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A Reliable Porcine Fascio-Cutaneous Flap Model for Vascularized Composite Allografts Bioengineering Studies

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TITLE:

A Reliable Porcine Fascio-Cutaneous Flap Model for Vascularized Composite Allografts Bioengineering Studies

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SUMMARY:

The present protocol describes the porcine fascio-cutaneous flap model and its potential use in vascularized composite tissue research.

ABSTRACT:

Vascularized Composite Allografts (VCA) such as hand, face, or penile transplant represents the cutting-edge treatment for devastating skin defects, failed by the first steps of the reconstructive

ladder. Despite promising aesthetic and functional outcomes, the main limiting factor remains the need for a drastically applied lifelong immunosuppression and its well-known medical risks, preventing broader indications. Therefore, lifting the immune barrier in VCA is essential to tip the ethical scale and improve patients' quality of life using the most advanced surgical techniques. *De novo* creation of a patient-specific graft is the upcoming breakthrough in reconstructive transplantation. Using tissue engineering techniques, VCAs can be freed of donor cells and customized for the recipient through perfusion-decellularization-recellularization. To develop these new technologies, a large-scale animal VCA model is necessary. Hence, swine fascio-cutaneous flaps, composed of skin, fat, fascia, and vessels, represent an ideal model for preliminary studies in VCA. Nevertheless, most VCA models described in the literature include muscle and bone. This work reports a reliable and reproducible technique for saphenous fascio-cutaneous flap harvest in swine, a practical tool for various research fields, especially vascularized composite tissue engineering.

INTRODUCTION:

Vascularized composite allografts (VCA) have revolutionized the treatment of hard-to-repair body part losses, such as hands, face, and penis[1-3]. Unfortunately, the first long-term outcomes[4] have shown that lifelong administration of high-dose immunosuppressive agents can lead to severe collateral medical conditions, including diabetes, infections, neoplasia, and reno-vascular dysfunction[5]. Lately, expert VCA teams have had to manage the risk of chronic rejection leading to graft loss and perform the first face retransplantation cases[6,7]. Different strategies have been described to overcome the limitations of immunosuppression in VCA. The first relies on establishing long-term graft tolerance by inducing an immune mixed chimerism state in the allograft recipient[8,9]. The second involves *de novo* creation of a patient-specific graft *via* tissue engineering.

Recently, perfusion decellularization of biological tissues has generated native extracellular matrix (ECM) scaffolds, allowing the preservation of the vascular network and tissue architecture of whole organs[10]. Hence, the recellularization of these ECM with recipient-specific cells would create a customized graft free of immune constraints. In research on VCA bioengineering, multiple teams have decellularized and obtained such ECM preserving the entire architecture[11-13]. However, the recellularization process remains challenging and has not been successful in large animal models[14,15]. Developing these breakthrough technologies creates a need for reliable and reproducible large animal composite tissue models. Swine models represent the utmost choice in the bioengineering developmental pipeline, as porcine skin presents the closest anatomical and physiological characteristics to human skin[16]. The use of fascio-cutaneous flaps (FCF) is ideal during the first steps towards the creation of 'tailored' vascularized composite tissue grafts. Indeed, FCF is an elementary VCA model containing skin, fat, fascia, and endothelial cells. A description of swine myocutaneous flaps[17] and osteomyocutaneous flaps[18] can be found in the literature. Nonetheless, there is a lack of focus on fascio-cutaneous flaps harvest techniques.

Hence, this study aims to provide researchers with a detailed description of a swine saphenous FCF procurement technique and depict all the flap's characteristics for its use in many research fields, especially in vascularized composite tissue engineering.

PROTOCOL:

All animals received human care following the National Institute of Health Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee approved the experimental protocol (IACUC- protocol #2020N000015). Seven female Yorkshire pigs (20-25 kg) were used for all experiments.

1. Preoperative care

1.1. Fast the animal for solid food 12 h prior to the surgery.

1.2. Sedate the animal with 4.4 mg/kg of Telazol, 2.2 mg/kg of Xylazine, and 0.04 mg/kg (IM) of Atropine sulfate (see **Table of Materials**).

1.3. Place an 18 G peripheral intravenous catheter in an ear vein.

1.4. Intubate the swine with an appropriate endotracheal tube (6-15 mm can be used for 10-200 kg pigs) and connect the tube to a ventilator. Administer pre-operative analgesia with buprenorphine (0.05 mg/kg, IM) (see **Table of Materials**).

2. Intraoperative monitoring

2.1. Maintain anesthesia with an inhalation mixture of 1.5%-3% isoflurane with 1.5 L/min oxygen flow.

2.2. Continuously monitor the heart rate, pulse oximetry, and end-tidal CO_2. Assess blood pressure and body temperature every 5 min.

NOTE: The target range for the heart rate is between 90-100 beats/min, the oxygen saturation must be higher than 93%, and the end-tidal CO_2 range is between 5%-6% of CO_2.

2.3. Administer 5-10 mL/kg per hour 0.9% saline throughout the procedure to regulate the mean arterial pressure between 60 mmHg and 90 mmHg.

3. Bilateral saphenous FCF procurement

3.1. Place the animal in a supine position. Shave and scrub both groins and hindlimbs, include the entire hindlimbs in the surgical site, and drape in a sterile fashion.

3.2. Palpate the pulse of the saphenous artery ~3 finger-widths medial from the patella and tag it.

3.3. Identify and draw the limits of the flap.

NOTE: The superior limit is an axis parallel to the inguinal crease 3 cm below it. The lateral limit is an axis from the anterior superior iliac spine to the medial part of the patella.

3.4. Draw a 10 cm diameter oval-like flap centered on the saphenous pedicle and contained in the previously described flap limits (step 3.3).

3.5. Make a 1.5 cm skin incision regarding the distal portion of the pedicle on the flap landmark.

3.6. Open the fascia and blunt dissect to expose the saphenous artery and its two venae comitantes. Perform a double ligature and separate in one bundle.

3.7. Incise the remaining skin of the flap with a blade.

3.8. Use cautery to open the subcutaneous tissue and the surrounding fascia. Perform thorough hemostasis using bipolar forceps (see **Table of Materials**).

3.9. Attach the skin component of the flap to the underlying fascia with 3-0 non-absorbable sutures to avoid inadvertent traction and disruption of perforating vessels.

3.10. Free the flap from the gracilis by dissecting the fascia away from the muscle.

NOTE: The distal part of the saphenous pedicle runs in a plane between the gracilis muscle and the fascia. Appropriate tension and cautious bipolar hemostasis of side branches are crucial elements to ease the pedicle dissection.

3.11. Use a knife to make a 12 cm incision in the inguinal crease. Perform a perpendicular incision joining the inguinal crease to the proximal part of the flap. Lift away the connecting skin and open the subcutaneous layer using cautery.

3.12. Continue the pedicle dissection by following the saphenous vessels down towards the femoral vessels.

NOTE: The proximal portion of the saphenous pedicle can either run through the intermuscular septum or dive into the gracilis muscle.

3.13. Skeletonize the femoral vessels and ligate them distally to the saphenous branch in two separate bundles. Continue the dissection of the femoral vessels from distal to proximal until reaching the level of the inguinal ligament. Use bipolar forceps to cauterize or vascular clips and 2-0 silk ties to ligate the deep femoral vessels, then cut.

NOTE: Vascular clips can also be used before cutting the vessels.

3.14. Repeat steps 3.2-3.13 on the contralateral hindlimb to harvest the second saphenous flap.

3.15. Heparinize the animal with an intravenous (IV) heparin injection (100 IU/kg) 5 min before step 3.16.

3.16. Ligate the femoral pedicle (artery and vein) as proximal to the inguinal ligament as possible and separate the flap from the donor pig.

3.17. Dilate the femoral vessel ends and insert a 20 G angiocatheter in both artery and vein. Use 3-0 silk ties to secure the catheter to the vessels.

3.18. Slowly flush the fascio-cutaneous flap artery with 10 mL of heparin saline (100 IU/mL) until a clear venous outflow is observed (**Figure 1**).

[Place **Figure 1** here]

3.19. Euthanize the animal with an IV injection of sodium phenobarbital (100 mg/kg). Confirm death by the absence of heartbeat and respiratory movements.

REPRESENTATIVE RESULTS:

This work on living animals was preceded by determining the saphenous perforasome on three cadaveric specimens (**Figure 2**). A colored filling solution was injected into the saphenous artery to opacify the specific vascular network coming from the artery. The solution is composed of 10 mL blue-colored glycerin agent mixed with 10 mL of the diluent agent (see **Table of Materials**). This generated a colored map of the skin vascularized by the saphenous artery and allowed drawing the limits of the saphenous FCF**.**

[Place **Figure 2** here]

A total of 14 saphenous fasciocutaneous flaps were harvested in this study (**Table 1**). The average flap procurement time was 47 (41; 62) min. The mean artery and veinous diameters were 2.25 mm (2; 2.5) and 3.56 mm (2.7; 3.9), respectively. Finally, the mean pedicle length was 10.8 cm (10.4; 12.6)**.**

[Place **Table 1** here]

An FCF angiography (**Figure 3**) was performed after each flap harvest through intraarterial injection of 10 mL contrast product immediately after the heparin saline flush. Thus, this step enabled to assess the vascularization of the skin paddle. All angiography images showed a dense and well-distributed vascular network on the flap.

[Place **Figure 3** here]

The flaps were then subjected to the custom decellularization protocol[11]. The flaps were perfused using pressure-controlled machine perfusion, delivering a continuous flow using this protocol.

With a target pressure of 80 mmHg, the flow of PBS, SDS, and Triton X was limited to a maximal speed of 3.1 mL/min. No oxygen consumption was noted as the perfusion system was dedicated to the flap cell detersion. This protocol resulted in effective decellularization of all tissues (**Figure 1**), as confirmed by the absence of DNA in all tissue samples.

FIGURE AND TABLE LEGENDS:

Figure 1: Native and decellularized saphenous fascio-cutaneous flap. (**A**) Isolated skin flap with a 20 G angiocatheter inserted in the femoral artery, allowing to wash the flap from the blood and proceed with different experiments (angiography, perfusion decellularization). (**B**) Decellularized skin flap. Perfusion decellularization yielding white, acellular scaffolds after 10 days of detergent perfusion. H&E-stained full-thickness cross-sections of (**C**) native skin flap and (**D**) decellularized skin flap.

Figure 2: Perforasome determination. A colored filing solution was injected in the Saphenous artery of cadaveric specimens to precisely determine the limits of the skin perfused by the Saphenous pedicle

Figure 3: Saphenous fascio-cutaneous flap angiography. A contrast product was injected through the femoral artery, showing a dense saphenous vascular network. Scale in centimeters.

Table 1: Saphenous flaps characteristics based on 14 flap harvests.

DISCUSSION:

This article describes a reliable and reproducible fasciocutaneous flap harvested on swine hindlimbs. Following this step-by-step surgical protocol will allow the procurement of two flaps on only one animal in less than 2 h. The most critical step of the surgery is the skeletonization of the vascular pedicle within the gracilis muscle fibers, which requires a thorough dissection by a skilled surgeon. Securing the skin to the fascia using cutaneous sutures is a crucial tip to avoid a shearing effect disrupting the perforator's vessels and a subsequent skin devascularization of the flap. The characteristics of the saphenous FCF (long vascular pedicle, decent calibers of vessels) and its reliability make it an ideal model for many research fields.

Several teams have demonstrated interest in this model in a skin bioengineering protocol by decellularization and recellularization[11]. The absence of muscle was a pivotal point in implementing a bioengineering protocol. Hence, we searched for fasciocutaneous flaps located either on the forelimb, midback, thigh, or groin where the panniculus carnosus (thin muscular layer dividing the superficial and deep fat layers in swine) is lacking[19]. In preliminary experiments, abdominal skin flaps based on the deep superior epigastric artery were harvested following previously published protocols[20-22]. However, the small diameter of the vessels, the more difficult harvesting technique, and the presence of the panniculus carnosus represented significant disadvantages. The experimental protocol by perfusion decellularization revealed inconsistencies in the skin perfusion through the perforators that appeared too small and/or injured during the surgery.

This flap has also been used to study the mechanistic pathways involved in the immune rejection of vascularized skin grafts, the skin being the most immunogenic component in VCA[8,23]. Using this model, the impact of the skin component in the transplant tolerance has been precisely evaluated.

Furthermore, this detailed procedure can also serve as a pre-clinical model in other realms of research. Saphenous FCF could evaluate ischemia-reperfusion injuries on a large animal skin model closer to a human. Finally, it could also be helpful for *ex-vivo* VCA machine perfusion preservation and help determine the best perfusion parameters to maintain skin viability before transplantation[24].

To conclude, this accurate description of a reliable and reproducible flap procurement technique offers a valuable tool for VCA bioengineering studies in swine.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

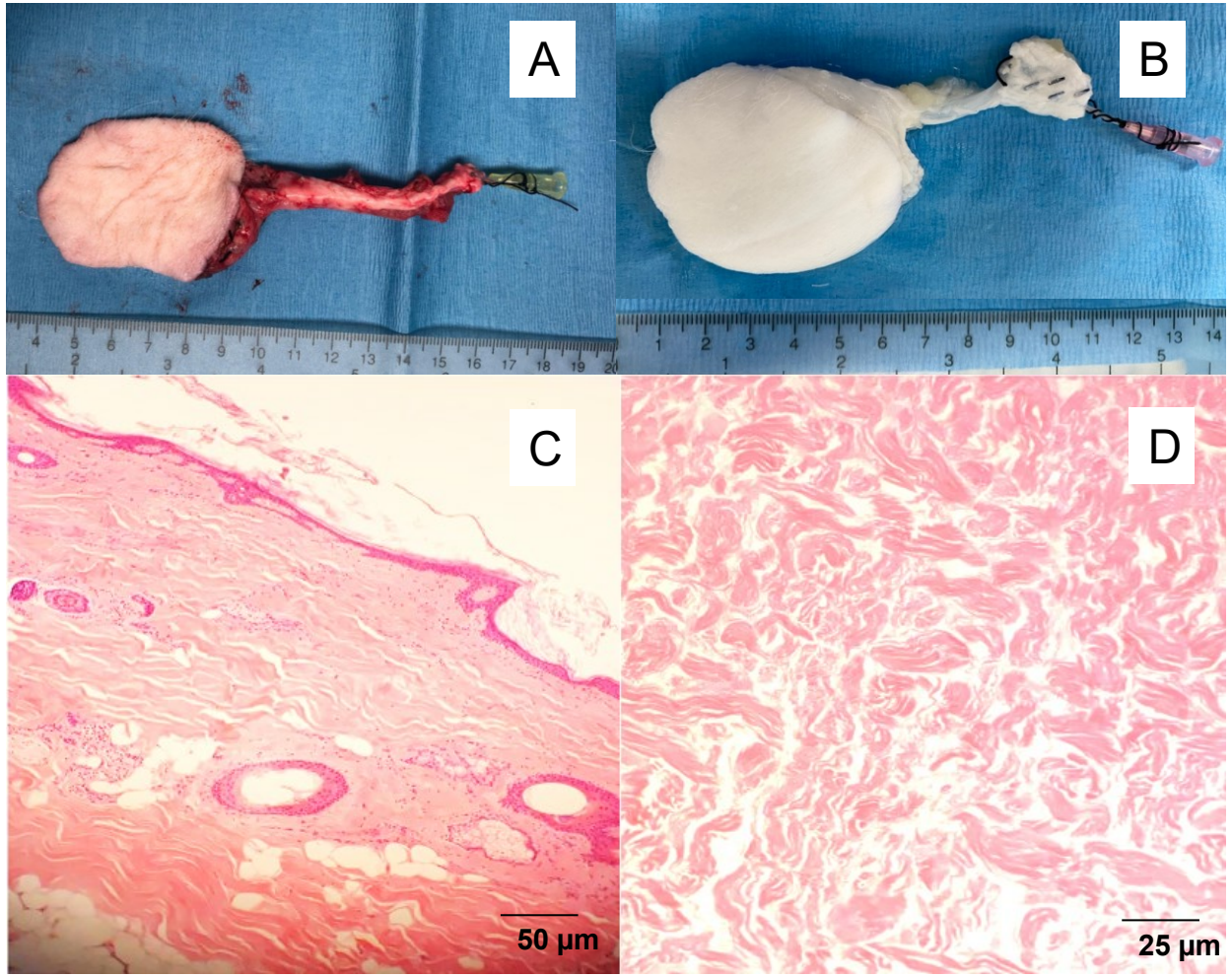

Figure 2

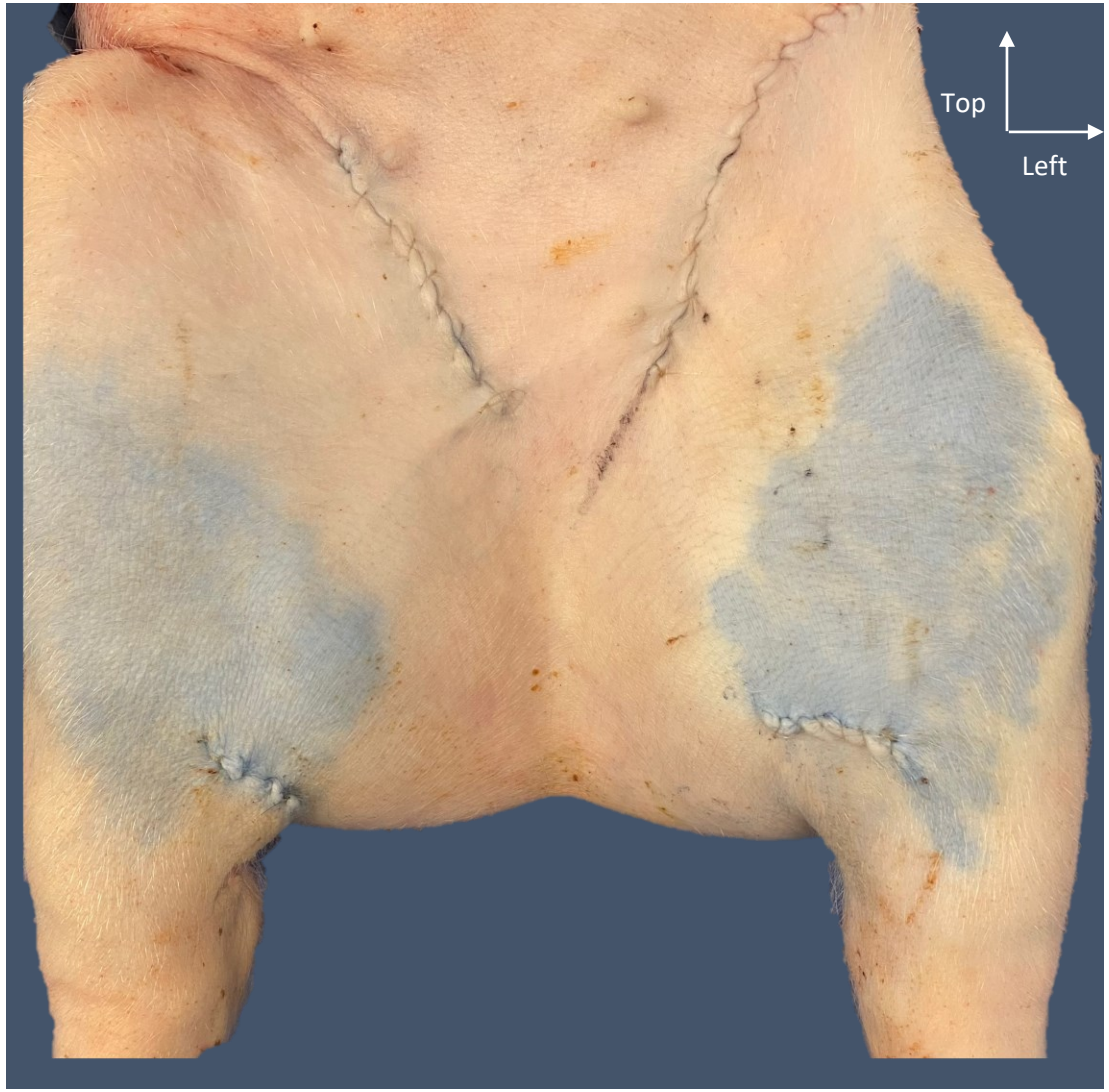

Figure 3

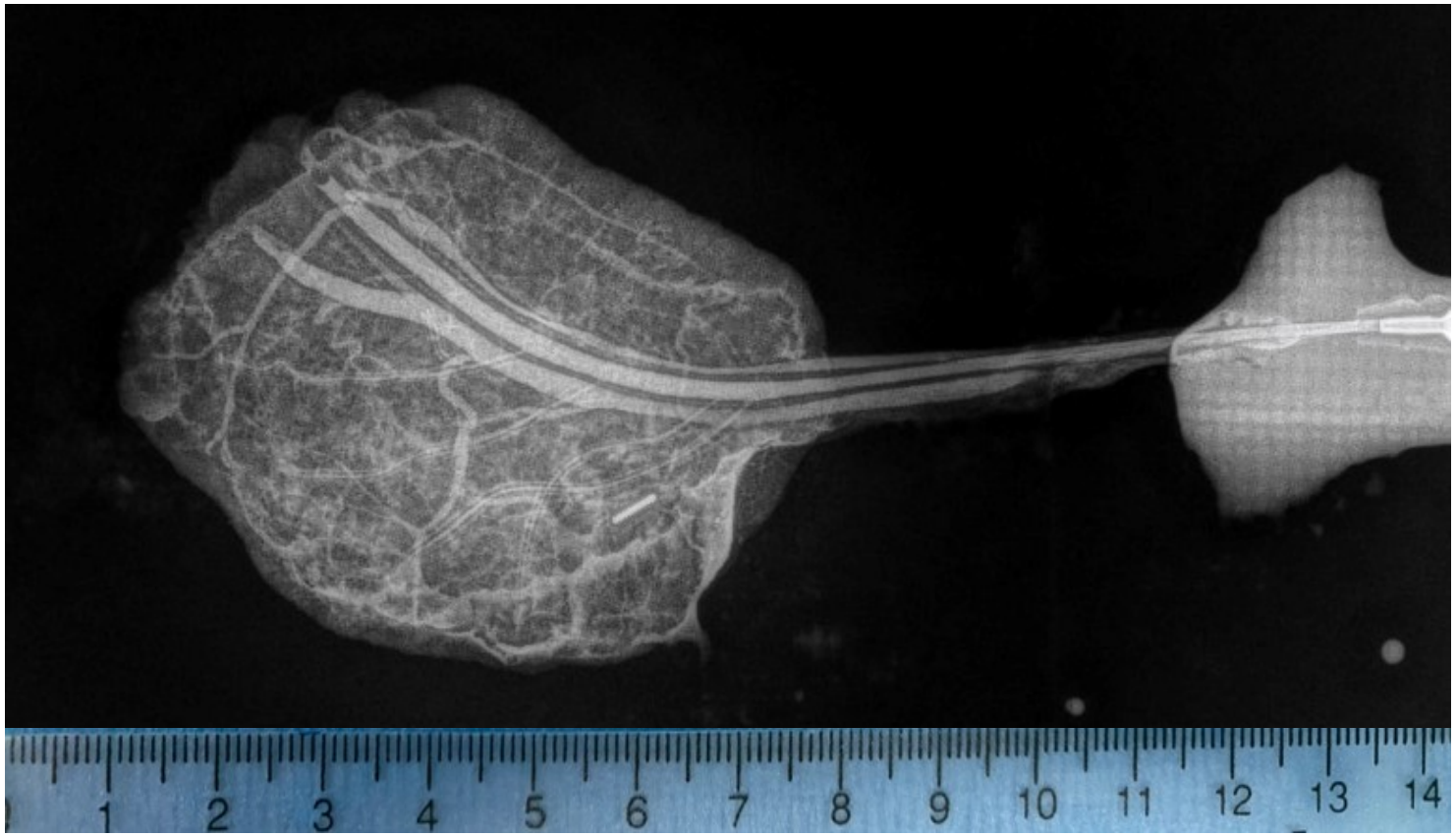

Table 1

Animal weight (kg)	**FCF harvest duration (min)**	**Pedicle length (cm)**
Mean *(min;max)*	Mean *(min;max)*	Mean *(min; max)*
23 (*20; 25*)	47 *(41; 62)*	10.8 *(10.4; 12.6)*

Artery diameter (mm)	**Venous diameter (mm)**
Mean *(min; max)*	Mean *(min; max)*
2.25 *(2; 2.5)*	3.56 *(2.7; 3.9)*

To: Nilanjana Saha
Review Editor
JoVE
nilanjana.saha@jove.com

Dear Nilanjana,

Thank you for giving us the opportunity to submit a revised and improved version of our manuscript titled "**A Reliable Porcine Fascio-Cutaneous Flap Model for Vascularized Composite Allografts Bioengineering Studies"**. We appreciate the time and effort that you and the reviewers have dedicated to providing your valuable feedback on our manuscript and are grateful to the reviewers for their insightful comments on the paper. We have addressed the reviewer's suggestions and enhanced the quality of our paper. Here is a point-by-point response to the editorial and reviewers' comments and concerns.

The revised manuscript has been resubmitted.

Best regards,

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Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been thoroughly proofread.

2. Please provide an email address for each author.

Email addresses have been added for each author.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Personal pronouns have been removed from the manuscript.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all

commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Flow Tech, Omnipaque, Microfil.

Commercial language has been removed from the manuscript.

5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. All action steps should be numbered.

The protocol steps have been numbered accordingly.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

The protocol answers the "how" question.

7. Please add more details to your protocol steps:
Line 88: Please include the details of the Atropine sulfate in the Table of Materials.
Line 118: Please provide the cautery details in the Table of Materials.
Line 151: Please note that anesthetization/euthanization steps cannot be filmed. So please remove the highlighting. Also, please add the details of sodium phenobarbital in the Table of Materials.

Atropine Sulfate, cautery details and Sodium Phenobarbital (Euthazol) have been added to the Table of Materials.

8. Please Reference all the Figures/Tables sequentially in the manuscript text.

Figures and Table have been sequentially referenced in the manuscript text.

9. As we are a methods journal, please ensure that the Discussion covers the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) Significance with respect to existing methods
e) Any future applications of the technique

As requested, we added more details in the discussion about the critical steps and the significance with respect to existing methods.

10. Please label the figure panels (A), (B), (C), etc., in capital letters. Accordingly, please upload revised Figures in your editorial manager account.

The figure panels have been labeled in the figure caption.

11. Please upload each figure individually in the Editorial Manager account. Please ensure a high resolution for each figure.

12. Figure 1: (C & D)-please include scale bars.

Scale bars have been included in the Figure 1

13. Figure 3: Please include a scale bar.

Scale bar has been included in the Figure 3

14. Please upload Table 1 as xls/xlsx file instead of embedding it in the manuscript text.

Table 1 has been uploaded as xlsx.

Reviewers' comments:

Reviewer #1:

Manuscript Summary: Authors submitted a simple surgical technique article that can be used in a variety of different studies including tissue engineering. Manuscript is written wel, subject is easy to understand and reproduce for a reconstructive microsurgeon. However, it is exceedingly difficult for reviewers to follow surgical steps of the manuscript in the absence of figures or the video. In that case, I assume that steps written under method is accurate and adequate to reproduce.

Every step of the procurement from 3.1 to 3.18 will be illustrated by the video. The video will allow the reader to easily reproduce the surgical technique.

Major Concerns: Since authors are recommending this flap specifically for tissue engineering studies (perfusion decellularization etc), it is helpful for the reader to know more about the perfusion aspect of the study. What parameters were used by authors to know that this flap was perfused better than others as stated under discussion? is this appropriate for ongoing machine perfusion, or one time manual injection? If appropriate for machine perfusion, is it continuous or pulsatile? what are the perfusion parameters, flow rate, oxygen consumption? In the end, success of this model requires a thorough understanding of the surgical anatomy and flap perfusion, if it was to be used in tissue engineering.

After procurement, the flaps were perfused using a pressure-controlled machine perfusion delivering a continuous flow. With a target pressure of 80mmHg, the flow of PBS, SDS and Triton X was limited to a maximal speed of 3.1ml/min. No oxygen consumption was noted as the perfusion system was dedicated to the flap cell detersion.

Minor Concerns: What are vessel diameters and pedicle length of the flap? What anatomic variations, if any, were observed during dissections? It will be useful to have the depiction of the arterial vascular tree as it will not be part of the video anyway and significantly adds to the value of the paper.

Flaps characteristics such as pedicle length, vessels diameters and time to harvest are described in the representative results section (lines, page) and details are provided in the **Table 1**. The main anatomical variation observed during our dissection was the path of the proximal portion of the saphenous pedicle which can either run through the intermuscular septum or dive directly into the gracilis muscle (lines 144-145, page 5). During our dissections, the saphenous artery and vein always raised respectively from the femoral artery and vein. The arterial vascular tree can be observed in the angiography (**Figure 3)**.

Reviewer #2:

Manuscript Summary:
This manuscript describes a porcine experimental model for free fasciocutaneous flap elevation that can be used, as the authors did, as a model for allografts or as a surgica model for free fasciocutaneous flap elevation or as a model for free flap studies about homeostasis, isquemia reperfusion, surgical training or some other intentions.
The objectives of the manuscript and justfication in the introduction are clearly stated.
The manuscript adds some knowledge in the field of experimental free flaps and allografts and seems appropriate for publication in this Journal.
The main advantage for this experimental model are reliability of the method, wich is a major objective of JoVe.
The manuscript is well written, short enough and easy to read, making it ideal for publication as sent for review.
The protocol for animal management during general anesthesia and bilateral free flap elevation is well described and allows an external observer to reliably reproduce the experiment.
The autor have highlighted and discussed the concerns on this experimental model and as a reviewer I have no other comments about the quality, accuracy or other concerns about the manuscript.
Tables and figures have good quality, legends permit self-explanation.
References are in the correct format and are relevant for this model descrption.
I have found no evidence of plagiarism, scientific fraud or ethical concerns in the study presented.

Minor Concerns:
In line 73-74 the authors state a lack of perforator-based free flaps elevation experimental models but there are some articles describing techniques for free skin flap elevation based on perforator vessels. There are also articles describing in detail the vascular anatomy of the region distal to the inguinal ligament for free flap elevation.

Indeed, Minqiang et al and Bodin et al described free skin flap elevation based on perforator vessels on swine. However, they mainly focused on abdominal skin flap based on the Deep Superior Epigastric Perforator artery. Those articles are quoted in our manuscript line 232, page 8.
Nevertheless, no literature was found on free fascio cutaneous flaps elevation on swine hindlimbs.