Original Article

In vitro Flow Perfusion Maintaining Long-term Viability of the Rat Groin Fat Flap: A Novel Model for Research on Large-scale Engineered Tissues

Yang An, Fang-Fei Nie, Ze-Lian Qin, Hong-Yu Xue, Lu-Jia Chen, Bi Li, Dong Li
Department of Plastic Surgery, Peking University Third Hospital, Beijing 100191, China

Abstract

Background: Large-scale muscle tissue engineering remains a major challenge. An axial vascular pedicle and perfusion bioreactor are necessary for the development and maintenance of large-scale engineered muscle to ensure circulation within the construct. We aimed to develop a novel experimental model of a large-scale engineered muscle flap from an existing rat groin fat flap.
Methods: A fat flap based on the superficial inferior epigastric vascular pedicle was excised from rats and placed into a perfusion bioreactor. The flaps were kept in the bioreactor for up to 7 weeks, and transdifferentiation of adipose to muscle tissue could have taken place. This system enabled myogenic-differentiation medium flow through the bioreactor at constant pH and oxygen concentration. Assessment of viability was performed by an immunofluorescence assay, histological staining, a calcein-based live/dead test, and through determination of RNA quantity and quality after 1, 3, 5, and 7 weeks.
Results: Immunofluorescence staining showed that smooth muscle around vessels was still intact without signs of necrosis or atrophy. The visual assessment of viability by the calcein-based live/dead test revealed viability of the rat adipose tissue preserved in the bioreactor system with permanent perfusion. RNA samples from different experimental conditions were quantified by spectrophotometry, and intact bands of 18S and 28S rRNA were detected by gel electrophoresis, indicating that degradation of RNA was minimal.
Conclusions: Flow perfusion maintains the long-term viability of a rat groin engineered muscle flap *in vitro*, and a large-scale vascularized muscle could be engineered in a perfusion bioreactor.

Key words: Culture *Ex vivo*; Flow Perfusion; Perfusion Bioreactor; Tissue Engineering

Introduction

The reconstruction of soft-tissue defects attributed to oncologic resections, complex trauma, or hereditary and congenital malformations still represents a major challenge in plastic and reconstructive surgery. Immunocompatible, transferred, or transplanted tissue; minimal or no donors item morbidity; and maintenance of the volume and function for a long period are considered the criteria of an ideal soft-tissue replacement. An *ex vivo* flow system can provide the conditions similar to those *in vivo* that can preserve the viability of a large-scale tissue for a long period by offering nutrients, removing waste, and mediating physiological shear stress.[1] Flow perfusion bioreactors are designed to meet requirements of large-scale tissue activity by means of a peristaltic pump to circulate a medium through tissue with a pedicle. In addition, flow perfusion has a potential to create an internal environment that offers the nutrient flow in native tissues. Thus, to enable delivery of nutrients, carry away waste, and provide sheer stress, these systems should be capable of maintaining the viability of explanted tissues.

Over the past few years, numerous research groups have presented such techniques for functional three-dimensional skeletal muscle tissue.[2] Nonetheless, the major challenge of popularizing the application of such constructs is scarcity

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Address for correspondence: Dr. Dong Li, Department of Plastic Surgery, Peking University Third Hospital, 49 North Garden Road, Haidian District, Beijing 100191, China
E-Mail: lidong9@sina.com

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of vascular supply. Therefore, an axial vascular pedicle is necessary for maintenance of functional engineered skeletal muscle. Accordingly, to maintain the vascularization and to enable preparation of larger volumes of engineered muscle tissues, many researchers have implanted vessel loops or pedicles into tissue-engineering chambers.[3] Enhancement of angiogenesis in axially vascularized tissues has been performed through an additional extrinsic vascular pathway, which allows for transplantation of the entire construct by means of the arteriovenous loop pedicle.[4] Furthermore, in another approach, addition of angiogenic factors may also facilitate vascularization of the engineered tissue grafts. For instance, the use of genetically modified myoblasts expressing vascular endothelial growth factor has been investigated to enhance neovascularization and tissue mass of engineered muscle *in vivo*.[5]

In addition, because muscle regeneration by adipose-tissue-derived adult stem cells proceeds in a time-dependent manner,[6] various attempts have been made to prolong tissue survival *ex vivo*. Suitable perfusion media, such as the Hannover solution, have been shown to increase flap viability.[7] In recent years, there were a few studies on animal models for large-scale tissue preservation *in vitro* over long periods. In the present study, we employed rat groin adipose tissue flaps on a vascular pedicle and incubated them in perfusion bioreactors without the addition of growth factors, an extracellular matrix, or a scaffold. We tested the suitability of a flow perfusion bioreactor for maintenance of long-term viability of this tissue. Flow perfusion bioreactors with rat groin adipose tissue flaps on a vascular pedicle may serve as a novel *in vitro* model for analysis of long-term preservation of a large-scale tissue. This novel model for maintenance of large-scale tissue viability can remain stable for up to 7 weeks *in vitro* and retains viability when the tissue is transferred to a muscle-like structure.

Methods

Ethical approval

The operating procedures were conducted according to the current regulations and principles of the Chinese law for animal welfare. The experimental animals were treated on the basis of humanitarian principles, with the approval of the Ethics Committee of our institution.

Animals

All the animals were kept in individual cages in a room with standard environmental conditions. The animals received water and stock diet *ad libitum*. A total of 24 adult male Lewis rats weighing 250–290 g served as fat flap donors in this study. They were used to elevate adipose flaps based on the superficial inferior epigastric vessels, a branch of the femoral vessels [Figure 1a]. The flaps consist of fat tissue and fascia but not skin.

Anesthesia and the surgical procedure

The groin of rats was shaved with an electrical shaver, and the operative site was disinfected with Octenisept

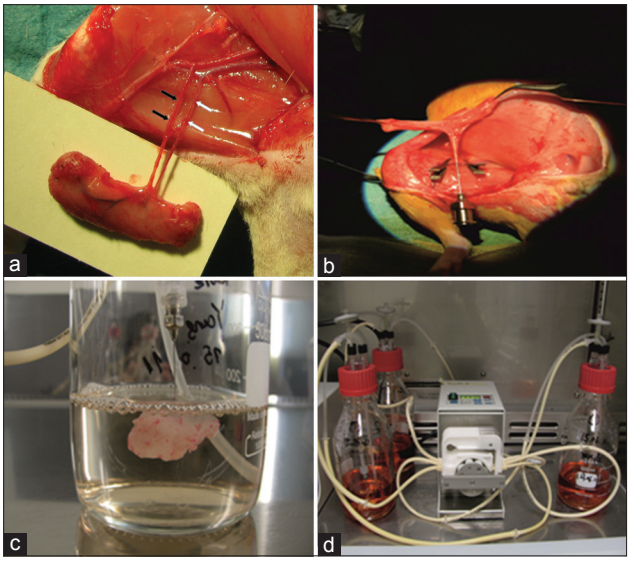

Figure 1: Rat groin adipose flap. Superficial inferior epigastric artery (black arrows), superficial inferior epigastric vein (white arrows a); A blunt tipped needle was inserted into the dissected epigastir cartery (b). The adipofascial flap in the perfusion bioreactor (c). The whole perfusion bioreactor device. The top has an access and an exit for an oxygen-permissible pipe, length 120 cm, and another access for air through a bacterial filter. Continuous perfusion is facilitated by a cable pump. All adipose flaps are connected to the apparatus (d).

under general isoflurane anesthesia by means of an animal narcotic unit (Euthanex Isoflurane). A 2 cm skin incision was made in the bilateral groin parallel to and adjacent to the inguinal ligament. Next, we exposed inguinal adipofascial flaps, and the complete preparation and freeing of the flaps from the surrounding tissue with bilateral ligation or coagulation of branches were performed under an operating microscope with microinstrumentation. The femoral vessel ligation was followed by dissection of the flaps and their flushing through the femoral artery with 10 ml of heparinized saline to wash out the blood. A blunt-tipped needle was inserted into the dissected epigastric artery, and the elevated flap was placed in the perfusion bioreactor [Figure 1b and 1c]. A sterile clamp was attached to the flap to prevent swimming of the fat flap at the solution's surface. All the dissected flaps with a vascular pedicle were connected to the same type of bioreactor in the same way, resulting in 48 flaps total (at two flaps per rat). The rats were euthanized under anesthesia by transecting the heart.

Medium

The composition of the myogenic differentiation medium used in this experiment is shown in Table 1.

Perfusion bioreactor

The bioreactor was constructed of a 500 ml Schott Duran bottle, with access and an exit for a 120 cm oxygen-permissible pipe and access for air through a bacterial filter at the top. The whole system was placed in a CO_2 incubator at 37°C and was run by a cable pump at a continuous flow of 2 ml/min through the Schott bottles filled

with 150 ml of the solution [Figure 1d]. The flaps were kept in the bioreactor such that the whole flap and the pedicle were completely covered with the solution. This system enables medium flow at constant pH and constant oxygen concentration through the bioreactor. Visual assessment of viability was performed after 1, 3, 5, or 7 weeks by histological staining, a calcein-based live/dead test, and RNA quantity and quality analyses.

Live/dead assay

A visual assessment of viability of thin adipose tissue slices of the inguinal flaps was performed by a calcein-based live/dead test (Invitrogen, Darmstadt, Germany) according to the manufacturer's protocol. These slices were prepared manually from adipose tissue as thinly as possible with a scalpel at room temperature, without any other workup. Microscopic analysis of the fresh slices was performed under an Olympus CK 40 microscope, Tokyo, Japan.

Immunofluorescence histological analysis

Tissue samples were studied and embedded in Tissue Freezing Medium® at an appropriate time point. After freezing in liquid nitrogen, they were sectioned on a Leica CM3050 cryostat at a thickness of 10 µm. Two sections per time point were subjected to immunofluorescence histological analysis. Immunofluorescent detection of smooth muscle actin was carried out with mouse monoclonal antibody clone ASM-1 (Temecula, CA, USA) at 1:100 dilution. The secondary antibody was an Alexa Fluor 488-conjugated chicken anti-mouse IgG antibody (Invitrogen, Eugene, Oregon USA). Counterstaining for nuclei was performed using the Vectashield mounting medium for staining with DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride, Vector Laboratories Inc., Burlingame, CA, USA). The protocol was as follows: the tissue slices were fixed with 4% paraformaldehyde (W/V) in phosphate-buffered saline (PBS) for 20 min at first. Next, they were washed with distilled water for 2 min and then unmasked with 0.1% Triton X-100 diluted in PBS for 4 min. The cross-sections were washed again three times with distilled water and then blocked with 2% fetal calf serum in PBS for 30 min. In the subsequent procedure, the primary antibody was incubated for 1 h. After that, the slices were washed again three times with PBS. Then, the secondary antibody was applied for 30 min. After 3 washes each with PBS and distilled water, the slices were dried, covered with a mounting medium, and finally fixed with a cover glass. Fluorescence microscopy was conducted using a Zeiss (Zeiss 200 M inverted microscope) Axio Vision microscope with Axio Vision software, version 4.6.3 (Carl Zeiss, Jena, Germany).

Table 1: Composition of myogenic medium

Component	Amount
DMEM/F12 (GIBCO, Invitrogen)	500 ml
FBS superior (Biochrom, Berlin, Germany)	50 ml
Donor horse serum (PAA, Austria)	25 ml
Penicillin/streptomycin (PAA)	5 ml
Dexametason (sigma)	928 µl
Hydrocortison (Pfizer-PHARMACIA, Berlin, Germany)	50 µl
HEPES buffer (PAA)	20 ml

DMEM: Dulbecco's Modified Eagle Medium; HEPES: 2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonic acid; FBS: Fetal bovine serum.

Tissue and total RNA preparation

At 1, 3, 5, and 7 weeks after incubation in perfusion bioreactors, large-scale tissue samples (approximately 100 mg) were cut into several pieces, and immediately frozen in liquid nitrogen.

Total RNA was isolated from the tissues using the TRIzol reagent (Life Technologies) and the NucleoSpin RNAII Kit according to the manufacturer's protocol. The RNA concentration was measured by spectrophotometry at 260 nm. RNA purity was determined by calculating ratios of absorbance at 260/280 nm and 260/230 nm. The quality of the total RNA was verified by assessing the integrity of 18S/28S ribosomal RNA via electrophoresis in 1% ethidium bromide-stained agarose gels.

Results

Visual assessment of viability by the calcein-based live/dead test

This assessment revealed viability of the rat adipose tissue preserved in the bioreactor system with permanent perfusion [Figure 2a-2d]. The samples of 1- and 3-week perfusion had almost no red staining, thus indicating the absence of dead adipocytes. Nevertheless, necrosis gradually appeared after 5 weeks, indicated by red staining. Besides, more cells turned red as a sign of broken cell membranes after 5 weeks. The increasing red staining visually denotes accumulation of nonviable cells. Although a little bit greater

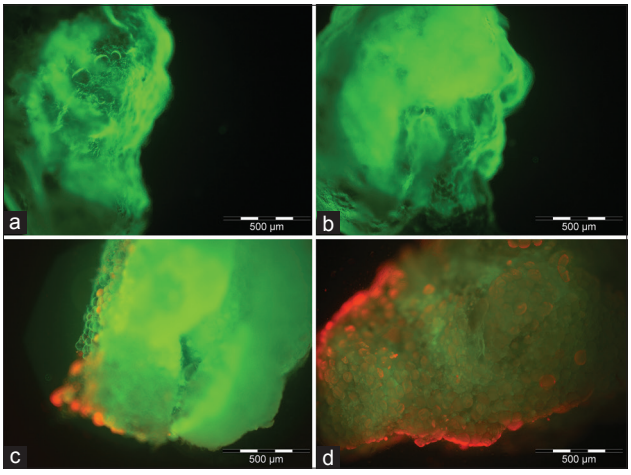

Figure 2: Visual assessment of viability by a calcein-based life/dead test. It revealed superior viability of the engineered muscle tissue preserved in perfusion bioreactors because fewer cells turned red (this color is a sign of broken cell membranes). Tissue from perfusion bioreactors after 1 week (a), 3 weeks (b), 5 weeks (c), and 7 weeks (d). The increasing red staining reveals visually the accumulation of nonviable cells.

number of nonviable cells was detected in the engineered muscle tissues at 7 weeks, the engineered muscle structures still maintained good long-term viability.

Histological examination of the large-scale tissue

Immunofluorescent analysis of the engineered muscle tissue preserved *ex vivo* was applied to study the smooth muscle around vessels in the adipose tissue of the rat fat flaps in the bioreactor systems. This analysis revealed that vessels in the bioreactor tissues were still intact. Furthermore, smooth muscle around vessels was detectable at all time points [Figure 3a-3d]. After 1- and 3-week, fluorescence was detected at fairly high levels in the bioreactor-cultivated tissues. Even though fluorescence intensity was found to be lower after 5 weeks, the smooth muscles around the vessels at 7 weeks were still viable, without signs of necrosis or atrophy.

RNA quantity and quality

It is well known that RNA quality and quantity are critical for successful analysis of gene expression.[8] This analysis requires quantification of the isolated RNA as well as an assessment of RNA integrity. In this study, RNA from different experimental groups was quantified by spectrophotometry and by electrophoretic analysis of rRNA subunits 18S and 28S [Figure 4]. The figure indicates that degradation of RNA was minimal.

Discussion

The rat is a preferred experimental subject for an animal model in experimental studies because it is inexpensive, readily available, reliable, and disease resistant. Research shows that many rat flap models have been described, and most of them have a clinical equivalent.[9-11] As a reproducible experimental model, the epigastric flap in the rats still used not only for microvascular surgical training[12-15] but also simply as a research model for laboratory studies. Although several flow-through flaps have been developed and widely used in clinical practice, there is still no effective model to further explore the long-term viability of a large-scale tissue *in vitro*. In our rat model, the epigastric vessels served as a graft to create an arteriovenous loop and to set up circulation in the large-scale groin adipose tissue in a flow perfusion bioreactor. The flow-through design of an epigastric flap (as a vascular source from the rat) based on the epigastric artery is described in this study to provide a method for organ tissue engineering research in the future.

In this study, we tested this model for maintenance of the long-term viability of the rat groin fat flap *in vitro*. The cultured tissue is autologous; therefore, there are no issues with immune rejection, and the model preserves the viability of the tissue around the preexisting vascular pedicle; therefore, it can allow for subsequent implantation. Numerous *in vitro* tissue-engineering models have shown successful regeneration of tissue.[16,17] Nevertheless, the newly engineered tissue cannot remain stable, and consequently, a transfer of the tissue is not feasible. This model has a potential to act as a large-scale structure for further research on tissue engineering. Based on this model, differentiation of the multilineage cells in a vessel-based fat flap may be possible. Perfusion through the natural vascular system of a flap offers the possibility to enable microcirculation throughout the entire tissue, which allows a variety of tissues before replantation.

Furthermore, mature adipocytes have a high metabolic rate and low tolerance of ischemia.[18] A number of experimental strategies are used to generate adipose tissue successfully, but its survival and long-term persistence remain unstable.[19] Vascular support of the construct is likely to be one of the most critical factors that constrains the size, maintenance conditions, and quality of an engineered tissue construct. Supply of nutrients to the fat flap not only derives from its pedicle but also is enriched by the myogenic medium from outside the bioreactor tissue. Moreover, we demonstrated that this technology preserves cellular and vascular architecture, maintains viability, preserves RNA for quantification, and enables large-scale myogenic differentiation in the rat groin adipose-tissue model. Our histological data indicate that vascular structures were still viable, and tissue architecture

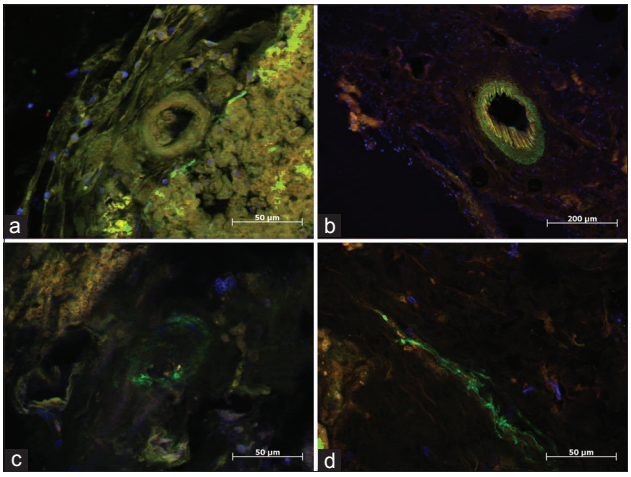

Figure 3: Immunofluorescent histological staining of newly formed engineered muscle tissue. Tissue from perfusion bioreactors after 1 week (a), 3 weeks (b), 5 weeks (c), and 7 weeks (d).

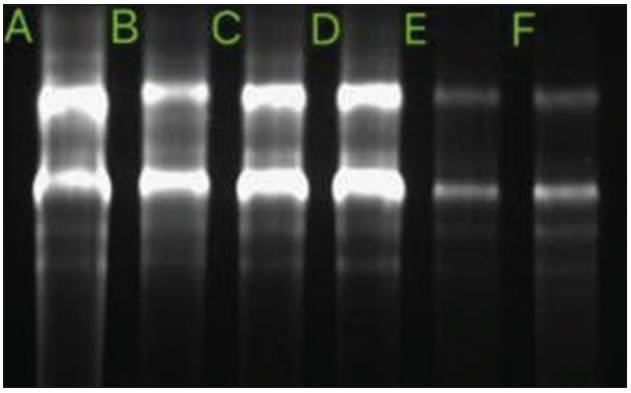

Figure 4: RNA isolation of newly formed engineered muscle tissue. A: Rat groin adipose tissue; B: Native muscle; C-F: Engineered muscle tissue after 1 week, 3 weeks, 5 weeks, 7 weeks, respectively.

was preserved where the medium infiltrated the artery network. The large-scale tissues in the bioreactor were filled with the myogenic medium evenly throughout the flow-perfused samples. Furthermore, the live/dead assay and RNA quantification results supported our histological observations with a demonstration of maintained metabolic activity and RNA preservation, respectively, thereby confirming viability after long-term flow perfusion *ex vivo*.

Moreover, histological evaluation showed that, besides the smooth muscles, which retained vitality around the vessels, there was also a significant increase in the number of nuclei, which is essential for the survival of the newly formed architectures. A lack of vascularity seems to be the common factor behind the limits on the size, on maintenance time, and on quality of an engineered tissue construct. Moreover, we demonstrated that we can preserve long-term a stable vascularized large-scale flap in a perfusion bioreactor without the addition of exogenous angiogenic or adipogenic growth factors or provision of biodegradable extracellular-matrix support. Furthermore, dissection of the vascularized adipose tissue flap is similar to that of human flaps.[20,21] Ongoing studies on larger tissue constructs in human models are evaluating an approach to a clinically applicable product. Thus, the strategy pursued in this study may be helpful for the reconstruction of soft-tissue defects after complex trauma or oncological resection as well as for nonimplant breast reconstruction. Indeed, this model may have much wider applications as a generic method of stimulating large-scale differentiation or formation of many different tissue types, including muscle, bone, and cartilage. In addition, this model will serve as a useful tool for studying large engineered tissues in regenerative medicine. This study illustrates a promising method of production of significant amounts of mature, vascularized, stable, and transferable large-scale tissue by tissue engineering techniques. This is an important step toward the development of a permanent autologous soft-tissue replacement.

Therefore, this study illustrates a promising method for large-scale fabrication of large, mature, vascularized, stable, and transferable tissue by tissue engineering methods.

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Conflicts of interest

There are no conflicts of interest.

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