study and no long term complications were noted one year post-study.

Subcutaneous silk sponge implants

Silk porous sponges with equal pore sizes but different degrees of crystallinity, providing two degradation time scales, were evaluated. Aqueous based sponges degraded within months (fig. 1A-C) while solvent based sponges had not begun to degrade (fig. 2A-C). The aqueous silk sponge readily absorbed the lipoaspirate (lipo) (fig. 1A). After 3 months of implantation, little to no silk was detectable when implanted without lipo (fig. 1B), however, when the sponge was seeded with lipo, 37 ± 10 % of the initial implant volume remained (fig. 1C). Although the silk sponge was not grossly apparent at the time of harvest, when palpated at 1 and 3 months prior to harvest, it had a noticeably different texture (still soft/supple but more resilient/compressible) than surrounding tissue. In both cases, blood vessels were seen macroscopically leading to the implants; additionally the implants had a red hue, yet no mature blood vessels were detected upon histological examination within the implant (fig. 1B). H&E staining indicated that more cells had populated the lipo seeded sponges than the unseeded sponges (fig. 1B). In both implants, remnants of the silk sponge were seen in histology sections, but it was evident that the pore walls were no longer intact as the silk structure degraded (fig. 1B, arrows). The aqueous silk sponges at 3 months were visible by ultrasound imaging and appeared well integrated with similar appearance as the surrounding tissue (fig. 1B, dotted circle).

The solvent based sponges were easily detected upon palpation and histologically at 6 months on explantation and they had not degraded significantly (fig. 2B), with $90\% \pm 22$ and $110\% \pm 16$ volume retained for the unseeded and lipo seeded sponges, respectively (fig. 2C).

Like the aqueous based sponges, the implants appeared vascularized macroscopically, but there was no evidence of mature blood vessels within the implant upon histological examination (fig. 2B). Also, like the aqueous based sponges, there was an increased cellularity within the lipo seeded sponge than the unseeded sponge (fig. 2B). There was a striking difference in the remaining solvent silk sponge pores when compared to the aqueous silk sponge group. The solvent silk sponge had relatively intact silk pore wall remaining at 6 months while the lipo seeded silk sponge pore walls had begun to fracture (fig. 2B, arrows). The solvent based silk sponges at 6 months were clearly visible by ultrasound imaging with distinct borders from the surrounding tissue (fig. 2B, dotted circle).

Subcutaneous silk foam implants

Another silk format implanted was silk foam (fig. 3A). While in appearance silk foams are similar to sponges, they were designed to be a solid state injectable scaffold; such that the foam can be deformed enough to pass through a needle or catheter but then regain close to its initial dimensions post-injection(43). In this study, the foams were implanted, not injected, to ensure initial implanted dimensions were consistent. Similar to the sponges, the foams readily absorbed the lipo (fig. 3A). At time of harvest, the foams were palpable through the skin and the foam structure was still apparent at 1 and 3 months (fig. 3A).

While there was no difference in volumes retained at individual time points between the unseeded and lipo seeded foam groups, both showed an increase in volume from 1 to 3 months (fig. 3D). At 1 month, $49\% \pm 18$ and $62\% \pm 19$ volume retained for the unseeded and lipo seeded foam, respectively (fig. 3D). At 3 months, $115\% \pm 24$ and $128\% \pm 24$ volume retained for the unseeded and lipo seeded foam, respectively (fig. 3D). At both time points, the unseeded foams were not well infiltrated with cells, except at the boundaries of the implant (fig. 3B-C). The lipo seeded foams visibly appeared more like that of native adipose tissue (fig. 3B-C). However, upon histological examination, no adipose tissue was detected within the implant (fig. 3B-C). The silk foams at 1 and 3 months were visible by ultrasound imaging (fig. 3B-C, dotted circle). When silk foams were pre-seeded with lipo, the borders between the foam and the surrounding tissue were less apparent than when foams were implanted alone (fig. 3B-C).

Subcutaneous silk gel implants

Upon explantation, silk gels were found to be completely resorbed and therefore they had not maintained their implant volume. Histological examination of the implantation site did not show any silk gel remnants.

Intramuscular defect model

At the time of explantation, all intramuscular implant sites had some fibrous tissue superficial to the underlying muscle. In the defect only site, scar tissue was visible with a 6.6 mm length void (not shown). When UMSCs were implanted alone there was a similar void at the defect site and a healthy appearance to the muscle below the superficial fibrous tissue (not shown). When lipo was implanted alone, the implant site had a healthy appearance without any detectable void (not shown).

We evaluated volume retention for aqueous based silk porous sponges, seeded with UMSCs or lipo, in an intramuscular defect model at 1 and 3 months (fig. 4A-C). At 1 month post-implantation, all groups had begun to degrade, with 17%, 12%, and 23% volume remaining for aqueous silk sponges alone, with UMSCs or with lipo, respectively (fig. 4F). Both the silk sponge alone and with UMSCs were infiltrated with inflammatory cells (fig. 4B-C). The silk sponge seeded with lipo contained more matrix and less cellularity than the other groups (fig. 4B-C, white arrow). At 3 months post-implantation, all groups had also continued to degrade, with 5%, 16%, and 31% volume remaining for aqueous silk sponges alone, with UMSCs or with lipo, respectively (fig. 4F). Similar to the subcutaneous implants, the residual silk seen in histology sections was no longer in its intact porous form as the sponges degraded (fig. 4B-C, black arrows). The silk sponge alone or seeded with UMSCs had less immune cell infiltration than seen at 1 month while the opposite trend was seen in the lipo seeded implant (fig. 4B). UMSCs were only detected at 1 month when seeded onto the silk sponge (fig. S2).

Next, we evaluated solvent based silk sponges, seeded with UMSCs or lipo, in an intramuscular defect model at 6 months. At 6 months, all groups had begun to degrade, with 29%, 18% and 22% volume retention for solvent silk sponges alone, with UMSCs or with lipo, respectively (fig. 4F). All implants had regions of inflammatory cells near the silk sponge pore walls with adjacent matrix rich regions (fig. 4D). In the lipo seeded silk sponge there was muscle-like tissue seen at the immediate boundary of the sponge (fig. 4D, inset-white arrow). Similar to the subcutaneous solvent silk sponge implants, the residual silk matrix was found with its more intact porous form as the sponges degraded (fig. 4D, black arrows). The defect alone site showed a collagen matrix dense scar area (fig. 4D). When UMSC cells were added without the silk sponge, regions of muscle were seen with scar tissue nearby (fig. 4D, brackets).

Ultrasound Results

Ultrasound imaging was performed throughout the study duration to track implant integration and tissue regeneration. Initially, in all implant groups there was a clear distinction between the implant and surrounding tissues. In all short-term subcutaneous implants the addition of lipoaspirate increased integration with tissue surroundings over time (fig. S3A). An improvement in tissue integration with the addition of lipoaspirate was not apparent in the long-term subcutaneous implants (fig. S3C). Implant dimensions were also acquired using electronic calipers (fig. S3B, S3D). Short-term (aqueous based) implants increased in length over implantation time (fig. S3B), while dimensions remained consistent for the long-term (solvent based) implants (fig. S3D). Silk gels were only detectable by ultrasound through 3 weeks (fig. S5).

Short-term intramuscular implants tended to remain more visible over time than their subcutaneous counterparts (fig. S4A). Pre-seeding with lipoaspirate here did not increase the apparent rate of integration and there was a negligible change in dimensions (fig. S4B). The long-term intramuscular implants remained visible throughout the 6 month implantation period (fig. S4C), and decreased by approximately 40% of their width, and 10% of their length upon implantation, after which no further decrease was seen (fig. S4D).

Discussion

Large animals are essential preclinical models for human medicine(41, 44). Horses are a preferred species for investigators because they frequently sustain clinical musculoskeletal injuries whose pathophysiology resembles that encountered in human clinical patients (41, 44). Consequently, the use of horses as large model animals for musculoskeletal injury in people has been approved by the US Food and Drug Administration (FDA) (41, 44, 45). In this preclinical study we were able to confirm that the horse's large size provides uncomplicated access to large subcutaneous adipose donor sites as well as extensive subcutaneous and intramuscular implant sites, all within a single animal. This allowed for multiple implant comparisons to be completed in fewer animals than would be the case with smaller species, a development which has welfare, cost and study design (minimization of variables) implications. Unlike the case with other large animal models previously reported, visceral fat depots accessed via laparotomy or laparoscopy were not required in this study as a source of adipose donor tissue (40).

Specifically, both short (aqueous sponges) and long (solvent sponges) implant degradation profiles were examined here. Our data imply that for applications where volume retention is crucial, the long-term degradation profile is preferred. In this case, the subcutaneous tissue could stably regenerate around, and into, the sponge as it degraded over time in a manner consistent with what has been previously demonstrated in a small animal model (38). However, it was important for us to also demonstrate that: a) the silk sponges can degrade completely and b) that degradation rate can be tailored to the application. Therefore, we chose to test a fast degrading silk matrix, i.e. lowest possible aqueous sponge silk protein concentration while still capable of forming a sponge scaffold. Conversely, for the solvent sponge, we chose to use the same silk protein concentration as was used for the previous small animal study wherein it degraded over a period greater than 18 months (38). For the subcutaneous tissue pocket model, our data imply the aqueous sponge (shorter degradation profile) may be better for smaller applications such as human facial dermal filling, whereas the solvent sponge (longer degradation profile, more robust mechanics due to increased physical crosslinking) may be better suited for reconstructive purposes or larger defects.

The silk foams implanted subcutaneously in this study displayed an increase in volume over time. These foams were originally designed for injectable applications, and as a result, must be collapsible such that they can be deformed to fit through a needle and then regain their shape upon injection. Therefore, the processing of the foams leads to them having an elongated, parallel pore structure. When the foam is stored in its dehydrated state, these parallel pores are collapsed. We propose that after being implanted for 3 months, these pores have opened and it is this change that may contribute to the increase in volume seen. This is in contrast to the sponge's spherical pores, which do not collapse when dehydrated.

In this study, we also implanted silk gels subcutaneously, yet at the time of explantation at 1 and 3 months, no gels were detected. However, using ultrasound they were visible through 3 weeks of implantation (fig. S5). Conversely, in small animal screening studies gels were consistently found out to 6 weeks (unpublished data). It is not clear whether this is the result of the different animal model (e.g., rodent versus equine).

For muscular volumetric defects we compared aqueous and solvent based silk sponges. Here we found that aqueous sponges degraded at a similar timescale to the aqueous subcutaneous implants. Yet, the solvent based sponges began to degrade more rapidly in an intramuscular space than in a subcutaneous space. In prior studies, aqueous sponges degraded approximately 4 times faster than solvent based sponges when implanted intramuscular, but there was no comparison undertaken to assess differences in degradation between subcutaneous and intramuscular (46).

Large animal models can be difficult to compare with standard small animal studies and many factors may exist why these models cannot be effectively comparable. In many cases, where allogeneic cells or tissue are implanted (such as the UMSCs here) an immunocompromised rodent model is employed, while our model subjects had intact immune systems. Specifically for studies that require subcutaneous adipose tissue depots, small animal models do not often have enough adipose tissue to harvest. Although as mentioned previously, access to subcutaneous adipose depots is limited in other large animals such as sheep and pig. Furthermore, it is not possible to do extensive long-term studies in small animals, required for volume stable soft tissue regeneration work, as their lifespan is relatively short (~2 years). The subjects in this study were adults, approximately equal to 35-45 years old in a human, and therefore are more relevant to a clinical case. Future studies using this model can be carried out much for longer periods (several years) without concern that subjects will have complications due or be lost to old age (as with rodents).

Given this is a proof-of-concept study and our use of only 2 subjects, we cannot readily draw statistically robust inter-subject conclusions (statistical data is based on multiple implants within 1 subject). Use of a large animal such as a horse could be cost-prohibitive if it requires many subjects to yield statistically sound data sets. Nevertheless, this can be off-set by the volume and size of implants able to be assessed at once (at least 40 subcutaneous sites, 6 intramuscular) and the ability to non-invasively track the implants by ultrasound-limiting the number of timepoints required. Furthermore, these procedures are non-terminal, allowing the horse to be adopted at study completion, an important ethics and welfare consideration. Our findings demonstrate that the gross anatomical observations at the time of explantation and the histology data matched well to the ultrasound images. It allowed the investigators to accurately and consistently detail implant size and location, as well as any integration between the implant and surrounding soft tissues. Moreover, this system gives us a unique insight to the degradation profile and integration of silk based implants in large animals to contrast with our previous small animal studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Machingal MA, Corona BT, Walters TJ, Kesireddy V, Koval CN, Dannahower A, et al. A tissue-engineered muscle repair construct for functional restoration of an irrecoverable muscle injury in a murine model. *Tissue Eng Part A*. 2011; 17:2291–303. [PubMed: 21548710]
2. Corona BT, Machingal MA, Criswell T, Vadhavkar M, Dannahower AC, Bergman C, et al. Further development of a tissue engineered muscle repair construct in vitro for enhanced functional recovery following implantation in vivo in a murine model of volumetric muscle loss injury. *Tissue Eng Part A*. 2012; 18:1213–28. [PubMed: 22439962]
3. Stosich MS, Moiola EK, Wu JK, Lee CH, Rohde C, Yoursef AM, et al. Bioengineering strategies to generate vascularized soft tissue grafts with sustained shape. *Methods*. 2009; 47:116–21. [PubMed: 18952179]
4. Patrick C. Tissue engineering strategies for adipose tissue repair. *Anat Rec*. 2001; 366:361–6. [PubMed: 11500812]
5. De Sousa A. Psychological issues in oral and maxillofacial reconstructive surgery. *Br J Oral Maxillofac Surg*. 2008; 46:661–4. [PubMed: 18771826]
6. Robinson E, Rumsey N, Partridge J. An evaluation of the impact of social interaction skills training for facially disfigured people. *Br J Plast Surg*. 1996; 49:281–9. [PubMed: 8774241]
7. Gomillion CT, Burg KJL. Stem cells and adipose tissue engineering. *Biomaterials*. 2006; 27:6052–63. [PubMed: 16973213]
8. Peer LA, Walker JC. The behavior of autogenous human tissue grafts; a comparative study. 1. *Plast Reconstr Surg* (1946). 1951; 7:6–23. [PubMed: 14807738]
9. Choi YS, Park S-N, Suh H. Adipose tissue engineering using mesenchymal stem cells attached to injectable PLGA spheres. *Biomaterials*. 2005; 26:5855–63. [PubMed: 15949551]
10. Kang S-W, Seo S-W, Choi C-Y, Kim B-S. Porous poly(lactic-co-glycolic acid) microsphere as cell culture substrate and cell transplantation vehicle for adipose tissue engineering. *Tissue Eng Part C Methods*. 2008; 14:25–34. [PubMed: 18454643]
11. Chung HJ, Park TG. Injectable cellular aggregates prepared from biodegradable porous microspheres for adipose tissue engineering. *Tissue Eng Part A*. 2009; 15:1391–400. [PubMed: 19327016]
12. Neubauer M, Hacker M, Bauer-Kreisel P, Weiser B, Fischbach C, Schulz MB, et al. Adipose tissue engineering based on mesenchymal stem cells and basic fibroblast growth factor in vitro. *Tissue Eng*. 2005; 11:1840–51. [PubMed: 16411830]
13. Patrick CW, Zheng B, Johnston C, Reece GP. Long-term implantation of preadipocyte- seeded PLGA scaffolds. *Tissue Eng*. 2002; 8:283–93. [PubMed: 12031117]
14. Zhu J. Bioactive modification of poly(ethylene glycol) hydrogels for tissue engineering. *Biomaterials*. 2010; 31:4639–56. [PubMed: 20303169]
15. Corona BT, Wu X, Ward CL, McDaniel JS, Rathbone CR, Walters TJ. The promotion of a functional fibrosis in skeletal muscle with volumetric muscle loss injury following the transplantation of muscle-ECM. *Biomaterials*. 2013; 34:3324–35. [PubMed: 23384793]
16. Vashi A V, Abberton KM, Thomas GP, Morrison W a, O'Connor AJ, Cooper-White JJ, et al. Adipose tissue engineering based on the controlled release of fibroblast growth factor-2 in a collagen matrix. *Tissue Eng*. 2006; 12:3035–43. [PubMed: 17518619]
17. Davidenko N, Campbell JJ, Thian ES, Watson CJ, Cameron RE. Collagen-hyaluronic acid scaffolds for adipose tissue engineering. *Acta Biomater*. 2010; 6:3957–68. [PubMed: 20466086]
18. Wu X, Black L, Santacana-laffitte G, Patrick CW. Preparation and assessment of glutaraldehyde-crosslinked collagen –chitosan hydrogels for adipose tissue engineering. *J Biomed Mater Res A*. 2007; 81:59–65. [PubMed: 17109417]
19. Flynn L, Prestwich GD, Semple JL, Woodhouse KA. Adipose tissue engineering with naturally derived scaffolds and adipose-derived stem cells. *Biomaterials*. 2007; 28:3834–42. [PubMed: 17544502]

20. Flynn L, Semple JL, Woodhouse KA. Decellularized placental matrices for adipose tissue engineering. *J Biomed Mater Res A*. 2006; 79:359–69. [PubMed: 16883587]
21. Choi JS, Yang H-J, Kim BS, Kim JD, Kim JY, Yoo B, et al. Human extracellular matrix (ECM) powders for injectable cell delivery and adipose tissue engineering. *J Control Release*. 2009; 139:2–7. [PubMed: 19481576]
22. Kimura Y, Ozeki M, Inamoto T. Adipose tissue engineering based on human preadipocytes combined with gelatin microspheres containing basic fibroblast growth factor. *Biomaterials*. 2003; 24:2513–21. [PubMed: 12695078]
23. Borzacchiello A, Mayol L, Ramires P a, Pastorello A, Di Bartolo C, Ambrosio L, et al. Structural and rheological characterization of hyaluronic acid-based scaffolds for adipose tissue engineering. *Biomaterials*. 2007; 28:4399–408. [PubMed: 17602736]
24. Jing W, Lin Y, Wu L, Li X, Nie X, Liu L, et al. Ectopic adipogenesis of preconditioned adipose-derived stromal cells in an alginate system. *Cell Tissue Res*. 2007; 330:567–72. [PubMed: 17922143]
25. Mandal BB, Kundu SC. Osteogenic and adipogenic differentiation of rat bone marrow cells on non-mulberry and mulberry silk gland fibroin 3D scaffolds. *Biomaterials*. 2009; 30:5019–30. [PubMed: 19577292]
26. Young DA, Ibrahim DO, Hu D, Christman KL. Injectable hydrogel scaffold from decellularized human lipoaspirate. *Acta Biomater*. 2011; 7:1040–9. [PubMed: 20932943]
27. Tan H, Rubin JP, Marra KG. Injectable in situ forming biodegradable chitosan-hyaluronic acid based hydrogels for adipose tissue regeneration. *Organogenesis*. 2010; 6:173–80. [PubMed: 21197220]
28. Chandler EM, Berglund CM, Lee JS, Polacheck WJ, Gleghorn JP, Kirby BJ, et al. Stiffness of photocrosslinked RGD-alginate gels regulates adipose progenitor cell behavior. *Biotechnol Bioeng*. 2011; 108:1683–92. [PubMed: 21328324]
29. Hong L, Peptan I, Clark P. Ex vivo adipose tissue engineering by human marrow stromal cell seeded gelatin sponge. *Ann Biomed Eng*. 2005; 33:511–7. [PubMed: 15909657]
30. Rossi CA, Flaibani M, Blaauw B, Pozzobon M, Figallo E, Reggiani C, et al. In vivo tissue engineering of functional skeletal muscle by freshly isolated satellite cells embedded in a photopolymerizable hydrogel. *FASEB J*. 2011; 25:2296–304. [PubMed: 21450908]
31. Ma J, Holden K, Zhu J, Pan H, Li Y. The application of three-dimensional collagen- scaffolds seeded with myoblasts to repair skeletal muscle defects. *J Biomed Biotechnol*. 2011; 2011:812135. [PubMed: 22203786]
32. Ahadian S, Ph D, Ostrovidov S, Kaji H. Engineered Contractile Skeletal Muscle Tissue on a Microgrooved Methacrylated Gelatin Substrate. *Tissue Eng Part A*. 2012; 18:2453–2465. [PubMed: 22963391]
33. Altman G, Diaz F, Jakuba C, Calabro T, Horan R. Silk-based biomaterials. *Biomaterials*. 2003; 24:401–16. [PubMed: 12423595]
34. Vepari C, Kaplan DL. Silk as a Biomaterial. *Prog Polym Sci*. 2007; 32:991–1007. [PubMed: 19543442]
35. Kim HJ, Kim HS, Matsumoto A, Chin I-J, Jin H-J, Kaplan DL. Processing Windows for Forming Silk Fibroin Biomaterials into a 3D Porous Matrix. *Aust J Chem*. 2005; 58:716–20.
36. Kang JH, Gimble JM, Kaplan DL. In vitro 3D model for human vascularized adipose tissue. *Tissue Eng Part A*. 2009; 15:2227–36. [PubMed: 19207036]
37. Mauney JR, Nguyen T, Gillen K, Kirker-Head C, Gimble JM, Kaplan DL. Engineering adipose-like tissue in vitro and in vivo utilizing human bone marrow and adipose-derived mesenchymal stem cells with silk fibroin 3D scaffolds. *Biomaterials*. 2007; 28:5280–90. [PubMed: 17765303]
38. Bellas E, Panilaitis BJB, Glettig DL, Kirker-Head C a, Yoo JJ, Marra KG, et al. Sustained volume retention in vivo with adipocyte and lipoaspirate seeded silk scaffolds. *Biomaterials*. 2013; 34:2960–8. [PubMed: 23374707]
39. Quinn KP, Bellas E, Fourligas N, Lee K, Kaplan DL, Georgakoudi I. Characterization of metabolic changes associated with the functional development of 3D engineered tissues by non-invasive, dynamic measurement of individual cell redox ratios. *Biomaterials*. 2012; 33:5341–8. [PubMed: 22560200]

40. Patrick C, Uthamanthil R. Animal models for adipose tissue engineering. *Tissue Eng.* 2008; 14:167–78.
41. Burk J, Badylak SF, Kelly J, Brehm W. Equine cellular therapy--from stall to bench to bedside? *Cytometry A.* 2013; 83:103–13. [PubMed: 23081833]
42. Kol A, Walker NJ, Galuppo LD, Clark KC, Buerchler S, Bernanke A, et al. Autologous point-of-care cellular therapies variably induce equine mesenchymal stem cell migration, proliferation and cytokine expression. *Equine Vet J.* 2013; 45:193–8. [PubMed: 22780195]
43. Bellas, E.; Kaplan, DL. Unpublished material n.d.
44. Patterson-Kane JC, Becker DL, Rich T. The pathogenesis of tendon microdamage in athletes: the horse as a natural model for basic cellular research. *J Comp Pathol.* 2012; 147:227–47. [PubMed: 22789861]
45. McLean, M. Spotlight On: Dr. Lynne Oliver, Office of New Animal Drug Evaluation. US Food Drug Adm; 2010.
46. Wang Y, Rudym DD, Walsh A, Abrahamsen L, Kim H-J, Kim HS, et al. In vivo degradation of three-dimensional silk fibroin scaffolds. *Biomaterials.* 2008; 29:3415–28. [PubMed: 18502501]
47. Rockwood DN, Preda RC, Yücel T, Wang X, Lovett ML, Kaplan DL. Materials fabrication from *Bombyx mori* silk fibroin. *Nat Protoc.* 2011; 6:1612–31. [PubMed: 21959241]

Fig. 1.

Aqueous silk sponge implants in subcutaneous pocket model. **(A)** Aqueous silk sponges prior to implantation, left- aqueous sponge alone, right- aqueous silk sponge seeded with lipo. Scale bar 5mm. **(B)** Implants after harvest at 3 months. Top row - macroscopic appearance of implants, scale bar 5mm. Implants have a natural tissue-like appearance and silk structure is not grossly detectable. Middle row - H&E sections of aqueous silk sponge (black arrowhead) no longer has pore structure as it degrades, scale bar 100 μ m, inset 200 μ m. Bottom row- representative ultrasound images of implant site with implant encircled in dotted line. **(C)** The aqueous silk sponge alone had degraded into small fractions after 3 months and therefore volume measurements were not possible, however the aqueous silk sponge with lipo retained 37 ± 10 % of its volume.

Fig. 2.

Solvent silk sponge implants in subcutaneous pocket model. **(A)** Solvent silk sponges prior to implantation, left- solvent sponge alone, right- solvent silk sponge seeded with lipo. Scale bar 5mm. **(B)** Implants after harvest at 6 months. Top row- macroscopic appearance of implants, scale bar 5mm. Implants retain a porous spongy appearance and silk structure is clearly visible in solvent sponge alone, whereas the solvent sponge with lipo has a more tissue-like appearance. Middle row- H&E sections of solvent silk sponge (black arrowhead) maintained pore structure, scale bar 100 μ m, inset 200 μ m. Bottom row- representative ultrasound images of implant site with implant encircled in dotted line. **(C)** Both solvent silk sponge groups maintained close to their initial volumes.

Fig. 3.

Subcutaneous silk foam implant volume increases with implant time. **(A)** Silk foams prior to implantation, left- foam alone, right- foam seeded with lipo. Scale bar 5mm. **(B)** Silk foam appearance after 1 month. Top row- macroscopic appearance of implants, scale bar 5mm. Silk foam implants alone still maintain their foam structure appearance. When foams are seeded with lipo the result is a more natural tissue-like appearance and silk structure is not as detectable. Scale bar 5mm. Middle row- Silk foam (black arrowhead) clearly visible in H&E sections, scale bar 100μm, inset 200μm. Bottom row- representative ultrasound images of implant site with implant encircled in dotted line. **(C)** Silk foam appearance after 3 months. Top row- macroscopic appearance of implants, scale bar 5mm. Silk foam implants alone maintain less of their foam structure appearance than at 1 month. When foams are seeded with lipo the result is a more natural tissue-like appearance and silk structure is even less detectable. Scale bar 5mm. Middle row- Silk foam (black arrowhead) clearly visible in H&E sections, scale bar 100μm, inset 200μm. Bottom row- representative ultrasound images of implant site with implant encircled in dotted line. **(D)** At 1 month only about half of the initial volume remained, yet at 3 months the initial volume was regained. Volume was unaffected by addition of lipo.

Fig. 4.

Intramuscular defect repair with silk sponges. **(A)** Sponge appearance prior to implantation (both aqueous and solvent silk sponges had same macroscopic appearance and therefore only aqueous sponges are shown for simplicity). Scale bar 20mm. **(B)** Aqueous silk sponge after intramuscular implantation at 1 month. H&E images of aqueous silk sponges at 1 month. The pore structure of silk sponges (black arrowhead) was no longer intact. When seeded with lipo, matrix-dense (white arrow) areas were seen. No muscle was seen. Scale bar 100 μ m, inset 200 μ m. **(C)** H&E images of aqueous silk sponges at 3 months. Fewer cells and more matrix were present than at 1 month. Scale bar 100 μ m, inset 200 μ m. **(D)** Intramuscular implants at 6 months. H&E images of solvent silk sponge groups. Pore structure of silk sponges clearly visible in all groups (black arrowhead). No muscle regeneration was seen within the boundaries of the silk implants. When silk sponges were seeded with lipo, there was a band (~100 μ m thick) of muscle present immediately adjacent to the implant (white arrow). **(E)** Intramuscular defect sites at 6 months without silk sponges. Defect alone (left image) had a scar like appearance, while UMSCs injected at the defect site (middle image) yielded some muscle regeneration (bottom bracket) and some scar tissue (top bracket). Improved muscle regeneration was visible in the lipo defect group. Scale bar 100 μ m, inset 200 μ m. **(F)** Aqueous silk sponges had begun to degrade at 1 month and 3 months. Solvent based silk sponges had degraded to a similar extent at 6 months as aqueous silk sponges did in 1-3 months.

Table 1

Experimental design of equine implant sites and harvest times. SQ – subcutaneous sites with original implant volumes of 1cm³, IM- intramuscular sites with initial implant volume of 24.5cm³.

Subject	Timepoint	SQ (1 cm ³)		IM (24.5 cm ³)	
		Implant type	Location	Implant type	Location
Subject 1	1 month	Foam alone (n=4) Foam +lipo (n=4)	Neck	Sponge (aqueous) alone (n=1) Sponge (aqueous) + lipo (n=1) Sponge (aqueous) + UMSCs (n=1)	Tricep, Semi-tendinosus, Gluteus
		Gel alone (n=4) Gel +lipo (n=4)	Dorsal paraspinal		
	3 month	Foam alone (n=4) Foam +lipo (n=4)	Dorsal paraspinal	Sponge (aqueous) alone (n=1) Sponge (aqueous) + lipo (n=1) Sponge (aqueous) + UMSCs (n=1)	Tricep, Semi-tendinosus, Gluteus
		Gel alone (n=4) Gel +lipo (n=4)	Dorsal paraspinal		
		Sponge (aqueous) alone (n=4) Sponge (aqueous) +lipo (n=4)	Neck		
Subject 2	6 month	Sponge (solvent) alone (n=4) Sponge (solvent) +lipo (n=4)	Dorsal paraspinal	Sponge (solvent) alone (n=1) Sponge (solvent) + lipo (n=1) Sponge (solvent) + UMSCs (n=1)	Tricep, Semi-tendinosus, Gluteus

Table 2

Ranking scheme for subjective tissue integration quantification of ultrasound images

Subjective ranking key	
0	Implant not visible
1	Implant barely visible (roughly 25% or less of margin visible)
2	Implant visible but margins are not well demarcated from adjacent tissue (roughly between 25-50% of margin visible)
3	Implant visible but margins not consistently demarcated from adjacent tissue (roughly between 50-75% of margin visible)
4	Implant is obvious with margins clearly demarcated (roughly between 75-100% of margin visible)
5	Implant extremely obvious with clearly defined margins