Video Article

Engineered Vascularized Muscle Flap

Dana Egozi*[1], Yulia Shandalov*[2], Alina Freiman[2,3], Dekel Rosenfeld[2], David Ben-Shimol[4], Shulamit Levenberg[2]

[1]Department of Plastic Surgery, Kaplan Medical Center

[2]Biomedical Engineering, Technion Israel Institute of Technology

[3]Interdepartmental Program in Biotechnology, Technion Israel Institute of Technology

[4]Medicine Department, Technion Israel Institute of Technology

*These authors contributed equally

Correspondence to: Shulamit Levenberg at shulamit@bm.technion.ac.il

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Abstract

One of the main factors limiting the thickness of a tissue construct and its consequential viability and applicability *in vivo*, is the control of oxygen supply to the cell microenvironment, as passive diffusion is limited to a very thin layer. Although various materials have been described to restore the integrity of full-thickness defects of the abdominal wall, no material has yet proved to be optimal, due to low graft vascularization, tissue rejection, infection, or inadequate mechanical properties. This protocol describes a means of engineering a fully vascularized flap, with a thickness relevant for muscle tissue reconstruction. Cell-embedded poly L-lactic acid/poly lactic-co-glycolic acid constructs are implanted around the mouse femoral artery and vein and maintained *in vivo* for a period of one or two weeks. The vascularized graft is then transferred as a flap towards a full thickness defect made in the abdomen. This technique replaces the need for autologous tissue sacrifications and may enable the use of *in vitro* engineered vascularized flaps in many surgical applications.

Video Link

The video component of this article can be found at https://www.jove.com/video/52984/

Introduction

Abdominal wall defects often arise following severe trauma, cancer treatment, burns and removal of infected mesh. These defects often involve significant tissue loss, requiring complicated surgical procedures and presenting a major challenge for plastic reconstruction surgeons [1-4]. Tissue engineering researchers seeking new sources for artificial tissues have explored different materials, cell sources and growth factors. Successful restorations of various tissues, such as trachea [5,6], bladder [7], cornea [8], bone [9]and skin [10], by implantation of engineered tissues were previously reported. However, fabrication of a thick vascularized engineered tissue, particularly for reconstruction of large defects, remains a significant challenge in tissue engineering.

One of the main factors limiting the thickness of a viable tissue construct is the control of oxygen supply to its constituent cells. When relying on diffusion, construct thickness is limited to that of a very thin layer. The maximum distance between oxygen- and nutrient-supplying capillaries *in vivo* is approximately 200 µm, which correlates with the diffusion limit of oxygen[11,12]. Insufficient vascularization can result in tissue ischemia and escalate to tissue resorption or necrosis [13].

In addition, the ideal material used for tissue reconstruction must be biocompatible and non-immunogenic. It must also be capable of promoting further integration of host cells with the biomaterial, and maintaining structural integrity. Various biological [14-16] and synthetic [1,17,18] matrices have been previously explored for tissue reconstruction, however their use remain limited due to lack of effective blood supply, infections or insufficient tissue strength.

In this study, a biocompatible, cell-embedded scaffold comprised of Food and Drug Administration (FDA)-approved poly L-lactic acid (PLLA)/ poly lactic-co-glycolic acid (PLGA), was implanted around the femoral artery and vein (AV) vessels of a nude mouse and separated from the surrounding tissue, ensuring vascularization from the AV vessels only. One week post-implantation, the graft was viable, thick and well vascularized. This thick vascularized tissue with the AV vessels, was then transferred as a pedicled flap to an abdominal full-thickness defect in the same mouse. One week post-transfer, the flap was viable, vascularized and well integrated with the surrounding tissue, bearing sufficient strength to support abdominal viscera. Thus, the engineered thick, vascularized tissue flap, bearing an autologous pedicle, presents a novel method for repairing full-thickness abdominal wall defects.

Protocol

All animal studies were approved by the Committee of the Ethics of Animal Experiments of the Technion. For this procedure, athymic nude mice were used to avoid immunological rejection. If using another type of mouse, the mice should be shaved prior to the surgical procedure and administration of cyclosporine (or another anti-rejection substitute) is recommended.

1. Scaffold Preparation and Cell Embedding

1. Prepare scaffolds composed of 1:1 mixture of poly-L-lactic-acid (PLLA) and polylactic-co-glycolic-acid (PLGA), in the following manner:
    1. Dissolve 500 mg of PLLA and 500 mg of PLGA in 10 ml chloroform.
    2. Add 0.24 ml of polymer solution to 0.4 g of sodium chloride particles gathered in a Teflon molds. Use a salt diameter of 212-600 µm. Allow the chloroform to evaporate O/N.
    3. Leach the salt out using distilled water leading to interconnected porous 3D scaffolds.
    4. Cut polymer pieces using a scissors, then soak in 70% ethanol (v/v) for 30 min and wash 3 times in PBS for 2 min before usage.
2. Prepare a tri-culture of the following cells in 1:1 endothelial cell medium (EGM-2 supplemented with the components of its bullet kit and muscle cell medium (Dulbecco's minimal essential medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2.5% HEPES buffer, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Pen-Strep Solution).
    1. Use 0.5 x 10^6 C2C12 myoblasts, 0.9 x 10^6 Human umbilical vein endothelial cells (HUVECs), and 0.2 x 10^6 Normal human dermal fibroblasts (NHDF).
3. Mix the cells together in a microcentrifuge tube and centrifuge at 200 x g for 4 min, aspirate the medium and re-suspend the pellet in a mixture of 4 µl ice cold extracellular matrix solution and 4 µl cell medium.
4. Cut a piece of 10 mm x 7 mm x 1 mm (length x width x thickness) PLLA/PLGA scaffold and seed the cell suspension in a 6-well plate, allow the extracellular matrix solution to solidify (30 min, 37 °C, 5% CO_2) before adding 4 ml cell medium.
5. Incubate the scaffolds for ten days before implantation and replace the medium every other day.

2. Before Starting the Surgical Procedure

1. Prepare and sterilize (autoclave) the following surgical tools: a small, fine, straight scissors, a spring scissors, a straight, fine-tipped forceps, a serrated forceps and a needle holder.
2. Prepare a mixture of ketamine:xylazine solution in an microcentrifuge tube by mixing 425 µl ketamine and 75 µl xylazine in a 1 ml syringe.
3. Dilute 0.3 mg/ml buprenorphine in sterile saline (1:20).

3. Flap Construction (Graft Implantation)

1. Using an insulin syringe, anesthetize the mouse by an intraperitoneal injection of ketamine:xylazine (35 µl per 20 g of body weight).
2. Subcutaneously inject buprenorphine (final concentration of 0.05-0.1 mg/kg) before the opperation.
3. Place the mouse on a stage heated to 37 °C, to ensure normal body temperature, and then secure the mouse using an adhesive bandage.
4. Using cotton tips, administer lubricating eye ointment to the mouse's eyes (to avoid dehydration).
5. Using cotton tips, clean the incision site with iodine and then with 70% ethanol, to establish an aseptic working field.
6. Using small scissors and forceps, make an incision through the skin, from the level of the knee to the inguinal ligament, parallel to the femoral vessels, until the femoral artery and vein (AV) vessels are exposed.
7. Hold the skin down using a retractor, to gain better access to the tissue.
8. Using spring scissors and forceps, carefully isolate the femoral artery and vein vessels from the surrounding tissue. Start from the level of the inguinal ligament to the bifurcation of the superior epigastric vessels. Leave the profunda untouched, in order to preserve blood flow to the leg and prevent subsequent ischemia.
9. Fold the graft (from Section 1) around the exposed femoral AV, below the profunda and above the bifurcation to the tibial and proneal AV, and join its ends using 8-0 silk sutures.
10. To ensure implant vascularization by the capillaries sprouting from the femoral AV vessels only, wrap a piece of sterilized latex around the graft, and suture with 6-0 sutures.
11. Close the overlying skin using 4-0 silk sutures.
12. Monitor the mouse closely until recovery from the anesthesia and every day thereafter, until the graft is transferred as a flap. Subcutaneously inject buprenorphine (final concentration of 0.05-0.1 mg/kg) twice a day for 2-3 days.
    NOTE: Use saline in any stage, to avoid dehydration of the exposed tissue.

4. Flap Transfer

1. At one or two weeks postimplantation, anesthetize the mouse by an intraperitoneal injection of ketamine:xylazine (35 µl/20 g), using an insulin syringe.
2. Subcutaneously inject buprenorphine (final concentration of 0.05-0.1 mg/kg) before the opperation.
3. Place the mouse on a stage heated to 37 °C, to ensure normal body temperature, and then secure the mouse using an adhesive bandage.
4. Using cotton tips, apply lubricating eye ointment to the mouse's eyes (to avoid dehydration).
5. Using cotton tips, clean the incision site (from the knee area to the abdomen) with iodine and then with 70% ethanol, to establish an aseptic working field.

6. Using a scissors and forceps, carefully open the sutures in the skin and, using a scissors, make an incision through the skin parallel to the inguinal ligament, continuing through the ventral skin.
7. Carefully remove the latex piece with forceps and expose the vascularized flap.
8. Using a scissors and forceps, dissect the tissue flap from the surrounding tissue.
9. Using a forceps and a needle holder, ligate the distal end of the femoral AV with 8-0 silk sutures to prevent bleeding from the AV. Then, cauterize the AV at the level of the knee, distally to the folded implanted tissue and the 8-0 sutures.
10. Gently transfer the femoral AV enveloped by the graft, towards the ventral abdominal wall, avoiding damage to the artery, to determine at which distance the full thickness defect should be made.
    CAUTION: Pulling the artery too far will damage the artery.
11. Using a fine scissors, make an incision in the ventral abdominal wall.
12. Using a spring scissors, make an incision in the rectus abdominus muscle. Remove an approximately 1 cm x 0.8 cm segment of the rectus abdominus muscle with the overlying skin.
13. Transfer the femoral AV, enveloped by the vascularized graft, as an axial flap, to the full-thickness defect in the ventral abdominal wall.
14. Suture the flap to the surrounding muscle tissue, using 8-0 silk sutures, to prevent hernia.
15. To avoid suturing the internal organs, raise the muscle with a forceps when suturing the flap to the abdominal muscle. Leave the ventral skin exposed to mimic the effect of a full abdominal wall defect.
16. Cover the wound in the skin with iodinated gauze and a sterile bandage to prevent contamination of the exposed area.
17. Suture the skin of the leg using 4-0 silk sutures.
18. Monitor the mouse closely until recovery from the anesthesia and every day thereafter, until retrieval of the flap. Subcutaneously inject buprenorphine (final concentration of 0.05-0.1 mg/kg) twice a day for 2-3 days.
19. NOTE: Use saline in any stage to avoid dehydration of the exposed tissue.

5. Ultrasound Determination of Vascular Perfusion of the Graft

NOTE: Before transfer, vascular perfusion of the graft is measured at one and two weeks after implantation, by ultrasonography.

1. Anesthetize the mouse using 2% isoflurane.
2. Place the mouse on a movable stage heated to 38 °C, to ensure normal body temperature, and then secure the mouse using an adhesive bandage.
3. Find the femoral artery and vein vessels with non-linear transducer set on the B-mode.
4. Examine the patency of the femoral vessels of the graft using the transducer set on the color Doppler mode.
5. Prepare non-targeted contrast agent according to the manufacturer's instructions.
6. Connect a tail vein catheter (12 cm tubing with a 27 G needle) to a 1 ml syringe filled with saline. Secure the syringe next to the mouse on the heating stage (to avoid movement of the syringe).
7. Connect the catheter to the mouse tail vein and secure the needle.
8. Inject some of the saline to both ensure that injections are being made into the vein and to fill the tubing with saline (to avoid air bubbles).
9. Fill a 1 ml syringe with 70 µl of contrast agent and replace the saline syringe with the contrast agent syringe.
10. Set up the ultrasound to image injection of the contrast agent. In properties of the non-linear mode set up the number of frames to 1,500 and the disruption pulse to 30% of the frames.
    NOTE: After the disruption pulse, the microbubbles re-fill the vessels at a rate that depends on the flow rate of the microbubbles in the capillaries and is independent of the injection rate of the microbubbles. The number of frames can be changed according to the filling time [19,20].
11. Slowly inject the contrast agent into the tail vein.
12. Capture images in the non-linear contrast mode of the high-resolution ultrasound system.
13. Analyze the results using the software, as previously described [19,20].
    1. Draw a region of interest (ROI) on the image obtained immediately after the disruption pulse in the contrast-mode. The ROI should outline the area filled by the contrast agent only.
    2. Calculate the peak enhancement (PE), which is the ratio of the mean intensity of the non-linear signal after the injection of the microbubbles versus after the disruption pulse, and is a measure of the perfusion volume.
    3. Calculate the flow rate, which is determined from the time (T) that elapses until the peak signal (P).

6. Determination of the Extent of Graft Vascularization

NOTE: The extent of tissue graft vascularization is determined one or two weeks after implantation.

1. Using an insulin syringe, anesthetize the mouse by an intraperitoneal injection of ketamine:xylazine (35 µl/20 g).
2. Using 1 ml syringe, intravenously inject 200 µl of 10 mg/ml fluorescein isothiocyanate-conjugated dextran (FITC-Dextran, MW = 500,000) into the tail vein.
3. Ten sec after completion of the injection, euthanize the mouse by cervical dislocation.
4. Open the leg of the mouse and expose the graft.
5. Wash the graft area with phosphate buffered saline (PBS) and apply 10% neutral buffered formalin.
6. Image the graft in the mouse leg, using a confocal microscope.
7. Quantify functional vessel density (FVD).
    1. Isolate the green channel (excitation = 488 nm) of the confocal microscopic images.
    2. Pass the resulting images through a high-pass filter, in order to accentuate the high-contrast structures.
    3. Use a despeckling filter to remove noise that might have been amplified by the high-pass filter.
    4. Set up the value of threshold on the resulting image; the threshold value is adjusted so that the features and structures in the original image are visible in the binary image.

5. Apply a size-threshold so that only groups of connected pixels larger than the defined size threshold remain in the binary image.
6. Outline the skeleton of the vessels in the graft using the Zhang-Suen's algorithm [21].
7. Calculate the FVD by summing the lengths of the midline of each vessel and dividing the result by the area of the ROI.

7. Immunohistological and Histological Staining of the Graft

1. Immunohistological Staining
    1. After confocal imaging, retrieve the graft using a small scissors and forceps. Dissect the AV vessels in the two ends of the graft and retrieve the graft.
    2. Fix the grafts in 10% neutral buffered formalin for 30 min - 2 hr.
    3. Place the grafts in histology cassettes and store in 70% ethanol until paraffin embedding.
    4. Embed in paraffin using a standard fixation and embedding protocol [22] and slice the paraffin embedded tissues into 5 µm slices using a microtome. Slice the whole tissue perpendicularly to the AV vessels.
    5. Deparaffinize the paraffin-embedded sections by immersion in 100% xylene twice, for 10 min each.
    6. Arrange the slides in a 250 ml plastic Coplin jar.
    7. Rehydrate by serial immersions in decreasing concentrations of ethanol (100%, 96%, 90%, 80%, 70%, 50%, 30% and double distilled water (DDW)) for 3 min each.
    8. Prepare antigen unmasking solution by diluting 2.35 ml of antigen unmasking solution with 250 mL of DDW. Heat the antigen unmasking solution for 2 min in a microwave set to 95 °C.
    9. Insert the slides into the pre-heated antigen unmasking solution and heat again for 3 min in a microwave set to 95 °C. Cool the slides to 50 °C.
    10. Heat again for 2 min and then cool to RT.
    11. Incubate the slides in 3.3% H_2O_2solution in methanol for 10 min, at RT to quench the activity of endogenous peroxidase and then rinse twice in PBS.
    12. Prepare an incubation chamber: place wet absorbent sheets inside a histology box and dry the slides using delicate task wipers.
    13. Circle the slice area on the slide using a hydrophobic pen and incubate for 30 min at RT, with approximately 50 µl of goat serum blocking solution (2%, prepared in PBS) per slice.
    14. Dilute the rabbit anti-CD31 antibody 1:50 in blocking solution, apply approximately 50 µl antibody per slice and incubate O/N, at 4 °C. Rinse 3 times with PBS, 5 min each.
    15. Dilute the biotinylated goat anti-rabbit secondary antibody 1:400 in PBS, place over slides approximately 50 µl of antibody per slice and incubate for 30 min at RT.
    16. Rinse 3 times with PBS, 5 min each.
    17. Dilute streptavidin-peroxidase 1:400 in PBS, apply approximately 50 µl per slice and incubate with slides for 30 min, at RT. Rinse 3 times with PBS, 5 min each.
    18. Apply approximately 50 µl of aminoethylcarbazole (AEC) substrate per slice, according to the manufacturer's instructions. Dip the slides in DDW to stop the reaction.
    19. Stain the nuclei in blue by immersing the sections in approximately 50 µl of Mayer's hematoxylin solution for 2 min. Gently rinse in water and then cover the sections with mounting medium.
    20. Quantify CD31-positive staining using a double-blind approach. CD31 staining appears as a brown stain. Choose 4-5 different fields in a 40X magnification viewing field under a binocular microscope. Count the number of CD31-positive lumens.
        1. Calculate the average number of CD31-positive lumens per scaffold by dividing the total number of counted lumens with the number of fields and the fieldarea (according to the binocular data).
        NOTE: The applied reagent volume must be sufficient to cover the entire outlined slice.

2. Histological Staining
    1. Fix grafts in 10% neutral buffered formalin.
    2. Embed grafts in paraffin using standard fixation and embedding procedures.
    3. Remove the paraffin from the paraffin-embedded sections by immersion in 100% xylene twice for 10 min.
    4. Fast wash twice for rehydration in 100% isopropanol.
    5. Fast wash twice 95% Ethanol.
    6. Wash three times in tap water.
    7. Immerse in Hematoxylin for 10 min.
    8. Wash three times in tap water.
    9. Immere in Eosin for 2 min.
    10. Fast wash dehydrate in 95% Ethanol twice.
    11. Fast wash 100% isopropanol twice.
    12. Fast wash 100% xylene twice.
    13. Cover with DPX glue with cover glass 1.5 mm thick.
    14. Image using microscope in 40X magnification.

8. Mechanical Properties Assessment

1. Retrieve the graft using a small scissors and forceps.
2. Rinse the graft in PBS.
3. Measure the width and thickness of the graft and calculate the cross-section area as width multiplied by thickness.
4. Use the test instrument to generate a stress-strain curve of hydrated grafts.

1. Mount the graft between the system tensile grips and measure the final length between the two grips (*i.e.*, initial length).
2. Apply a strain rate of 0.01 mm/sec, until failure.
3. Record the developed force and displacement using the manufacturer's protocol.
4. Generate a stress-strain curve in Matlab.
    1. Calculate strain according to displacement divided by the initial length. Stress is calculated as the measured force divided by the cross-sectional area.
    2. Determine stiffness as the slope of the linear-region in the stress-strain curve and the maximum point of the curve is the ultimate tensile strength.

Representative Results

Graft vascularization and perfusion *in vivo*

The grafts were implanted one or two weeks prior to their transfer as axial flaps. At one and two weeks post-implantation, gross observation of the graft area revealed viable and vascularized tissue grafts. These grafts proved to be highly vascularized, as determined by positive CD31 immunostaining (**Figure 1A**), and highly perfused, as evidenced by FITC-dextran tail vein injection and ultrasound measurements. Many vessels were already observed at one-week post-implantation, a number that rose significantly after an additional week in the vicinity of the AV vessels. FITC-dextran-based determination of the functional vessel density (FVD) (**Figure 1B**) showed that the graft was highly vascularized at one-week postimplantation and that no significant changes took place in the additional week *in vivo*. Vessel patency and perfusion were demonstrated by ultrasound imaging (the FVD was 5.71 mm^{-1} ± 0.51 $mm^{-1}$and 10.28 mm^{-1} ± 2.71 mm^{-1}, one and two weeks post-implantation, respectively). **Figure 1C** confirmed host femoral vessel patency and integrity after graft implantation. Moreover, ultrasonographic examination revealed perfusion within the graft area, which was slightly higher two weeks post-implantation as compared to one week post-implantation (**Figure 1D**).

Flap properties

Gross examination of the flaps one week post-transfer revealed a viable, vascularized and well integrated tissue. The flaps underwent firm attachment to their surroundings. When comparing the pre-vascularized, cell-embedded flaps to control acellular, nonvascularized grafts, the former showed superior mechanical properties, as manifested by increased stiffness and strength (**Figure 2**). We also observed that wound dehiscence and herniation occurred less frequently in mice treated with cell-embedded flaps, when compared to animals treated with control grafts, which can be attributed to the increased mechanical strength of the transplanted tissue.

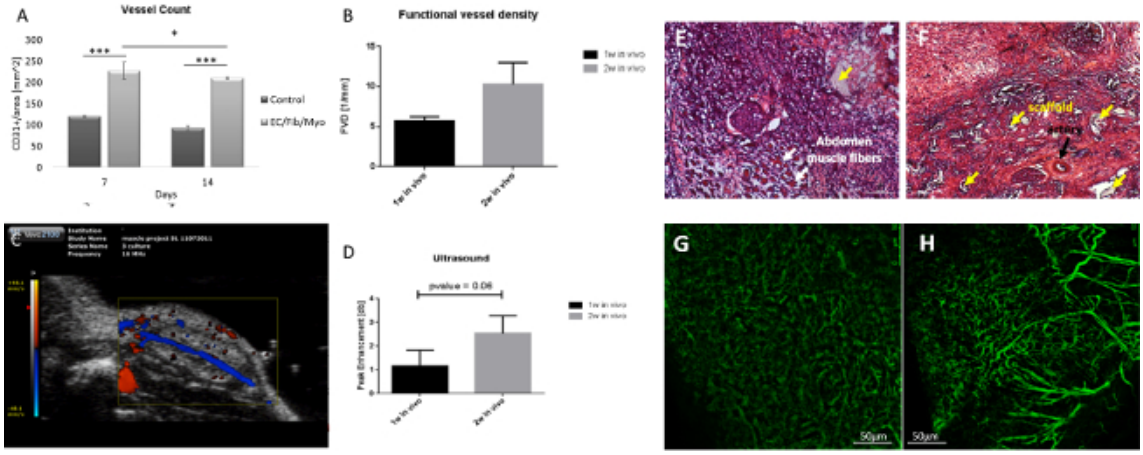

Figure 1. Graft vascularization. (**A**) The density of CD31-positive vessels, measured at one and two weeks postimplantation compared to control group. All values are normalized to the graft area (mm^2). *= T-test p <0.05. (**B**) Functional vascular density (FVD) at one and two weeks postimplantation. No significant difference was observed, p = 0.08 (T-test). (**C**) Patent AV vessels as imaged using ultrasound in the color Doppler mode. Blue and red represent the blood flow in the graft. The yellow quadrant outlines the graft area. (**D**) Graft perfusion at one and two weeks postimplantation. (**E** and **F**) H&E staining of the flaps integrated with the host tissue: black arrow point viable artery; white arrows point abdominal muscle fibers; yellow arrows point scaffold remains. (**H** and **G**) FITC dextran distribution as shown by confocal microscopy images. For all determinations, the sample size was n ≥ 3 and all values are represented as mean ± standard error of the mean. Please click here to view a larger version of this figure.

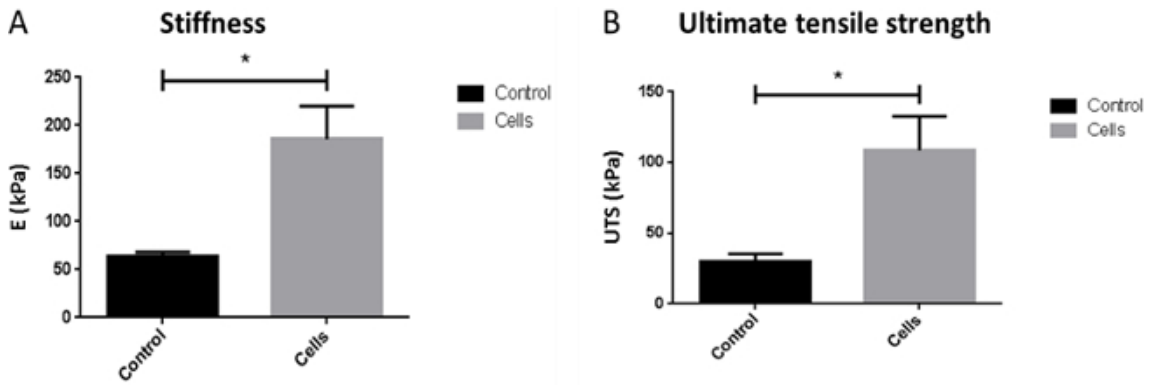

Figure 2. Mechanical properties of the flap one week post-transfer. (**A**) The stiffness and (**B**) the ultimate tensile strength (UTS) of the flaps. Control stands an empty graft without cells, cells stands tri-culture grafts. * = T-test p <0.05, n = 3. Values are represented as mean ± standard error of the mean. Please click here to view a larger version of this figure.

Discussion

The advances in tissue engineering have been met with a growing demand for substitute tissues for reconstruction of various tissue types. A variety of synthetic [1,17,18] and biological [14-16] materials as well as fabrication methods have been assessed for their capacity to address these demands. However, despite the progress in clinical care and in tissue engineering, the restoration of full-thickness abdominal wall defects remains a challenge. A tissue adequate for reconstruction of such massive defects must be (1) thick and (2) vascularized, and demonstrate (3) mechanical integrity, and (4) viability over time [23].

Here, we present a step-by-step detailed protocol for creating a thick, viable, and vascularized tissue flap, which can serve as an alternative to autologous tissue flaps. The fabricated flap was constructed in two steps: (1) A PLLA/PLGA scaffold was implanted around the AV vessels of the mouse hindlimb and separated from the surrounding tissue to allow vascularization by the AV vessels only. (2) The created vascularized, thick tissue flap was then transferred with the AV vessels, which served as its pedicle, into a full-thickness abdominal wall defect.

Proper flap vascularization is essential for its successful integration within the host [17,18,24]. Various approaches to create vascularized engineered tissue in order to improve oxygen supply and diffusion in thick tissues, have been discussed in the literature. Among these were methods involving seeding of endothelial cells (ECs) on various scaffold types [13,20,25-30], various techniques to supply angiogenic factors to the implantation site (either by direct administration by injection, use of transformed cells expressing the factors [31-34] or slow release from different scaffolds [35,36]), use of bioreactors to ensure engineered tissue perfusion [37] and employment of AV loop chambers [38,39]. Here, the tissue graft was vascularized *in vivo*, by exploitation of autologous vessels. The graft, which proved viable, vascularized and perfused, was then transferred as a thick tissue flap to repair a full-thickness abdominal wall defect. One week post-transfer, the flap was viable and featured sufficient mechanical strength to support the abdominal viscera. Here, we used athymic nude mice, which bear an impaired immune system; when using any other type of mouse or other animal, the possibility of immune reactions should be taken into consideration (especially when seeding the scaffolds with cells). Other limitations of this technique include (1) the transfer of femoral AV vessels, which supply the blood flow to the hindlimb. However, the technique leaves the profunda and the deep femoral vessels untouched and no hindlimb ischemia was observed after flap transfer. Moreover, in larger animals and in humans, the use of peripheral vessels will be advised as they are redundant in the body; (2) the time required to achieve adequate vascularization prior to flap transfer, can be a limiting factor in urgent cases; and (3) flap creation is performed *in vivo*, which can limit its relevance in humans. In the future, we aim to develop a means of creating this flap *ex vivo*. The advantage of the presented method lies in the capacity to enhance formation of an autologous tissue (with autologous cells) around the scaffold. The resulting tissue presents a suitable alternative to an autologous tissue flap. Moreover, the scaffold can be seeded with autologous cells prior to implantation, to further improve graft vascularization and integration within the host tissue. Use of autologous cells and a biodegradable and biocompatible scaffold, can circumvent the substantial risk of immuno-rejection reactions and present an alternative to autologous flaps while avoiding harvesting and postoperative scarification.

The described method can translated to experiments in large animals and further expanded to clinical trials in humans. Moreover it can be translated to repair various damaged tissues within the body. In humans and in large animals, the flaps can be generated around peripheral vessels instead of on the femoral AV vessels.

Disclosures

The authors declare that they have no competing financial interests.

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