Journal of Visualized Experiments

A Modified Cuff Technique for Mouse Cervical Heterotopic Heart Transplantation Model

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE63504R2
Full Title:	A Modified Cuff Technique for Mouse Cervical Heterotopic Heart Transplantation Model
Corresponding Author:	Jun Li CHINA
Corresponding Author's Institution:	
Corresponding Author E-Mail:	herrleej@foxmail.com
Order of Authors:	Xin Mao
	Peng Xian
	Hang You
	Gang Huang
	Jun Li
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TITLE:

A Modified Cuff Technique for Mouse Cervical Heterotopic Heart Transplantation Model

AUTHORS AND AFFILIATIONS:

Xin Mao[1#], Peng Xian[1#], Hang You[2], Gang Huang[3], Jun Li[1]*

[1]Department of Urological Oncology Surgery, Chongqing University Cancer Hospital, 400030 Chongqing, China

[2]School of Medicine, Chongqing University, Chongqing 400030, China

[3]Department of Biochemistry and Molecular Biology, College of Basic Medical Science, Army Medical University, 400038 Chongqing, China

Email addresses of the authors:

Xin Mao	(1467155960@qq.com)
Peng Xian	(pengx@vip.qq.com)
Hang You	(13541339873@163.com)
Gang Huang	(cqhuanggang@aliyun.com)
Jun Li	(lijunmed@cqu.edu.cn)

*Email address of the corresponding author:

Jun Li (lijunmed@cqu.edu.cn)

[#]These authors contributed equally to this work

SUMMARY:

In the present protocol, a mouse heart transplantation model is used for investigating the mechanism of cardiac allograft rejection. In this heterotopic heart transplantation model, operation efficiency is improved, and the survival of cardiac grafts is ensured by a cervical end-to-side anastomosis of heart implantation using a modified Cuff technique.

ABSTRACT:

Cardiac allograft rejection limits the long-term survival of patients after heart transplantation. A mouse heart transplantation model is ideal for investigating the mechanism of cardiac allograft rejection in preclinical studies because of their high homology with human genes. This understanding would help develop unique approaches to improving patients' long-term survival treated with cardiac allografts. In a mouse model, abdominal donor heart implantation is commonly performed with an end-to-side anastomosis to the recipient's aorta and inferior vena cava using stitches. In this model, the donor's heart is implanted by end-to-end anastomosis to the recipient's carotid artery and jugular vein by the modified-Cuff technique. The transplantation surgery is performed without stitching and thus may increase the survival of the recipient since there is no interference with the blood supply and venous reflux of the lower body. This mouse model would help investigate the mechanisms underlying the immunological and pathological (acute/chronic) rejection of cardiac allografts.

INTRODUCTION:

Heart transplantation has become the standard treatment for terminal heart failure. More than 5,500 heart transplantations per year are performed in the organizations registered under the International Society for Heart and Lung Transplantation. Among the allogeneic heart transplant recipients, the 1-year rejection rate is still >10%, while the 3-year rejection rate increased to 36%[1,2]. However, effective prophylactic treatments for patients with cardiac allograft rejection are lacking. Therefore, animal model studies are warranted that elucidate the physiological mechanisms underlying the immunological and pathological rejection of cardiac allografts. Such studies would contribute to the investigation of novel targets required to develop efficacious drugs, which would help prevent cardiac allograft rejection and improve survival rates in those patient populations.

Some potential immunological and pathophysiological mechanisms of cardiac allograft rejection have been proposed recently in mouse model studies of heterotopic heart transplantation[3-5]. Consequently, mouse heterotopic heart transplantation became an ideal preclinical model to investigate the mechanisms of immune rejection and pathological injury occurring in cardiac allografts after heart transplantation because of their high homology with human genes. The prevalent concept is to perform heterotopic transplantation in a mouse model by an abdominal end-to-side anastomosis in the recipient aorta and inferior vena cava using stitches, similar to the normal human anatomy. However, this procedure may interfere with the recipient's blood supply and venous reflux of the lower body[6]. Therefore, a modified heterotopic heart transplantation procedure in a mouse model is proposed here.

The donor's heart is implanted with the recipient's carotid artery and jugular vein by an end-to-end cervical anastomosis using a modified Cuff technique. This modified procedure facilitated the operative feasibility and ensured the survival of the cardiac graft without interfering with the blood supply and venous reflux of the lower body.

PROTOCOL:

All animal handling procedures were conducted in compliance with the NIH Care and Use of Laboratory Animals guidelines. All experimental protocols were approved by the Animal Care and Use Committee of the Chongqing University Cancer Hospital, Chongqing, China. Male BALB/c and C57BL/6 mice weighing 20–30 g, obtained from commercial sources (see **Table of Materials**), were used for allogeneic heart transplantation study. The C57BL/6 mice were used as donors and syngeneic recipients, while the BALB/c mice served as allogeneic recipients. A schematic of the protocol is shown in **Figure 1**.

1. Recipient procedure

1.1. Induce general anaesthesia *via* inhalation of 5% isoflurane through a 15 x 10 x 10 cm induction chamber connected with a hood (see **Table of Materials**).

1.2. Fix the recipient mouse on the operating table with a heating pad. Maintain anaesthesia with continuous inhalation of 2% isoflurane through a face mask over the nose and mouth.

NOTE: Slow respiratory rate and rhythm, the disappearance of the corneal reflex, and the absence of the pedal reflex in the toes indicate the effectiveness of anaesthesia.

1.3. After shaving the hair, disinfect the skin of the neck with 75% ethyl alcohol, and incise the skin by 1.5-2 cm in parallel to the cervical midline from the right mandibular angle to the tail-end.

1.4. Dissect ~1 cm of the right external jugular vein using an electro-coagulator and micro-forceps. Clip the vein at the proximal end with an atraumatic microvascular clamp and ligate it at the distal end.

1.5. Pass the distal end of the vein through a 22 G polyurethane barbed cuff (see **Table of Materials**) with a bevel end and superficial grooves. Fix the vein with the handle of the cuff using a microvascular clamp.

1.5.1. Remove the 8-0 ligation suture at the distal end, turn the lumen over the cuff hooked by the superficial barb inside out and fix with a 10-0 surgical suture in the grooves of the surface.

1.6. Resect the right sublingual gland to form a fossa for implanting the cardiac graft, and reserve the right lobe of the submaxillary gland and the right sternocleidomastoid.

1.6.1. Dissect the right common carotid artery for ~1 cm using micro forceps, and clip the artery with an atraumatic microvascular clamp at the proximal end. At the distal end, ligate and cut off the artery.

1.7. Pass the distal end of the artery through a 26 G polyurethane barbed cuff (see **Table of Materials**) with a bevel end and grooves on the surface. Fix the artery with the cuff's handle using a microvascular clamp.

1.7.1. Remove the ligation suture at the distal end, turn the lumen inside out over the cuff, and fix with a superficial barb and grooves with a 10-0 surgical suture.

1.8. After preparing the recipient's vessels, drop 100 IU/mL of heparin saline on the vessels to prevent thrombosis. Cover the cervical incision with wet saline gauze for subsequent implantation.

2. Donor procedure

2.1. Employ the same anaesthetic procedure (step 1.1) for the donor mouse.

2.2. Shave the abdominal hair using an electric razor, and sterilize the skin using 75% ethyl alcohol.

2.3. Incise the abdomen (2-3 cm) with a scissor along the midline from the symphysis pubis to the subxiphoid, and expand the incised area with a retractor.

2.4. Dissect 1 cm of the abdominal aorta and inferior vena cava using an electro-coagulator and a micro-forceps, and perform heparinization by injecting 1 mL of physiological saline supplemented with 250 IU/mL of heparin through the inferior vena cava. After this, excise the abdominal aorta and inferior vena cava.

2.5. Excise the thorax along the anterior axillary line on both sides using a surgical scissor to separate the chest wall. Ligate the superior vena cava with an 8-0 surgical suture.

2.6. Insert a scalp needle at the suprahepatic inferior vena cava. Then, inject ice-cold physiological saline supplemented with 100 U/mL of heparin through the scalp needle from suprahepatic inferior vena cava to perfuse the donor heart until the blood color fades.

2.7. Re-perfuse the donor heart with 2-3 mL of ice-cold histidine-tryptophan-ketoglutarate (HTK) solution (see **Table of Materials**) using a scalp needle from the aortic arch to protect the donor myocardium. The mean warm ischaemia time is 5 min.

2.8. Ligate the superior and inferior venae cavae and the pulmonary vein with a 5-0 surgical suture. Dissect and cut off the donor aorta and pulmonary artery before their branching. After that, divide the superior and inferior venae cavae and the pulmonary vein to remove the donor's heart.

3. Implantation

3.1. Implant the donor heart into the cervical pocket of the recipient mouse in an inverted position.

3.2. Pull the cuff with an everted recipient jugular vein into the lumen of the donor pulmonary artery to perform end-to-end anastomosis of the donor pulmonary artery to the recipient external jugular vein. Ligate the cuff using the grooves on the surface through a 10-0 surgical suture to fix the anastomosis.

3.3. Employ a similar procedure for end-to-end anastomosis of the donor aorta to the recipient carotid artery.

3.4. Release the atraumatic microvascular clamp of the jugular vein followed by the carotid artery to re-perfuse the donor's heart. The mean cold ischaemia time is 15 min.

3.5. Fix the cardiac graft and suture it properly to prevent twisting of the graft.

3.6. Close the cervical incision with continuous sutures using a 5-0 polyamide monofilament suture (see **Table of Materials**).

3.7. Retain the recipient mouse inside a warm, dry, and clean cage until it recovers from anaesthesia.

NOTE: It takes 5-10 min to recover.

3.8. Inject buprenorphine (0.05 mg/kg) subcutaneously into the recipient mouse every 6 h for 48 h for postoperative analgesia.

REPRESENTATIVE RESULTS:

In this mouse cervical heterotopic heart transplantation model, the survival rate of recipient mice was approximately 95.2% (20 out of 21 mice survived). The primary cause of death was postoperative bleeding. The fast heartbeat with a regular rhythm serves as an indicator of the survival of the implanted donor heart.

C57BL/6 and BALB/c mice were MHC (H-2b) and MHC (H-2d) types in this model, respectively[7,8]. These two strains differ by the H-2, which causes acute T-cell–mediated rejection[9]. Of all the cardiac allografts, 62.5% were lost within 7 days after transplantation, as assessed by palpating the heartbeat. All cardiac allografts were lost within 8 days after transplantation. In contrast, all the isogeneic heart transplants survived beyond 4 weeks (**Figure 2**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of the mouse cervical heterotopic heart transplantation model. (**A**) Protocol for preparing the recipient: after clipping the common carotid artery and external jugular vein at the proximal end, the vascular lumen of vessels is everted and fixed after passing through the barbed cuff with a bevel end and grooves on the surface. The dashed square shows the structure and usage of the cuff. (**B**) Donor heart resection: after the donor heart's perfusion with heparin and HTK solution from the inferior vena cava and aorta, the superior and inferior venae cavae and pulmonary vein are ligated with sutures. The donor's heart is then resected by incising the vascular vessels. (**C**) Implantation of the donor's heart. The donor pulmonary artery and aorta is anastomosed to the recipient's external jugular vein, and carotid artery *via* the cuff with the recipient's vasculature turned inside out in an end-to-end pattern.

Figure 2: Survival curve for cardiac grafts. The survival curve of cardiac grafts shows that allogeneic heart transplants are lost within 8 days after transplantation, which was assessed by palpation of the heartbeat. A total of 10 recipient mice underwent the modified cervical heterotopic heart transplantation in each group. All the isogeneic heart transplants survived more than 4 weeks.

Table 1: Comparison of heart transplantation techniques. The current mouse cervical heterotopic heart transplantation technique is modified from Oberhuber, R. et al.[10] and possesses additional advantages for cardiac graft survival.

DISCUSSION:

The mouse heart transplantation model contributes to the investigation of rejection mechanisms after heart transplantation, contributing to the development of unique approaches to improve the long-term survival of cardiac allograft recipients. However, heart transplantation in mice is a complex and challenging task, requiring a high level of microsurgery techniques, especially in vascular anastomosis[11-13]. The mouse abdominal heterotopic heart transplantation model is performed using stitches by anastomosis of the donor aorta and pulmonary artery to the recipient aorta and inferior vena cava. The recipient's aorta and inferior vena cava need to be blocked in this operation. Therefore, ischaemia of the lower body and thrombosis of the inferior vena cava can increase the disability and death of recipient mice. To reduce the difficulties of vascular anastomosis during transplantation, Matsuura et al. first introduced a cervical heart transplantation model in mice using the cuff technique in 1991[14]. In this model, the oversleeve-like everted anastomosis of vessels by ligation with cuff increased the anastomosis efficiency. In contrast to the anastomosis of vessels with sutures in an abdominal heart transplantation mouse model, it reduced the bleeding probability post-procedure. Therefore, the improvement of the anastomosis efficiency reduced the ischaemia time of cardiac muscle and increased the survival rate of cardiac grafts. Additionally, cervical implantation of the donor's heart does not interrupt the circulation of the recipient aorta and inferior vena cava compared with abdominal implantation[15]; therefore, the survival of recipient mice is increased.

A unique experimental mouse heterotopic heart transplantation model is described here, established by Rupert Oberhuber et al.[10]. The procedure involves an end-to-end cervical anastomosis of the donor aorta and pulmonary artery to the recipient carotid artery and jugular vein, following a modified Cuff technique. In this model, the systemic circulation of the recipient mice does not interfere with[10], and the donor's heart was perfused from the inferior vena cava and aorta with heparin and HTK solutions for better myocardial protection. However, the critical component of this model differed from that of Oberhuber et al.[10], which employed the modified barbed cuff with a bevel end and grooves similar to that of Finsterer et al.[16]. The bevel end facilitates an oversleeve-like evert of the vascular lumen. The grooves on the surface facilitate the fixation of everted vessel walls with a cuff using sutures, and the barb outside the cuff reduces the slippage of anastomosed vessel walls from the cuff (**Figure 1**). These modifications shorten the surgical time by 20% and improve cardiac grafts' implantation efficiency and survival. Furthermore, the modified barbed cuff is produced from the most common polyurethane catheter used for scalp acupuncture, thus significantly reducing the cost of the procedure. A comparison of the current technique with that of Oberhuber et al.[10] is shown in **Table 1**.

The unique features of this model need to be noted. First, the length and calibre of the cuff are essential for successful anastomosis. The suitable length of the cuff was ~3 mm with a handle (1 mm) (**Figure 1**). The suitable calibre of the cuff is 26 G and 22 G for artery and veins, respectively. The unsuitable length and calibre of the cuff would result in twisting or excessive tension of the anastomosed vessels. Second, the suitable lengths of the recipient's vessels are 1.5 to 2-folds of the cuff. Third, the donor's heart is not perfused with excessive pressure, potentially damaging the graft. Fourth, the cardiac graft is fixed, and the cuff is anastomosed in a suitable position by suturing after implantation to avoid movement or twisting of anastomosed vessels or grafts. Fifth, preservation of the submaxillary gland and sternocleidomastoid contributes to reducing twirling or twisting of the anastomosed vessels or graft when resecting the right sublingual gland to produce a fossa for cardiac graft. Sixth, to facilitate the oversleeve-like evert of the vascular lumen and reduce thrombosis after surgery, heparin solution (100 U/mL) can be provided to the anastomosed vessels while performing anastomosis.

This cuff technique facilitates the anastomosis of donor and recipient vessels during implantation; however, the hardness of the cuff may, in turn, increase the risk of twisting the anastomosed vessels, resulting in an increase in thrombosis after transplantation. Optimization of the cuff material is warranted to reduce complications, increase graft survival, and increase the utilization rate of the models in subsequent experiments. Furthermore, the fibrous scar of the cut may limit the space for the cardiac graft and affect its long-term survival. In addition, ejection of the cardiac graft may interfere with recipient mice's normal haemodynamic blood flow. Finally, this model is non-functional and cannot be used to evaluate the cardiac function of grafts. Nevertheless, this study provides knowledge regarding heart transplantation's immunological and pathological functions.

ACKNOWLEDGMENTS:

This work was supported by the National Natural Science Foundation of China (81870304) to Jun Li.

DISCLOSURES:

The authors have nothing to disclose.

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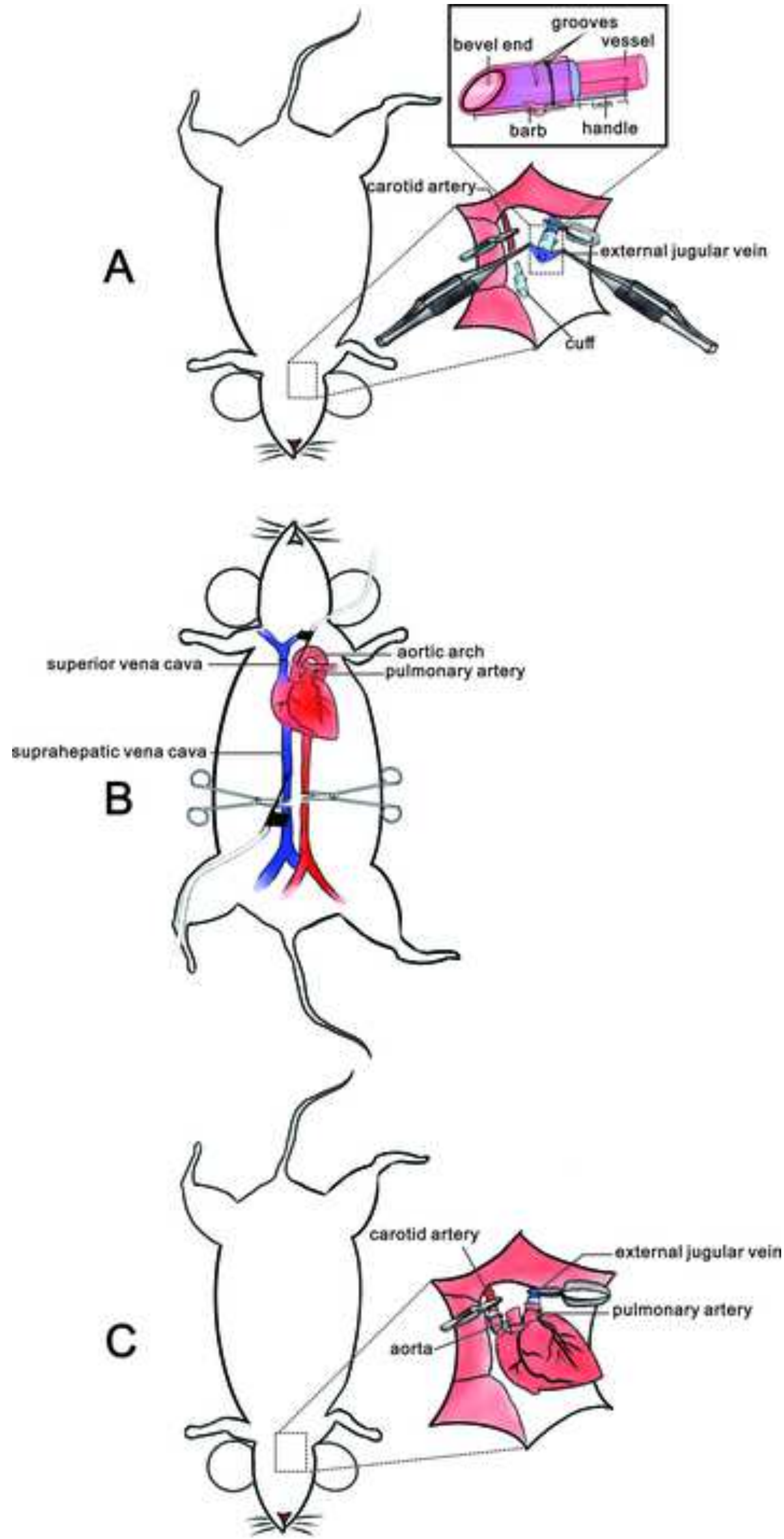
A
grooves
bevel end
vessel
barb
handle
carotid artery
external jugular vein
cuff
B
superior vena cava
aortic arch
pulmonary artery
suprahepatic vena cava
C
carotid artery
external jugular vein
pulmonary artery
aorta

Figure 2-revised

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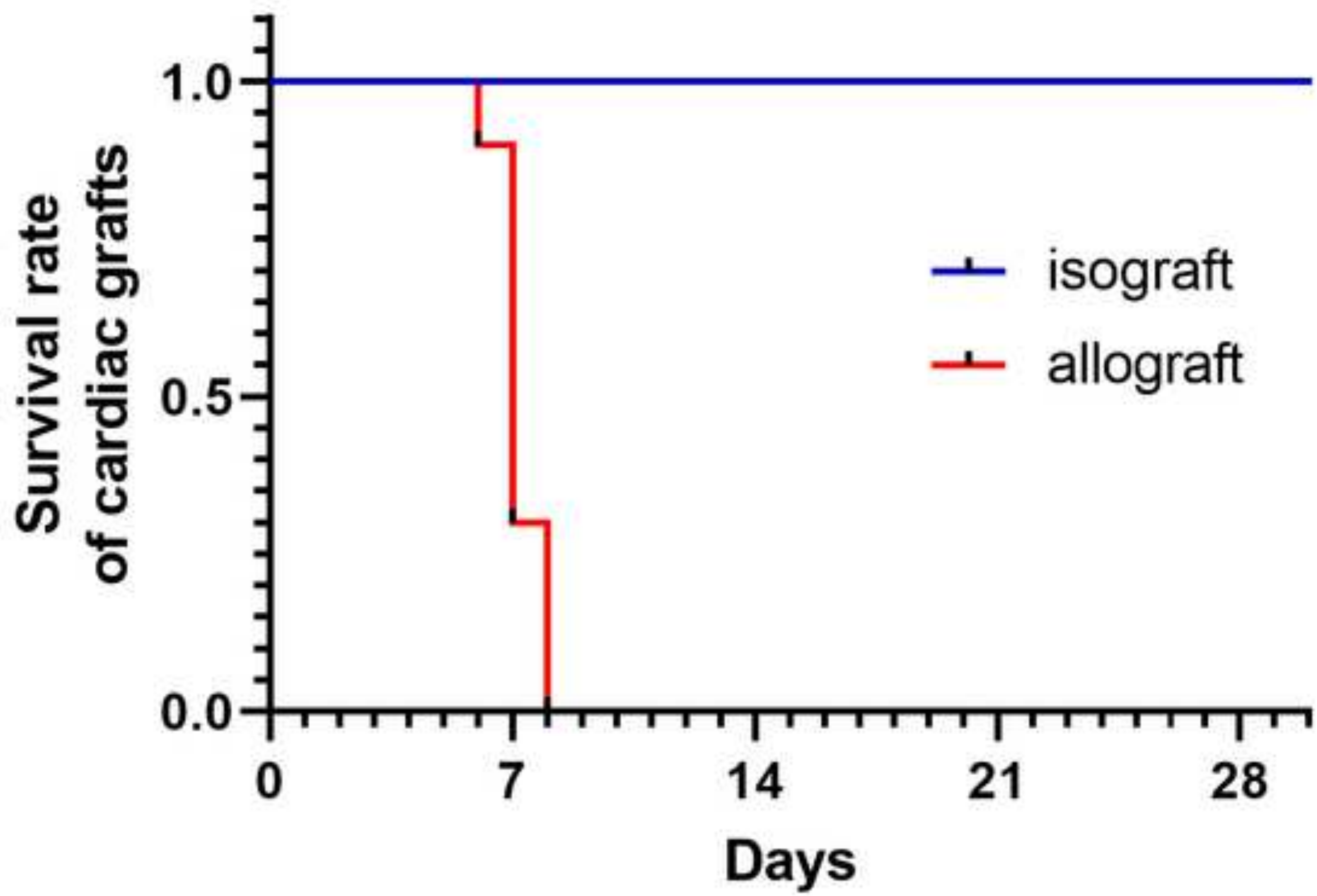

Number at risk

isograft arm	10	10	10	10	10
allograft arm	10	9	0	0	0

Anaesthesia
Right lobe of the submandibular gland
Right sternocleidomastoid
Cardiac perfusion
Cuff

Rupert Oberhuber et al.[10]
xylazine and ketamine
removal
removal
Retrograde perfusion with 4 °C HTK solution from the aortic arch
blunt end, with handle

Xin Mao et al (present work)
isoflurane (Safe, continuous and stable)
preservation (Reduce twirling of grafts)
preservation (Reduce twirling of grafts)
1. Anterograde perfusion with ice-cold physiological saline supplemented with 100 U/ml heparin solution from the suprahepatic vena cava. 2. Retrograde reperfusion with ice-cold HTK solution from the aortic arch. (Reduce coagulation and increase myocardial protection)
bevel end, with handle, barb and grooves on the surface (Facilitate eversion and fixation)

Click here to access/download
Table of Materials
63504_R2_Table of Materials.xlsx

Dear Editor,

We have revised the manuscript as required and address all the concerns. All the changes are highlighted by red in the revised manuscript. There is also a revision in the Table of Materials and all the revisions are marked by red.

It is highly appreciated if the manuscript can be accepted.

Best regards.

Sincerely yours,

Dr. Jun Li

Point-by-point Response

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Response: We have carefully proofread the manuscript again and the manuscript is edited by a native speaker to correct the spelling and grammar errors. We also have defined all abbreviations at first use in the revised manuscript. All the revisions are marked with yellow.

2. In your in-text citations, please remove the brackets around the superscripted numbers.

Response: We have removed the brackets around the superscripted numbers of in-text citation.

3. Line 81: what do you mean by "Allogeneic heart transplantation is performed between male C57BL/6 and BALB/c mice"?

Response: C57BL/6 and BALB/c mice are allogeneic. Allogeneic heart transplantation means that the heart from C57BL/6 donor mice is transplanted into BALB/c recipient mice.

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

Response: We have carefully edited and removed the personal pronouns in the revised manuscript.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc. For example, step 1.1: Induce general anesthesia in the mouse by 5% isoflurane inhalation using an induction chamber OR Use an induction chamber to induce general anesthesia in the mouse by 5% isoflurane inhalation.

Response: We have carefully edited the protocol using the imperative tense without phrases such as "could be," "should be," and "would be".

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. Please ensure the inclusion of specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: These concerns are important. We carefully check the protocol again and ensure that the protocol is detailed enough to produce the video which is easily to be replicated.

7. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep, and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

Response: We have edited format of the manuscript as the required.

8. Please make sure you include results to support your claim that your method is better than other methods in ensuring the survival of cardiac grafts.

Response: These concerns are important. We side-by-side compare the previous technique of Oberhuber et al with our technique in Table 1 to support our claim.

9. As we are a methods journal, please add the following to the Discussion:
10. As we are a methods journal, please add limitations of your technique to the Discussion.

Response: We fully understand the characteristic of JoVE. We have discussed the technique including critical steps in the protocol, modifications and troubleshooting of the method, the significance of the method with respect to existing methods and potential applications of the method in specific research areas. Especially, we add the limitations of the method in the discussion as below: "This cuff technique facilitates the anastomosis of donor and recipient vessels during implantation, however, the hardness of cuff may increase the twisting of anastomosed vessels and result in increase of thrombosis after transplantation. Optimization of the material for cuff could reduce the complications, increase the survival of graft and the utilization rate of the models in subsequent experiments. Furthermore, the fibrous scar of the cut may limit the space of cardiac graft and affect its long-term survival. In addition, ejection of the cardiac graft may interfere with the normal hemodynamics of recipient mice. At last, this model is non-functional and cannot be evaluated the cardiac function of graft, nevertheless, this model provides a reference to scientists who study heart transplantation immunity and pathology".

11. Please add all items (plasticware, glassware, buffers, solvents, equipment, software etc) in the Table of Materials so that it serves as a handy reference for users to get everything ready for the protocol. Please sort the Materials Table alphabetically by the name of the material.

Response: These concerns are important. We have added all the materials in the Table of Materials so that the technique can be easily replicated.

12. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

Response: We have edited the format of references as required.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors have described a technique for sutureless cervical hetertopic murine transplant. This is not a novel technique and has been well described in the literature. The manuscript suffers from several grammatical errors and requires extensive English language review.

Response: These concerns are important. We have carefully proofread the manuscript again and the manuscript is edited by a native speaker to correct the spelling and grammar errors.

Major Concerns:

The authors do not state how their technique is materially different than that reported by Oberhuber, or by Fukunaga et al (Transplantation 2018 and 2019).

Response: These concerns are important. We side-by-side compare the technique of Oberhuber et al. with our technique in Table 1.

The authors do not state how many mice underwent transplantation using this technique. Figure 3 needs to include the grafts at risk. What is the operative mortality of this *technique? We have achieved survival >95% with our cuff technique and any modifications would need to show similar or better survival.*

<u>Response</u>: We appreciate the outstanding outcome of the reviewer's technique, and these concerns are important. In our experiment, Ten recipient mice received heart transplantation from ten donor mice in each isogeneic and allogeneic group. We add the grafts at risk below the survive curve in revised Figure 2. The operative mortality of our technique is approximately 4.8% and the survival rate of recipient mice is approximate 95.2% with our cuff technique.

Reviewer #2:

Manuscript Summary:

The authors present a modified cuff technique to perform cervical heterotopic heart transplantation in mice. The modified cuff technique combines various features of other known cuffs with some modification to facilitate vessel eversion and anastomosis. The cuff has a barb, a bevel/slanted end, a handle, and grooves. By combining all these cuff features it should facilitate the anastomosis and reduce the ischemia time heart grafts. The manuscript is well organized and presented in a logical manner. However, throughout the text there are numerous grammatical errors. Also, some of the terminology needs editing. The authors present some "Representative results" and only provide percentages. It is difficult to assess the meaning of the percentages without reporting the animal numbers that were used per study groups.

Major Concerns:

Line 179, the legend of Figure 2 indicates that the "Data are expressed as the mean ± SEM of each group (n=8) from 3 separate experiments." The Kaplan-Meier survival analysis presented in Figure 2 only shows the number of animals per group that survive at each time point. This type of graph does not use mean values nor SEM error bars. Also, it is not clear what is meant by "from 3 separate experiments". Was the experiment repeated 3 times and the results presented an average of the three experiments? Or is the data a representative experiment from the three experiments performed? The authors should clarify the meaning of this sentence. Also, the authors should include in the Figure 2 legend, the number of recipients that were used in the isogeneic and allogeneic groups.

<u>Response</u>: We appreciate the comments of the reviewer. We are sorry to use the wrong statement in the legend of Figure 2. In our experiment, ten recipient mice received heart transplantation from eight donor mice in each isogeneic and allogeneic group. We have added the grafts at risk below the survive curve in revised Figure 2 and also corrected the wrong legend of Figure 2.

Minor Concerns:

1. In the "Recipient procedure" the authors should describe the creation of a "pocket" in the cervical area to accommodate the transplanted heart.

Response: Thanks for the reviewer's suggestion. We have added the procedure to create the fossa for cardiac graft in step 1.6.

2. Lines 140 and 141, this sentence is not clear, please clarify how the groves and suture were used to perform the anastomosis.

Response: These concerns are important. We produce a revised figure 1a to show the structure of the cuff and its usage in the anastomosis.

3. Lines 144 and 145, it is not clear what is meant by the term "noninvasive" in this sentence, please clarify.

Response: The "noninvasive" means that the vascular clamp has no injury to the vessels. We change the inappropriate "noninvasive" into "atraumatic" in the revised manuscript.

4. In the results section the percent survival rate of recipient mice is reported (line 156). The authors should include the number of procedures that were performed to arrive at this percentage.

Response: These concerns are important. We performed twenty-one heart transplantation in mice with our cuff technique and one recipient mice died because of post-operative bleeding. The survival rate of recipient mice is approximate 95.2% (20 survival / 21 total) with our cuff technique.

5. Figure 1 labels are difficult to read. The authors should use a larger font size.

Response: We have changed the labels in figure 1 using a larger font size.

6. The authors in the discussion section (lines 206 - 208) should reference relevant papers in rodent models of cervical heterotopic heart transplantation in which cuffs had a handle, a barb to reduce slippage of everted vessels, and a cuff with a slanted end (which authors refer to as a "bevel end") to facilitate vessel eversion. Other investigators have reported cuffs for quick vessel anastomosis that have some of these features.

Response: Thanks for the reviewer's suggestion. We have cited the 16th reference (Fensterer et al, *Comp Med* 2014; 64:293-299) in the Discussion section.

7. Some suggestions for surgical terminology:
Line 92, replace "response to the toe pinch" with "absence of pedal reflex"
Line 103, replace "disassociate" with "dissect"
Line 132, replace "removed by cutting off the superior" with "removed by dividing the

superior"
Lines 136 and 137, replace "Implant the donor heart into the cervical incision of the recipient mouse in an upside down position." with "Implant the donor heart into the cervical pocket of the recipient mouse in an inverted position."

<u>Response</u>: Thanks for the reviewer's suggestion. We have revised the surgical terminology as suggested.

Reviewer #3:
Manuscript Summary:
In this manuscript, Mao et al describe a novel modified cuff technique for heterotopic (cervical) mouse heart transplant that is based on the use of a barbed cuff with a bevel end and grooves. The authors provide a framework of previous work being done, and include important technical details on how to avoid common pitfalls with using their novel technique.

Major Concerns:
None

Minor Concerns:
The discussion could benefit from adding 1 paragraph discussing complications, limitations, surgical times and costs associated with the new technique. It would also be good if the authors can add a table comparing side-by-side previous techniques with their new technique.

<u>Response</u>: Thanks for the reviewer's suggestion. We discuss the complications and limitations in the last paragraph of the Discussion section. We also discuss that the modified barbed cuff with a bevel end and grooves shortens 20% of the surgical time. Because the bevel end of the modified cuff facilitates the oversleeve-like evert of vascular lumen and the grooves on the surface facilitate the fixation of everted vessel walls with cuff using sutures, and the barb outside cuff reduces the slippage of anastomosed vessel walls from cuff (Figure 1). Furthermore, the modified barbed cuff is produced from the most common polyurethane catheter of scalp acupuncture, thus significantly reduces the cost of the procedure.
We also side-by-side compare the technique of Oberhuber et al. with our technique in Table 1.