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To
The Editors/Reviewers of JOVE

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Submission of a manuscript

Dear Editors/Reviewers,

First of all, we want to thank you for the invitation to present our work.

Transplantation models in experimental animals, such as rats, are still an import tool in immunological research. In our manuscript, we describe a model of heterotopic abdominal heart transplantation in rats implying own modifications of current strategies, which lead to a simplified surgical approach. Additionally, we describe a novel rejection model by in-ear injection of vital cardiac muscle cells allowing further transplant immunological analyses in rats.

We think that our contribution might be of interest for researchers working in the field of transplantation immunology, especially since the introduction of commercial cloning technologies in rats will most likely lead to a recurring interest in experimental rat models, and hope that the topic is of interest for your Journal.

Best regards from Germany,

Oliver Beetz

TITLE:

An Immunological Model for Heterotopic Heart and Cardiac Muscle Cell Transplantation in Rats

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KEYWORDS:

heart transplantation; rat organ transplantation; cell transplantation; transplant model; rejection model; experimental microsurgery

SUMMARY:

We describe a model of heterotopic abdominal heart transplantation in rats, implying modifications of current strategies, which lead to a simplified surgical approach. Additionally, we describe a novel rejection model by in-ear injection of vital cardiac muscle cells, allowing further transplant immunological analyses in rats.

ABSTRACT:

Heterotopic heart transplantation in rats has been a commonly used model for diverse immunological studies for more than 50 years. Several modifications have been reported since the first description in 1964. After 30 years of performing heterotopic heart transplantation in rats, we have developed a simplified surgical approach, which can be easily taught and performed without further surgical training or background.

After dissection of the ascending aorta and the pulmonary artery and ligation of superior and inferior caval and pulmonary veins, the donor heart is harvested and subsequently perfused with ice-cold saline solution supplemented with heparin. After clamping and incising the recipient abdominal vessels, the donor ascending aorta and pulmonary artery are anastomosed to the recipient abdominal aorta and inferior vena cava, respectively, using continuous running sutures.

Depending on different donor-recipient combinations, this model allows analyses of either acute or chronic rejection of allografts. The immunological significance of this model is further enhanced by a novel approach of in-ear injection of vital cardiac muscle cells and subsequent analysis of draining cervical lymphatic tissue.

INTRODUCTION:

Heterotopic heart transplantation is a frequently used experimental model for different investigations regarding transplantation tolerance, acute and chronic allograft rejection, ischemia-reperfusion injury, machine perfusion or cardiac remodelling. Among other advantages, the graft function can be monitored noninvasively by palpation and graft failure does not lead to a vital impairment of the recipient in contrast to other organs, such as kidneys or livers.

In 1964, Abbott et al. initially described heterotopic abdominal heart transplantation in rats[1]. Later, in 1966, the end-to-side technique for anastomoses was described by Tomita et al.[2]. The groundwork for the currently used model was reported by Ono and Lindsey in 1969[3]. During the last decades, several modifications have been published to create different types of unloaded, partially loaded or loaded left ventricular heart grafts including combined heterotopic heart-lung transplantation[4–6]. For immunological analyses a non-volume loaded heart graft transplantation is most commonly performed. In this case, the blood flow retrogradely enters the donor ascending aorta and subsequently the coronary arteries. The venous drainage occurs along the coronary sinus into the right atrium and ventricle (**Figure 1A-B**). Therefore, the left ventricle is excluded from blood flow, apart from marginal amounts of blood from Thebesian veins. This also makes it a useful model for studying the pathophysiological mechanisms during left ventricular assist device therapy[7].

Heterotopic heart transplantation has been performed in various species including mice, rabbits, pigs and has even been used as a uni- or biventricular assist device in humans[8–11]. The rat still represents a popular experimental animal for transplant models, especially since the graft survival times for different rat strain combinations have been well-defined in the past and a large number of immunological reagents are accessible[12,13]. Unlike mice, rats are larger making surgery and access to lymphatic tissue for immunological analyses more feasible[12]. Furthermore, the introduction of commercial cloning technologies in rats in recent years will most likely lead to a recurring interest in experimental rat models[14].

In general, heterotopic heart grafts can be attached to the recipient vessels either by performing cervical or abdominal anastomosis. However, a few studies suggest that a femoral anastomosis facilitates improved monitoring due to better access for manual palpation or transfemoral echocardiography and thus allows a more precise detection of graft failure[15,16].

It has been shown that there is no difference regarding operation time, complication rate, outcome and graft survival time between both anastomosis techniques[17]. Clearly, the availability of a sufficient number of draining lymph nodes must be mentioned as a benefit of cervical anastomosis; however, longer training periods are required. In contrast, the abdominal

anastomosis is less complicated and equally valuable for immunological investigations, especially when combined with results from a novel method of in-ear injection of allogenic cardiac muscle cells and subsequent cervical lymphadenectomy. A combination of both models offers a broad spectrum of post-interventional immunological analyses.

The following protocol refers to operating in pairs of surgeons in order to reduce ischemia time. However, all experiments can be performed by a single person. The setup of instruments and materials for heart explantation and implantation is displayed in **Figure 2A,B**.

PROTOCOL:

All animal experiences have been performed according to the guidelines of the local Ethics Animal Review Board of the regional authorities for consumer protection and food safety of Lower Saxony (LAVES, Oldenburg, Germany) with the approval IDs 12/0768 and 17/2472.

1. Heart explantation and perfusion

NOTE: As graft donors, female or male rats at an age of 7-22 weeks were used.

1.1 Anesthetize the donor rat by isoflurane inhalation (induction at 5% and maintenance at 3% with an O_2 flow of 1 L/min). Inject 5 mg of Carprofen subcutaneously per kg of bodyweight for perioperative analgesia.

1.2 Remove the abdominal and thoracic fur using a mechanical clipper and sterilize the skin with 70% ethanol or another sufficient alternative.

1.3 Place the donor in a supine position, fix the limbs at the base of the operation table with an elastic band, and apply eye lubricant.

1.4 After checking for the absence of the toe pinch withdrawal reflex, perform a median laparotomy by using scissors.

1.5 Insert retractors, mobilize the intestine to the left of the donor, and expose the inferior vena cava with sterilized cotton swabs.

1.6 For anticoagulation, inject 500 I.U. of heparin dissolved in 1 mL of ice-cold isotonic saline solution intravenously by puncturing the inferior vena cava. Stop the bleeding at the puncture site by light compression with a cotton swab after retraction of the needle (**Figure 3A**).

1.7 Incise the diaphragm and perform lateral thoracotomy to both sides of the donor.

1.8 Pin the mobilized ventral wall of the thorax onto the operation table.

1.9 Remove the pericardium and the vagal nerve by blunt preparation using two micro-needle holders.

1.10 Perform transection of abdominal vessels in order to exsanguinate the donor and unload the heart.

1.11 Insert the blunt branch of a probe pointed scissors into the transvers pericardial sinus and separate the ascending aorta and pulmonary artery as distal as possible under light caudal traction of the heart with a wetted compress (**Figure 3B**).

1.12 Place a single 5-0 ligature around the superior and inferior vena cava and the pulmonary veins and tighten it as dorsal as possible (**Figure 3C**).

1.13 Sever the tissue dorsal to the ligature and extract the heart (**Figure 3D**).

1.14 Perfuse the explanted heart with an 18 G cannula from an intravenous catheter through the ascending aorta and the pulmonary artery with 30 mL of ice-cold, isotone saline solution supplemented with 1000 I.U. of heparin and place the heart in a 15 mL tube filled with saline solution on ice (**Figure 3E-F**).

2. Heart implantation

NOTE: As recipients, 10-14 weeks old female or male rats were used. Donors and recipients were approximately weight matched.

2.1 Perform anesthesia of the recipient rat by also using isoflurane inhalation (induction at 5% and maintenance at 1.5-2% with an O_2 flow of 1 L/min). Inject 5 mg of Carprofen subcutaneously per kg of bodyweight for perioperative analgesia.

2.2 Remove the abdominal fur, sterilize the skin, apply eye lubricant and fix the limbs analogously to the donor preparation. For optimal postoperative outcome, perform the operation on a heating mat to prevent intraoperative hypothermia.

2.3 After longitudinal incision of the skin, apply a local anesthetic, such as lidocaine (0.2%), on the abdominal fascia. Open the abdominal cavity by median laparotomy and insert retractors.

2.4 Mobilize the intestine to the upper left side of the recipient and place it in a warm, wetted compress.

2.5 Open the retroperitoneal space using the surgical microscope (or magnifying spectacles) with a 5-7x magnification and expose the abdominal aorta and inferior vena cava by blunt preparation with cotton swabs. Do not separate the abdominal vessels.

2.6 Elevate the abdominal vessels using two micro needle holders without injuring the lumbar veins and position the Cooley vascular clamp (**Figure 4A**).

2.7 Puncture the abdominal vessels with a 30-45° arched 27 G cannula (**Figure 4B**).

2.8 Enlarge the puncture site using Potts scissors to create a longitudinal incision that matches the size of the lumen of the donor vessels (**Figure 4C-D**) and perfuse the recipient vessels with saline solution in order to remove clots and prevent postoperative thrombosis.

2.9 Place the graft into the situs and fixate the donor ascending aorta to the recipient abdominal aorta by two simple interrupted stitches (8-0 monofilament non-resorbable suture) at the cranial and caudal corner of the longitudinal incision (**Figure 4E**).

2.10 Anastomose the ascending aorta of the donor with the abdominal aorta of the recipient by a running 8-0 monofilament suture in two steps: first, place the graft to the right of the recipient vessels and perform the first half of the anastomosis (**Figure 4E**). Subsequently, place the graft to the left of the recipient vessels and perform the second half of the anastomosis (**Figure 4F**).

2.11 Fixate the donor pulmonary artery to the inferior vena cava analogously to the aortal anastomosis (8-0 monofilament non-resorbable suture). Suture the first half of the venous anastomosis from the intraluminal side of the vessel (**Figure 4G,H**).

2.12 Flush the anastomoses with saline directly before tightening the knots to prevent peripheral embolism.

2.13 Place a hemostatic gauze around both anastomoses and carefully release the Cooley vascular clamp so that the reperfusion of the graft can begin. Handle bleeding along the anastomoses by light compression with sterilized cotton swabs.

NOTE: The graft should start beating after around 60 s.

2.14 Replace the intestine in a meander like fashion. Make sure that there are no malrotations of the mesenteric radix to prevent intestinal necrosis or mechanical obstruction.

2.15 Close the abdominal muscles/fascia and skin separately using continuous 3-0 polyfilament running sutures.

3. Postoperative care

3.1 For postoperative analgesia, supply the recipients with an additional subcutaneous injection of 5 mg of Carprofen per kg of bodyweight on the first postoperative day (POD). Additionally, add 1 g of Metamizol to 500 mL of drinking water until the third POD.

3.2 Start monitoring the heart graft function by daily abdominal palpation on the third POD.

NOTE: In case of graft failure before the third POD, a surgical rather than an immunological failure should be considered. However, this of course depends on the chosen strain combination and the respective immunological model (e.g., hyperacute rejection after prior immunization).

3.3 Following graft rejection, extract tissues like the draining retroperitoneal lymph nodes cranial of the anastomoses, the spleen, the blood, the thymus and the graft for further immunological analyses via flow cytometry or immunohistochemistry.

4. Enzymatic digestion of the heart and subcutaneous injection of heart cells in the ear

4.1 Perform heart explantation and perfusion analogously to heterotopic heart transplantation (see step 1).

4.2 Shred the heart in 3 mm x 3 mm blocks using sterile scissors and incubate it for 30 min at 37 °C in culture medium containing 0.5 mg/mL collagenase.

NOTE: It is important to use culture medium containing penicillin, streptomycin and glutamine without fetal calf serum (FCS) particularly as FCS inhibits collagenase digestion.

4.3 Mince the digested tissue through a large-pored sieve to get a suspension of vital cardiac muscle cells, mostly dead single heart cells and remaining blood cells.

4.4 Filter the suspension using a 40 µm cell strainer and collect the vital cell congeries by flushing the cell strainer with 5-10 mL of isotonic saline solution.

4.5 Draw 1 x 10^5 cardiac muscle cells dissolved in 200 µL of saline solution up into a 1 mL syringe.

4.6 Perform anesthesia analogously to the protocol described for the recipient narcosis (see step 2) for heterotopic heart transplantation.

4.7 Place the recipient in a lateral position and fix the ear with a finger using double sided tape (**Figure 5A**).

4.8 Inject 20 µL of the cardiac muscle cell solution (containing 1 x 10^4 cells) via a 27 G cannula *s.c.* close to the visual capillary vessels into the recipient's ear (**Figure 5B**).

4.9 After a defined observation period (depending on the chosen strain combination and strength of rejection), extract the draining cervical lymph nodes and perform further analyses such as flow cytometry or co-cultures (**Figure 5C**).

NOTE: Furthermore, histological analysis of the pinna can be performed to determine cell infiltration.

REPRESENTATIVE RESULTS:

In the past, different immunological issues have been addressed on the basis of the model, which was validated in the work group by more than 500 transplants with a survival rate of more than 95%[13,18–24]. Total operating times (including graft explantation and implantation) usually did not exceed 60 minutes, whereas combined cold and warm ischemia times were around 30 minutes. The strain combinations applied were mainly based on Lewis (Lew) background. Syngenic transplants survived up to 100 days without signs of graft failure, but significant weight and size reduction upon graft explantation. Most recently, we performed heterotopic heart transplantation in two different donor-recipient combinations simulating a fast and a prolonged rejection model: Lew.1a $\rightarrow$ Lew wt leading to fast rejection (mean survival time of 7.4 days) and Lew.1u-7B $\rightarrow$ Lew.1a leading to a more prolonged rejection (mean survival time of 42.5 days) (**Figure 6**). Macroscopically, the rejected grafts showed a thrombosis accompanied by a livid discoloration and swelling, whereas non-rejected grafts show distinct atrophy, most likely as a consequence of an unloaded left ventricle. Furthermore, we drew up cryostat sections of transplanted hearts in order to detect cell infiltration using an alkaline phosphatase-antialkaline phosphatase (APAAP) staining method. Single frames with a 50x magnification were merged to one composite image, giving an overview of the complete graft and the distribution of infiltrating cells. Histological analysis showed an increased infiltration of (e.g., $CD4^+$, TCR^+, or NKR-P1A/B^+) immune cells in the allogenic grafts, whereas syngenic grafts were largely free of cell infiltration (**Figure 7A,C**).

Cervical lymphadenectomy and re-stimulation assays of draining lymph node cells after in ear injection of cardiac muscle cells in the above mentioned strain combinations revealed distinct strain-specific immune responses towards allogenic cardiac tissue and allowed further immunological analyses, such as cytokine profiling (**Figure 8A-C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic presentation of the end-to-side anastomoses and the resulting blood flow through the heart. After anastomosing the donor ascending aorta end-to-side to the recipient abdominal aorta and analogously the pulmonary artery to the recipient inferior vena cava (**A**), blood flow enters the coronary arteries via the ascending aorta. The venous drainage occurs via the sinus coronarius into the right atrium and ventricle and through the pulmonary artery into the recipient inferior vena cava (**B**).

Figure 2: Required surgical instruments and materials. (**A**) Explantation: 1: elastic limb band, 2: retractors, 3: 5-0 ligature, 4: probe pointed scissors, 5-6: micro needle holders, 7: scissors, 8: surgical forceps, 9: micro forceps, 10: eye lubricant, 11: compresses, 12: cotton swabs, 13: perfusion base, 14: saline solution on ice. (**B**) Implantation: 1: elastic limb band, 2: retractors, 5-6: micro needle holders, 7: scissors, 8: forceps, 9: micro forceps, 10: eye lubricant, 11: compresses, 12: cotton swabs, 15: micro scissors, 16: micro forceps, 17: Potts scissors, 18: needle holder, 19: arched cannula, 20: Cooley vascular clamp, 21: 8-0 monofilament sutures,

22: hemostatic gauze, 23: 3-0 polyfilament sutures, 24: Petri dish, 25: Carprofen (5 mg/mL), 26: local anesthetic (lidocaine 0.2%), 27: saline solution

Figure 3: Heart explantation. After heparinization (**A**), thoracotomy is performed and ascending aorta and pulmonary artery are severed as distal as possible (**B**). With one single ligature pulmonary and both caval veins are occluded (**C**) and the heart is removed from the thoracic cavity (**D**). (**E**) shows the heart before and (**F**) after perfusion with 30 mL saline solution containing heparin.

Figure 4: Heart implantation. After exposition of abdominal vessels and placement of a Cooley vascular clamp (**A**) the vessels are cannulated (**B**) and a longitudinal incision is performed using Potts scissors (**C-D**). The donor ascending aorta is fixated with one knot each at the cranial and caudal corner of the incision of the recipient abdominal aorta (**E**) and anastomosis is performed by continuously running sutures (**E-F**). Note that the graft is placed on the right side of the vessels for the first half of the anastomosis (**E**) and onto the left side of the vessels for the second half of the anastomosis (**F**) and the subsequent venous anastomosis. The first half of the venous anastomosis is performed by an intraluminal suture (**G**). After finishing the second half of the venous anastomosis, the graft is ready for reperfusion (**H**).

Figure 5: In-ear injection of allogenic cardiac muscle cells. After fixing the recipient's ear on a finger using double-sided tape (**A**) allogenic vital cardiac muscle cells are injected subcutaneously close to visual capillary vessels (**B**). After an observation period draining cervical lymph nodes (*****) are extracted (**C**).

Figure 6: Heart survival in different syngenic and allogenic donor-recipient combinations. Kaplan-Meier analysis shows the survival of syngenic (n=10 fast rejection model; n=5 prolonged rejection model) and allogenic grafts (n=11 fast rejection model; n=14 prolonged rejection model). In the prolonged rejection model six of 14 recipients reached the end of the observation period (60 days) without graft failure, leading to a prolonged graft survival in this group.

Figure 7: Histological analysis of syngenic and allogenic heart grafts. (**A,B**) show the infiltration of $CD4^+$ cells using the APAAP staining method in a syngenic graft (**A**) and an allogenic graft upon rejection (**B**). (**C**) presents the increase of cell infiltration in allogenic grafts when compared to syngenic grafts (serving as reference group) for different immune cell subsets. The classification applied to quantify cell infiltration was modified from Hirschburger et al.[25]: 0 = infiltration comparable to syngenic grafts; 0.5 = slight increase of stained cells in isolated tissue sections; 1 = increase of singular stained cells over the whole tissue section; 1.5 = increase of stained cell clusters uniformly distributed over the whole tissue section; 2 = strong; 2.5 = very strong; 3 = strongest increase of stained cell clusters throughout the whole tissue section. The histological sections of the grafts were analyzed using a 50x magnification. Five grafts per group (syngenic and allogenic, respectively) were included in the analysis.

Figure 8: Analysis of draining lymph node cells after in-ear injection of allogenic cardiac

muscle cells. Specific re-stimulation (with 2 x 10^5 splenocytes of the respective donor strain) of 2 x 10^5 lymphocytes harvested from either draining cervical or mesenteric lymph nodes (LN) of fast rejecting Lew wt and prolonged rejecting Lew.1a recipients showed significantly reduced proliferation of draining lymph node cells in Lew.1a recipients (**A**), whereas proliferative capacity in general was still observable after unspecific stimulation with CD3/CD28 antibody (**B**). Surprisingly, cytokine profiling revealed an increase of inflammatory cytokines in the lymph nodes of prolonged rejecting recipients (**C**). Results are presented as mean ± SEM of at least 4 animals per group. Significance is indicated with * for p-values ≤ 0.05 and **** for p-values ≤ 0.0001. (This figure has been modified from Beetz et al.[24]).

DISCUSSION:

The previously described method of heterotopic cardiac transplantation in rats is mainly based on the description of Ono and Lindsey in 1969[3]. Since then, several modifications have been introduced in various species leading to a wide diversity of this model. Combining several of these modifications and introducing our own experience resulting from over 30 years of performing heterotopic heart transplants in the laboratory, we created a feasible surgical approach, which does not require long training periods or surgical background. In the following, we will discuss general limitations of this model and underline critical steps of the protocol. Furthermore, we will emphasize the benefits of combining heterotopic heart transplantation with a novel method of in-ear injection of cardiac muscle cells.

Anesthesia and general complications

Although it has been reported that isoflurane anesthesia is superior to injection anesthesia regarding early survival after heterotopic heart transplantation, intraoperative respiratory depression still represents one of the most common complications and thus requires a careful narcosis management[26]. Instead of serial graft explantation and implantation, respectively, we advise beginning the preparation of the recipient and the abdominal vessels immediately after thoracotomy of the donor animal, particularly because operation times of less than one hour are associated with a better outcome regarding graft and recipient survival[26,27]. Besides already mentioned complications such as hypothermia due to long operating times and missing heating mats, and intestinal necrosis or obstruction by unphysiological placement of the intestine, a paresis of hinder limbs represents a further complication, which can be prevented by atraumatic vascular clamping and thorough flushing of the anastomoses to avoid peripheral embolism[28,29].

Ligature of pulmonary and caval veins and bleeding complications

In order to reduce warm ischemia time, we use one single ligature for both caval and all four pulmonary veins. As a possible complication resulting from a too proximal/ventral placement of the ligature, the disruption of venous backflow by occlusion of the sinus coronarius has to be mentioned. In case of severe bleeding after removal of the Cooley clamp and visible beating of the graft, the ligature has to be checked for insufficiency immediately. We observed this type of complication especially if the dissection of the pulmonary veins was performed too close to the ligature while removing the graft from the thoracic cavity. Otherwise, severe bleeding is mainly caused by insufficiency of the vascular anastomoses. Additionally, a coronary artery running

along the ascending aorta which is often severed during explantation is described to cause lethal bleeding upon reperfusion[27].

Ischemia time and perfusion

Regardless of the transplantation model, it is always indispensable to reduce ischemia time, especially as the heart is considered as a vulnerable organ regarding ischemia damage. By performing surgery with two surgeons, we are able to achieve a minimum warm and cold ischemic time and therefore forgo the usage of cardioplegic solutions in order to reduce ischemia-reperfusion damage[30]. Generally, the perfusion of the graft plays a key role and is essential to cool the graft and remove blood cells, which could result in thrombosis or embolism. Whereas low perfusion pressures lead to insufficient perfusion and thus to an incomplete removal of blood cells, high perfusion pressures can cause endothelial damage[31,32]. We advise perfusion of the pulmonal artery as well as the ascending aorta until the coronary arteries are visibly flushed clear.

Incision of recipient vessels

A critical step in the protocol constitutes the incision of the recipient vessels without causing damage to the vessels posterior wall: Schmid et al. described the benefit of aortotomy or venotomy performing a small transverse incision followed by longitudinal enlargement in cranial and caudal direction, which further leads to a decreased stenosis rate of aortal anastomosis[33]. In the model, the recipient vessels are punctured using a small cannula. Afterwards, the puncture site is enlarged by using Potts scissors to create a longitudinal incision. For a better feasibility, we recommend bending the tip of the cannula to an angle of 30-45° leading to a decreased risk of damaging the vessels posterior wall. We did not observe clinically relevant vascular stenoses in any of our recipients. Similar benefits of opening recipient vessels by puncturing the abdominal aorta and the inferior vena cava with a cannula have been described by Shan et al.[34].

Mastering the model of heterotopic heart transplantation

During the last decade, the model has been performed by researchers in the department with little or no surgical background. As stated above, we operate in pairs of surgeons, whereas the more experienced researcher is in charge of guaranteeing the success of the transplantation and at the same of gradually improving the skill set of the unexperienced researcher. After a short training period of approximately ten graft explantations and implantations in dead animals, the unexperienced researcher is in charge of performing the heart explantation and assisting the graft implantation in approximately ten live animals. Subsequently, the unexperienced researcher actively performs the vascular anastomoses, so that after approximately ten further transplantations, the former unexperienced researcher is usually capable of performing all critical steps of the model.

Applying this training concept, past publications from our department using heterotopic heart transplantation in rats showed no differences regarding morbidity, mortality or graft function despite several different teams of surgeons[13,18–24].

Of note, performing the vascular anastomoses represents the most critical step in this protocol and solid organ transplant models in general. We, therefore, recommend prolonged training periods using dead animals until anastomoses are performed accurate and fast, especially if an experienced researcher is not available for guidance in live animals.

General advantages and disadvantages of the model

Whereas spontaneous tolerance induction is often described as a phenomenon in liver transplantation and also observed in kidney transplantation, the heart is considered as a rather immunogenic organ and thus enables reliable rejection in transplant models[35,36]. Contrary to these findings, we could also notice long-term survival and absence of rejection after heterotopic heart transplantation in certain donor-recipient combinations despite complete major histocompatibility complex disparity.

An often-mentioned criticism of heterotopic heart transplantation is the subjectivity of graft monitoring by manual palpation. Therefore, the model has been extended to femoral anastomosis techniques in order to facilitate the access for palpation and introduce further monitoring techniques such as transfemoral echocardiography[15,16]. On the other hand, Mottram et al. demonstrated that the monitoring of the graft via palpation correlates well with electrocardiographic measurements[37]. Thus, manual palpation in heterotopic heart transplants seems sufficient for monitoring graft function in an acute rejection model.

As a consequence of heterotopic placement and left ventricular unloading, the heart does not function under anatomical or physiological conditions assuming that this does not impact immunological analyses. Contrary to this assumption, it had been shown that cardiac remodeling resulting from left ventricular unloading during left ventricular assist device therapy leads to a decreased cytokine release[38,39]. On the other hand, Tang-Quan et al. described the unloaded setting as a more appropriate approach for immunological analysis, since long-term ischemic damage of the graft resulting from perfusion with partially deoxygenated blood in the left ventricular loaded model was observed[40].

Although abdominal placement of the grafts offers surgical benefits in terms of practicability, it is difficult to harvest sufficient numbers of draining retroperitoneal lymph nodes upon graft rejection impairing further analyses. For this reason, we introduced a novel method of in-ear injection of allogenic cardiac muscle cells. Originating from parasitological research, this concept has not been applied for immunological analyses in transplantation, despite its feasibility[41,42]. The advantage of this model is the possibility of identifying and harvesting a significant number of draining lymph nodes, which offers the possibility of performing complex immunological analyses. Of note, both models could be combined in one recipient offering further insights into the mechanisms of rejection and tolerance in cell and organ transplantation in rats.

Our models of rat heart and cardiac muscle cell transplantation represent a practicable and well-studied approach and can be performed without further surgical training or background. Facing the fact that new cloning technologies for rats have been introduced and developed

recently, these models offer vast opportunities for transplant immunological researchers.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

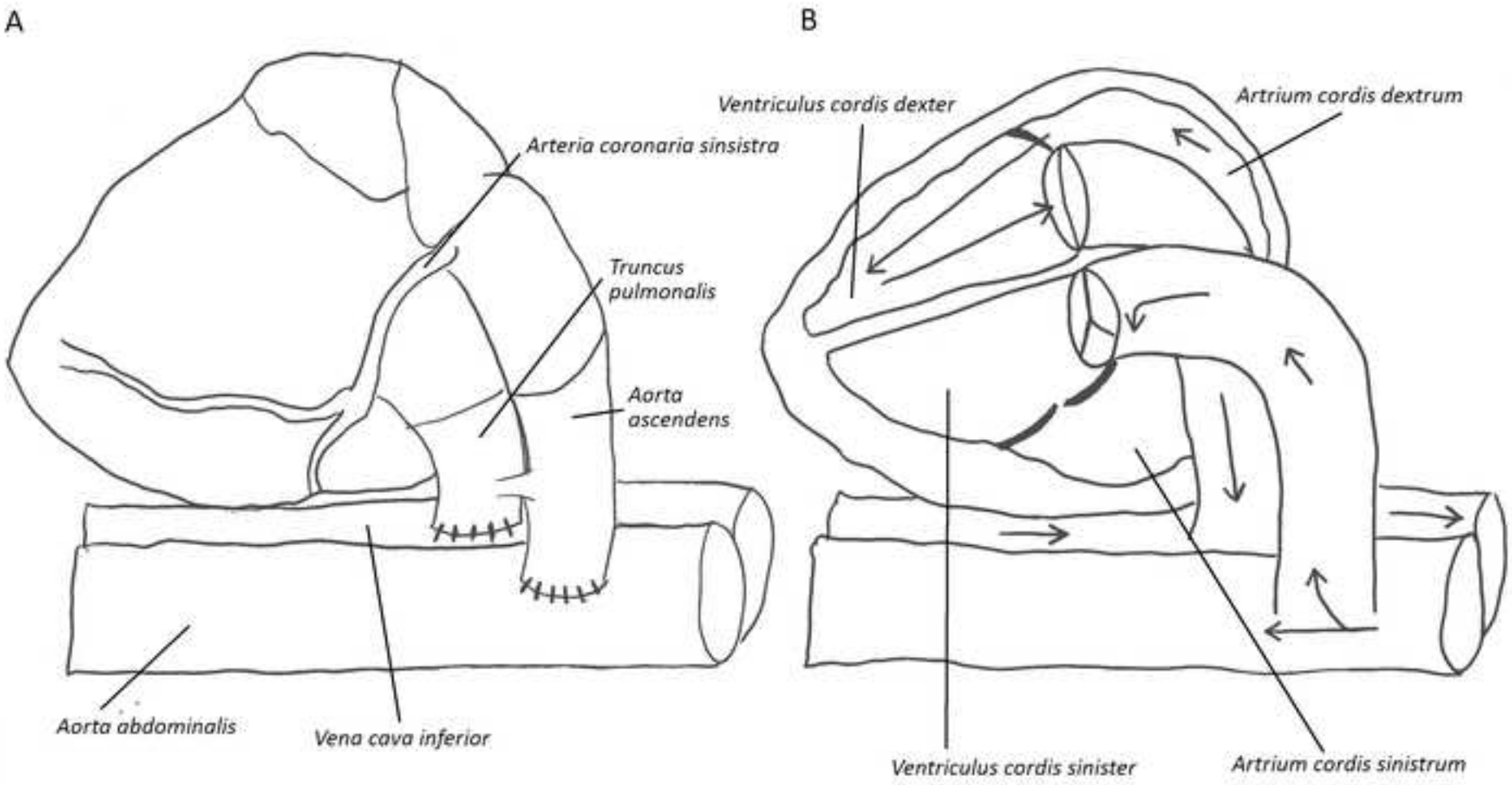

Figure 2

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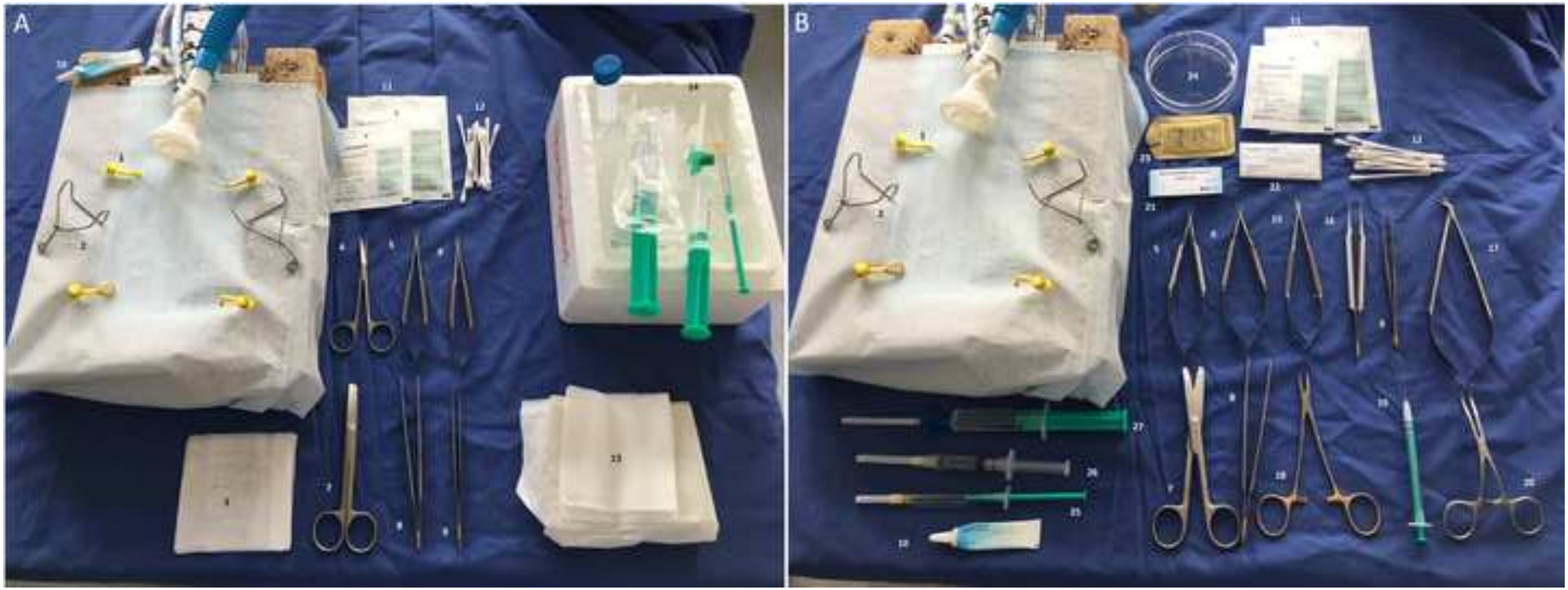

Figure 3

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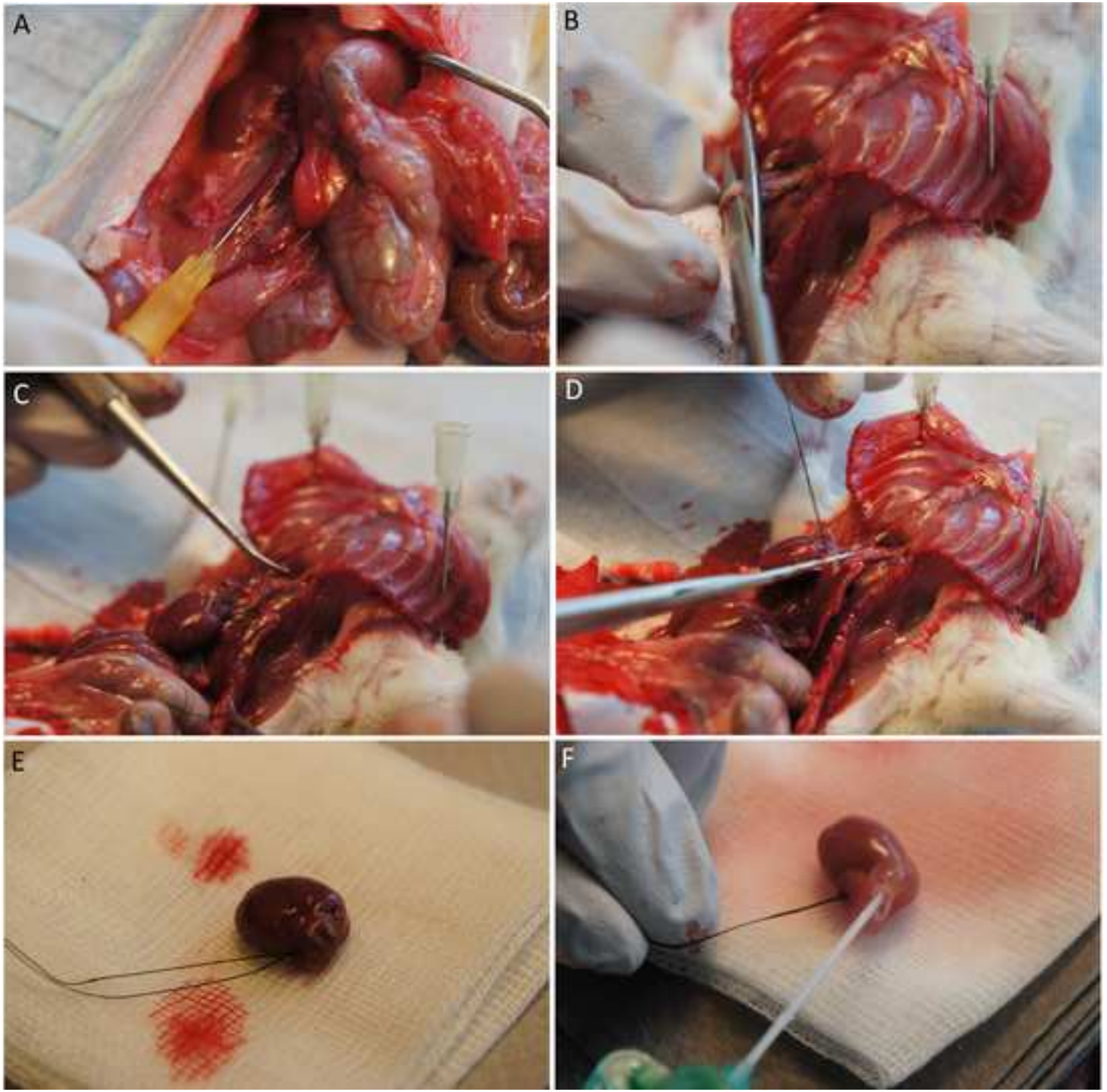

Figure 4

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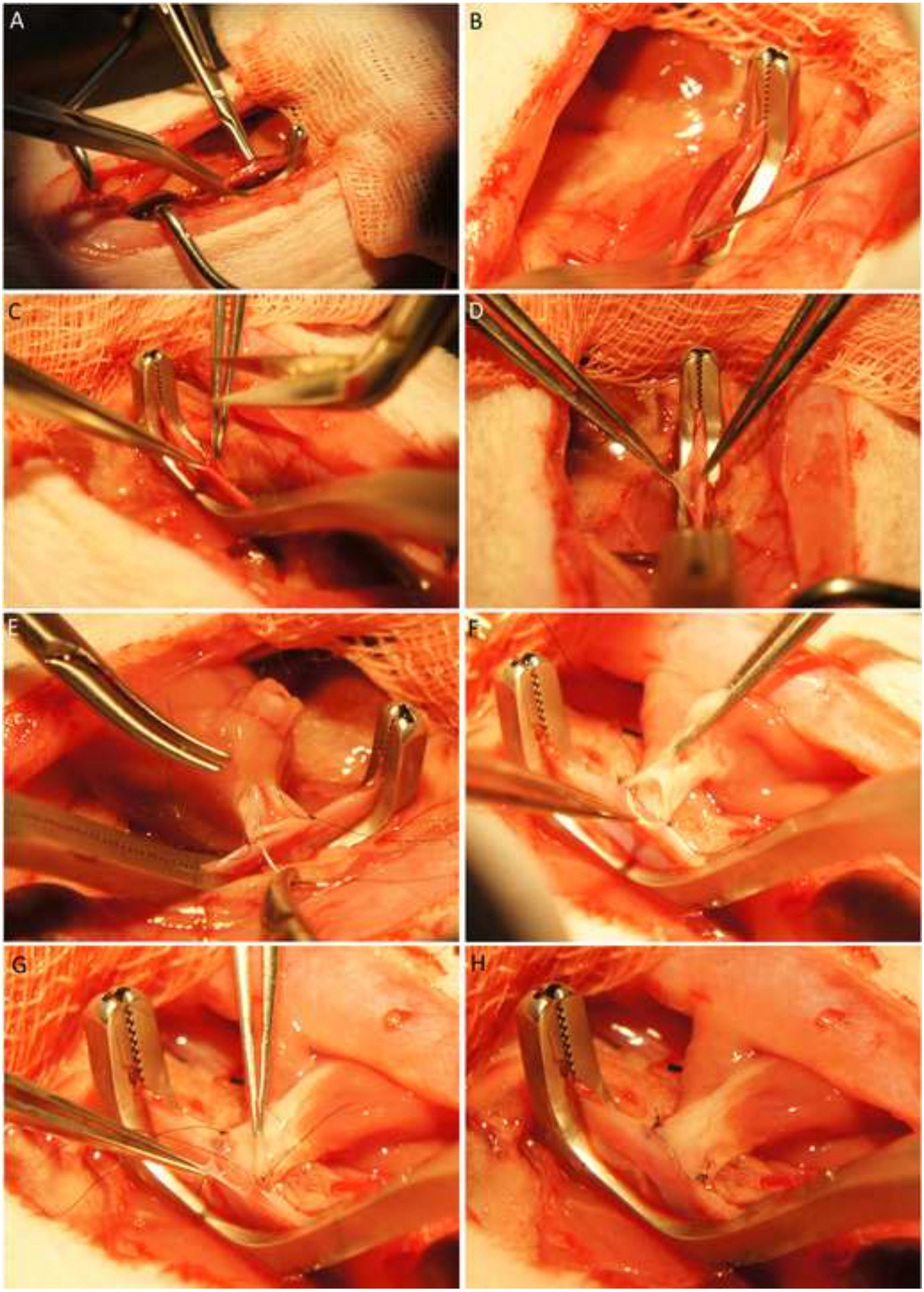

Figure 5

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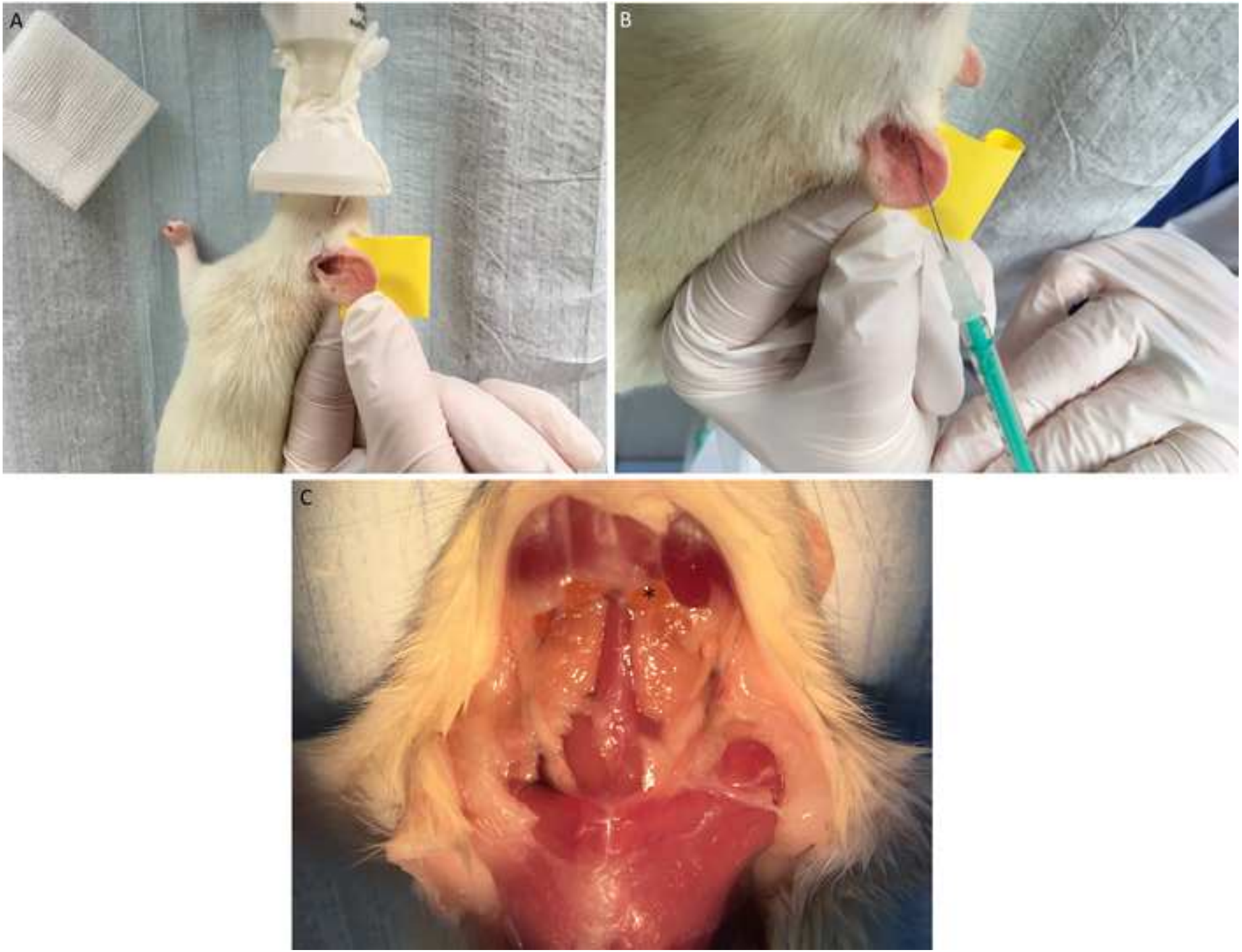

Figure 6

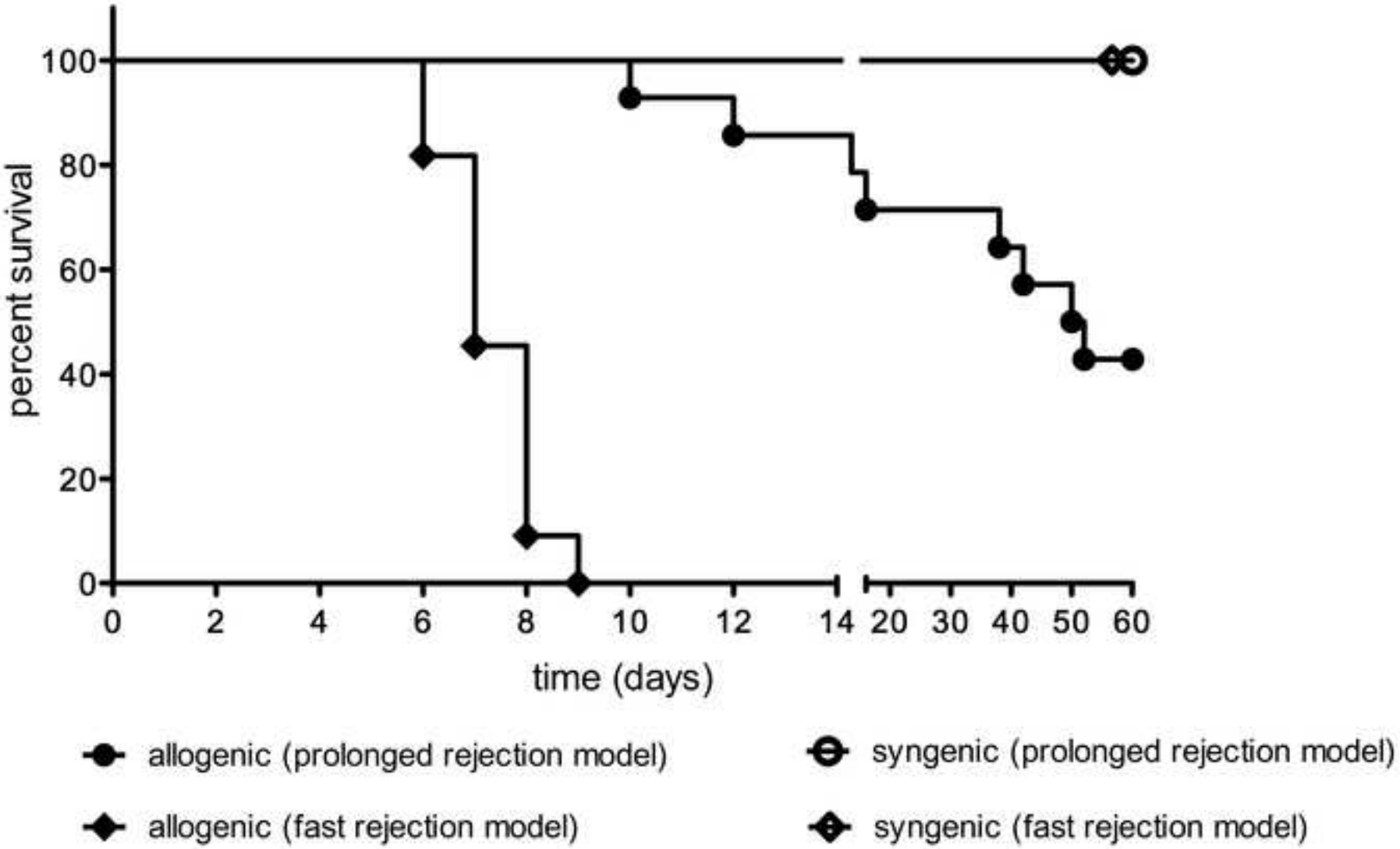

Figure 7

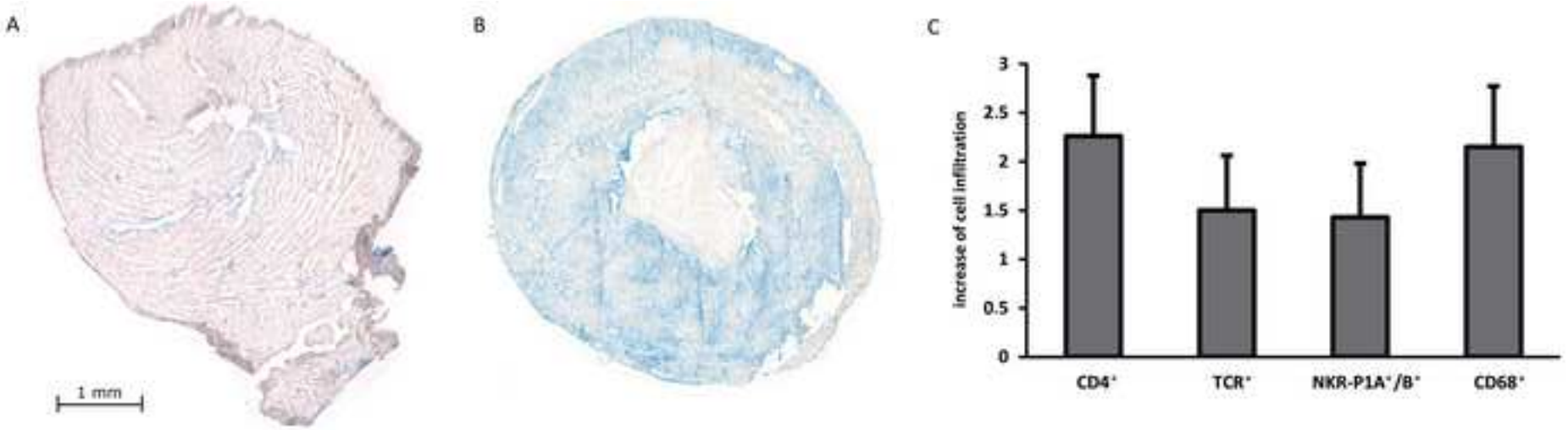

Figure 8

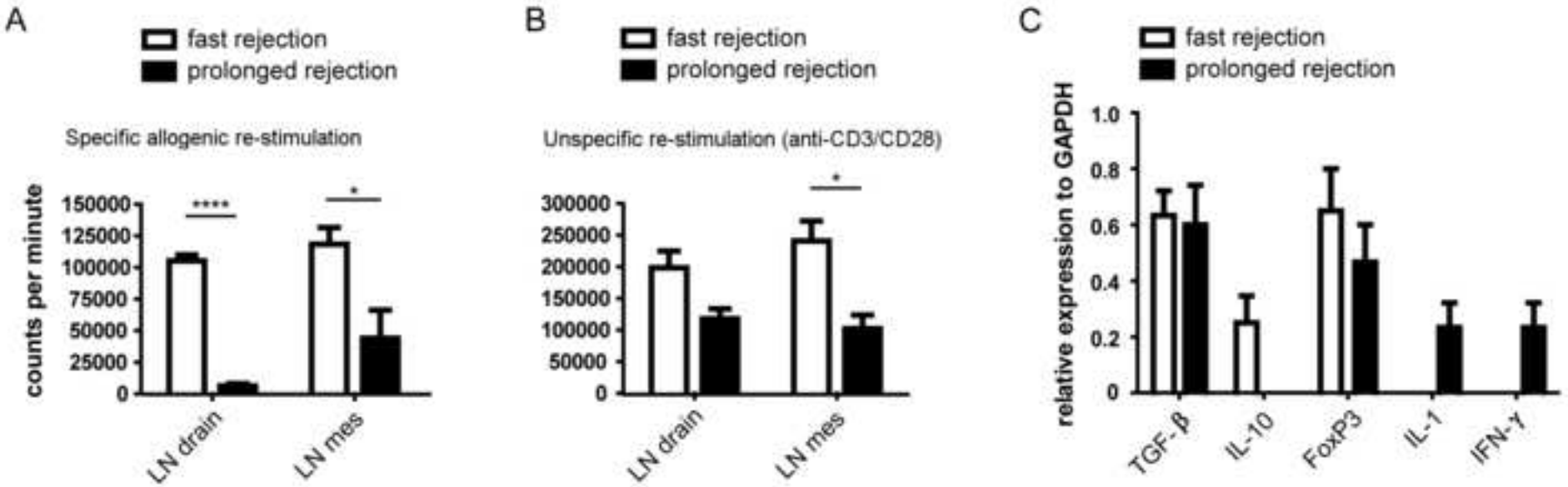

Table of Materials

Click here to access/download;Table of Materials;Table of Materials.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Anesthesia device (including isoflurane vaporizer)	Summit Anesthesia Solutions	No Catalog Number available	
Cannula (27 G)	BD Microlance	302200	
Carprofen	Pfizer	Rimadyl 50 mg/mL	
Cellstar Tubes (15 mL)	GreinerBioOne	188271	
Cell strainer (40 µm)	BD Falcon	2271680	
Collagenase Type CLSII	Biochrome	C2-22	
Compresses 5x5 cm	Fuhrmann	31501	
Compresses 7.5x7.5 cm	Fuhrmann	31505	
Cotton swabs	Heinz Herenz Medizinalbedarf	1032128	
Dexpathenol (5 %)	Bayer	"Bepanthen"	
DPBS BioWhittaker	Lonza	17-512F	
Forceps	B. Braun	Aesculap BD557R	
Forceps	B. Braun	Aesculap BD313R	
Forceps	B. Braun	Aesculap BD35	
Heating mat	Gaymar Industries	"T/Pump"	
Hemostatic gauze	Ethicon	Tabotamp	
Heparin-Natrium 25 000 I.E.	Ratiopharm	No Catalog Number available	
Isofluran CP	CP-Pharma	No Catalog Number available	
Large-pored sieve (stainless steel)	Forschungswerkstätten Hannover Medical School	No Catalog Number available	
Lidocaine	Astra Zeneca	2 % Xylocain	
Metamizol-Natrium	Ratiopharma	Novaminsulfon 500 mg/mL	
Micro forceps	B. Braun	Aesculap BD3361	
Micro needle holder	Codman, Johnson & Johnson Medical	Codmann 80-2003	
Micro needle holder	B. Braun	Aesculap BD336R	
Micro needle holder	B. Braun	Aesculap FD241R	
Micro scissors	B. Braun	Aesculap FD101R	
Micro scissors	B. Braun	Aesculap FM471R	
Needle holder	B. Braun	Aesculap BM221R	
Penicillin/Streptomycin/Glutamine (100x)	PAA	P11-010	
Peripheral venous catheter (18 G)	B. Braun	4268334B	
Peripheral venous catheter (22 G)	B. Braun	4268091B	

Probe pointed scissors	B. Braun	Aesculap BC030R
Retractors	Forschungswerkstätten Hannover Medical School	No Catalog Number available
RPMI culture medium	Lonza	BE12-702F
Saline solution (NaCl 0.9 %)	Baxter	No Catalog Number available
Scissors	B. Braun	Aesculap BC414
Surgical microscope	Carl-Zeiss	OPMI-MDM
Sutures (anastomoses)	Catgut	Mariderm 8-0 monofil
Sutures (ligature)	Resorba	Silk 5-0 polyfil
Sutures (skin, fascia)	Ethicon	Mersilene 3-0
Syringe (1 mL)	B. Braun	9166017V
Syringe (10 mL)	B. Braun	4606108V
Syringe (20 mL)	B. Braun	4606205V
Vascular clamp	B. Braun	Aesculap FB708R

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To
The Editors/Reviewers of JOVE

December the 1st, 2019

Letter of Response regarding manuscript
"Heterotopic heart and cardiac muscle cell transplantation in rats – a simplified and a novel immunological model" (JoVE60956)

Dear Editor, dear Reviewers,

First of all, thank you for the fast review and the advice regarding our manuscript. We appreciate the possibility of re-submitting a revised and improved manuscript.
In the following sections we provide point-by-point replies to all issues raised in the review.
You will receive two revised versions of the manuscript (with and without track-change-function) with this letter as well as the edited figures and table.
We feel that the manuscript has profited considerably from the questions raised by the editor and the reviewers and the implemented changes and hope to meet your approval on the updated version.

If new information given in our replies – not yet integrated into the revised manuscript – should be added to the final version of the manuscript, we would be happy to comply.

If you need any further information, please let us know.

Best regards from Germany,

O. Beetz

Oliver Beetz

Point-by-point replies (*Page references refer to the clean version of the revised manuscript)

Editor:

1. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

Response:

All figures were transformed to a .psd format in a resolution of 300 DPI. The table containing the material list was converted to a .xlsx file.

2. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response:

The complete manuscript was reread by the authors to ensure no spelling or grammar issues.

3. Please include at least 6 keywords of phrases.

Response:

We provided six updated keyword phrases containing two or more words.

4. 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.

Response:

The formatting of the manuscript was implemented as requested.

5. 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 and Reagents.
For example: Axiovision Release 4.8.2.0.

Response:

Commercial language was removed or exchanged by generic terms.

6. 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."

7. Please ensure you answer the "how" question, i.e., how is the step performed?

Response to 6+7:

We reassessed the manuscript and addressed the above mentioned points.

8. 1.1., 2.1: Age sex strain of the animal used.

Response:

Information regarding the age and sex of the donors (Note above 1.1) and the recipients (Note above 2.1) have been added. The respective rat strain used depended on the chosen strain combinations and rejection model. We provided further information on the matter in the section "REPRESENTATIVE RESULTS".

9. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Response:

We have ensured that we do not exceed the 2.75 page limit for filmable content. We apologize for the inconvenience.

10. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Response:

Since PLOS ONE works under an Open Access license, the copyright permission for previous publications is part of the editorial policy if the content is cited appropriately (see also https://journals.plos.org/plosone/s/licenses-and-copyright). We have provided the link to the PLOS ONE Licenses and Copyright section in an additional .docx file which we will upload separately. If you would like us to obtain an additional letter from the editor, we are of course happy to comply.

11. Please do not abbreviate the journal titles in the references section.

Response:

We have removed abbreviations of journal titles by applying the "JOVE Reference Output Style" to our bibliography in the "REFERENCES" section via Mendeley Software.

12. Please sort the materials table in alphabetical order.

Response*:*

The materials table has been alphabetically ordered.

Reviewer #1:

-

Reviewer #2:

1. The author thinks their models represent a practicable and well-studied approach and can be performed without further surgical training or background. However, we all know anastomose is very difficult for a freshman. So, we need more data to prove the models is easy to learn.

Response:

This is indeed a valid point; we would therefore like to provide some general remarks concerning the learnability of the model (which were also partially included into the "DISCUSSION" section): During the last decade our model has been performed by medical or PhD students in our department with no surgical experience to begin with. Since it is a general policy of our work group, we operate in pairs of surgeons, whereas the more experienced "surgeon"

is in charge of guaranteeing the success of the transplantation and at the same of gradually improving the skill set of the "freshman".
After a short training period of approximately ten graft explantations and implantations in dead animals, the "freshman" is in charge of harvesting the graft and assisting the graft implantation in approximately ten live animals. Subsequently, the "freshman" actively performs the vascular anastomoses, so that after approximately ten further transplantations, the "freshman" is usually capable of performing all critical steps of the model.
Applying this training concept, past publications from our department using heterotopic heart transplantation in rats showed no differences regarding morbidity, mortality or graft function despite several different teams of surgeons caused by a high turnover of work group members [1–7].
We agree with Reviewer #2, that performing vascular anastomoses is indeed the critical step of solid organ transplantation models in general, however, the presented modifications of our heterotopic heart transplantation model simplify the surgical steps that have to be undertaken before performing the vascular anastomoses and allow less room for critical errors.
Unfortunately, we cannot present statistical data on learning curves to measure the feasibility of our model, since our training concept for mastering the heterotopic heart transplantation represents a gradual and guided process, as stated above, impeding the collection of reliable and comparable statistical data.
Regardless of this fact, statistical data reflecting the degree of difficulty of surgical models in general, especially for transplantat models, are rarely presented in the current literature, making it difficult to compare the degree of feasibility of different models.
If it is necessary to mitigate the statements regarding the feasibility of our model due to missing statistical data, we would of course comply. Nonetheless, we hope that our newly included general statements on the matter ("Mastering the model of heterotopic heart transplantation", page 11, lines 435-452) provide relevant information when assessing the learnability and feasibility of our model.

2. The paper concluded histological analysis showed an increased infiltration of (e.g. TCR +, CD4 + or NKR-P1A/B +) cells in the allogenic grafts, whereas syngenic grafts were largely free of cell infiltration (Figure 6.A-B). We need more data to prove the results.

Response:

We agree that the presented figure (6.A-B) displaying two exemplary histological sections of a syngenic and an allogenic heart graft are not sufficient to statistically prove an increase of cell infiltration in allogenic grafts. We therefore provided further data on the matter and modified Figure 6 by adding a further figure (Figure 6.C) displaying the increase of cell infiltration in allogenic grafts by $CD4^+$ (T helper cells, monocytes), TCR^+ (T cells), NKR-$P1A/B^+$ (Monocytes/macrophages, NK cells, NKT cells) and $CD68^+$ (Monocytes/macrophages) cells by blinded evaluation of sections from five grafts of each group (syngenic reference group and allogenic group, respectively). The classification system used to quantify cell infiltration is described in the edited legend of Figure 6 (page 8-9, lines 344-352).

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6. Timrott, K. *et al.* Application of allogeneic bone marrow cells in view of residual alloreactivity: Sirolimus but not cyclosporine evolves tolerogenic properties. *PLoS ONE*. **10** (4), 1–16, doi: 10.1371/journal.pone.0119950 (2015).
7. Beetz, O. *et al.* Recipient natural killer cells alter the course of rejection of allogeneic heart grafts in rats. *Plos One*. **14** (8), e0220546, doi: 10.1371/journal.pone.0220546 (2019).

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