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ORIGINAL ARTICLE

An alternative model of composite tissue allotransplantation: groin–thigh flap

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Summary

This study focuses on development of a simpler and nonfunctional model that includes all the same tissue components as the traditional hind limb allotransplantation in rats. Adult male inbred Wistar rats (WF, $RT1^u$) weighing 250–300 g were used as syngeneic ($n = 12$) donors and recipients of a new experimental model for composite tissue allotransplantation. In the allogenic group ($n = 4$), adult male Brown Norway rats (BN, $RT1^n$) weighing 200–250 g were used as donors. A groin–thigh osteo-myocutaneous flap, composed of skin (groin), muscle (thigh), and bone (2/3 femur), based on the femoral vessels and superficial epigastric vessels, was developed for composite tissue allotransplantation. All the flaps were successful except for two dying soon postoperatively. Histology confirmed vessel patency in the syngeneic group and acute rejection in the allogenic group. The total operative time was shortened compared with the standard and other modified models of rat hind limb allotransplantation. Advantages of this new model include its simplicity, relative purity, and the more humanistic fact that it does not cause claudication to the animals as does standard orthotopic hind limb transplantation, or extra-deformity to the recipients as does the heterotopic hind limb model.

Introduction

Recently performed clinical face allotransplantations have evoked widespread interest and revolutionized the field of surgery [1], and the majority of surgeons agree that the ultimate aim of composite tissue allotransplantations is to serve as ideal restorations for tissue defects after tumor ablation, traumatic loss or inherited impairment.

Although the immune systems of rats and humans have absolute differences, such as the lack of expression of class II antigens in the endothelial cells [2–4], most experiments on composite tissue allotransplantation to date have been performed on rats. Rats offer many advantages, including simpler breeding, lower costs, and ease in manipulation. The orthotopic hind limb of the rat is thought of as the standard model [5,6]. However, it might result in great suffering to the recipient rats, and mortality rates as reported in the literatures are in the range of 20% [7–11].

As Ulusal *et al.* [12] have stated, in standard orthotopic hind limb allotransplantations, intramedullary fixation with a metallic stent has resulted in much bleeding, an increased risk of fat embolus and a higher risk of infection, which are contributing factors for the high morbidity and mortality rates. They also thought that anastomosis failure due to mismatch of the osteotomy levels and rigid fixation is common, especially when

discrepancies of femoral bone length exist. Therefore, different modified models with a combination of various composite tissues have been designed as research tools [13–16].

Our purpose was to design an effective rat model, which could help investigate, in immunologic research, about composite tissue allotransplantation without inducing undue suffering to the experimental animals. The simpler, nonfunctional rat model comprising all components needed would offer a new means of studying transplantation immunity. Furthermore, experimental animals would experience less stress and mortality, because there would be no sacrifice of normal ambulation. The purpose of this study was to develop a new, reliable, less traumatic model for rat composite tissue transplantation.

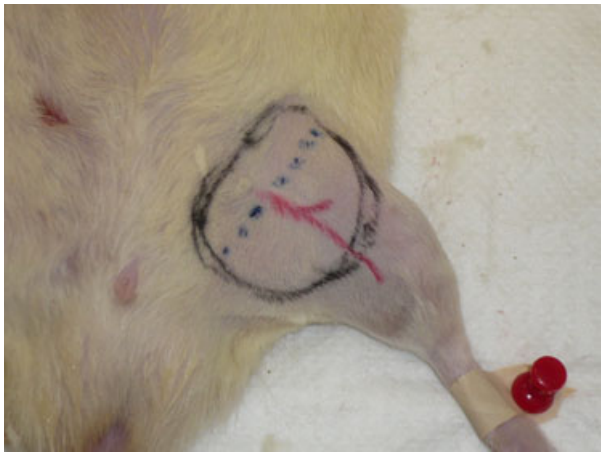

Figure 1 Flap design: dot line indicates inguinal ligament, and solid line shows vascular pedicle.

Materials and methods

All procedures were performed using Wistar (WF, $RT1^u$) and Brown Norway rats (BN, $RT1^n$) obtained from the National Science Council, and approved by the Institutional Animal Care and Use Committee of Kaohsiung Medical University.

Experimental groups

Sixteen syngeneic ($n = 12$) and allogenic ($n = 4$) groin–thigh osteo-myocutaneous flaps were performed in two groups. For syngeneic transplants, we used male Wistar rats (WF, $RT1^u$) weighing 250–300 g as donors and recipients. For the allogenic group, male Brown Norway rats (BN, $RT1^n$) weighing 200–250 g were used as donors and male Wistar rats (WF, $RT1^u$) weighing 250–300 g were used as recipients. All experimental rats were fed and housed at Kaohsiung Medical University, following the abovementioned guidelines. Two teams worked simultaneously for timesaving, with one to harvest donor flaps and the other to prepare recipients. Following transplantation, the recipients were returned to their independent cages and cared for intensively for 24 h postoperatively. They were sacrificed on day 7 (syngeneic group) or when signs of rejection were noted (usually 3–5 days for the allogenic group). The transplants, irrespective of whether they were viable or rejected, were sent for histologic examination.

Donor surgery

All donor animals were anesthetized with 50 mg/kg of sodium pentobarbital by intraperitoneal injection prior to surgery and maintained with 5 mg of sodium pentobarbital in each subsequent shot, if needed. The midline abdomen, left groin, and medial thigh of the donor rat were shaved, and the skin was prepped. The groin cutaneous flap (Fig. 1) was designed in a circular shape along the lateral aspect of the thigh from the knee to the groin, based on the superficial epigastric artery and vein. Kinking of pedicles was avoided as much as possible. The femoral vessels were mobilized individually from the knee to the inguinal ligament. The deep branch that supplies the knee joint was ligated and divided, and the popliteal artery further ligated distally in the popliteal fossa. At this level, the thigh muscle cuffs were harvested along the femoral vessels, preserving the feeding artery to the femur bone. The bones were divided with a small mechanical saw at the thinnest section of the distal femur bone and proximal femur neck, taking care not to traumatize the vascular pedicle. Fat tissue was put into the osteotomized wound to prevent hematoma formation, if bleeding developed. Larger branches were ligated with 9-0 suture and divided, and smaller branches were cauterized with bipolar forceps. The vessels remained connected to the donor to keep the flap perfused in case the recipient was not yet fully prepared. When the recipient was completely prepared for transplantation, the donor's femoral vessels were severed very close to the inguinal ring, with the cutting of the inguinal ligament. It was unnecessary to dilate the femoral vessels by gentle hydrostatic pressure or to drain any blood remaining in the flap. After flap harvests, the donor animal was immediately euthanized by CO_2.

Recipient surgery

Preparation of the recipient vessels was the same as all donor transplants. The left groin was shaved circularly, and the skin was prepped. A horizontal skin incision along the inguinal ligament was made. The femoral vessels were exposed, and the artery and vein were

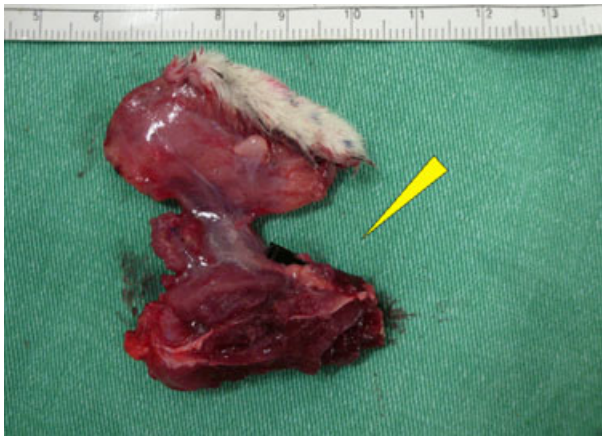

Figure 2 Harvested flap with vascular pedicle clamped (arrow).

individually mobilized just distal to the superficial epigastric vessels and proximally up to the inguinal ligament. Usually one or two anterior branches of the femoral artery were seen distal to the inguinal ligament. These branches could be ligated, unlike those of the recipient rats. Microvascular clamps were used for proximal control of the recipient vessels and distal ligation with the suitable size of vessel diameter was carried out simultaneously. In such circumstances, the femoral nerve was usually kept intact. At this time, the groin–thigh flap (Fig. 2) was brought into the field and simply placed in the inguinal area without bone fixation, with either the artery or vein anastomosed first (Fig. 3). In our series, an interrupted anastomosis in an end-to-end fashion was first performed on veins, using 10-0 interrupted nylon sutures. Usually 10–12 interrupted sutures were required for vein anastomosis.

The following arterial anastomosis was also performed in an end-to-end fashion using 10-0 nylon. However, 6–8 interrupted sutures were usually sufficient to secure vessel patency. Direct intra-luminal dilatation of the vessels was avoided because their size was already adequate and to prevent possible injury to the endothelial wall. Once anastomosis of the vessel was completed, the arterial clamp was released, and venous filling was noted in the donor vein. The proximal venous clamp was then released, followed by the distal venous clamp. At this time, the venous blood was passing promptly through the anastomosis into the recipient vein. Anastomotic bleeding was controlled by the application of a small piece of wet gauze and light pressure, or by one or two additional stitches-whenever the bleeding was more significant. After stabilizing vessel patency, the groin–thigh flap was sutured to approximate the recipient defect with 4-0 absorbable stitches.

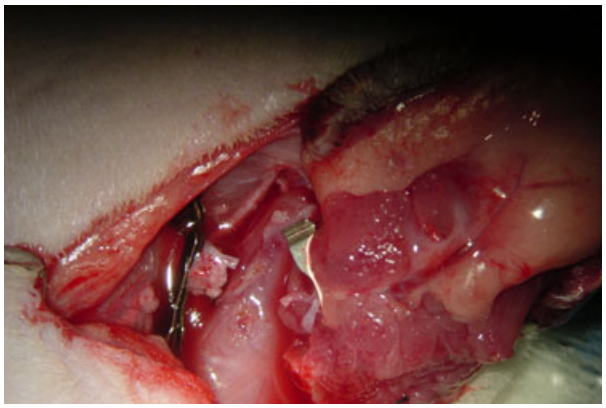

Figure 3 Flap inset *in situ* and end-to-end anastomosis is performed.

Results

Operative time

The total operative time ranged from 55 to 100 min, which consisted of a harvesting time of 30–65 min (mean, 47 min) and a warm ischemia time of 20–50 min (mean, 32 min) as shown in Table 1. There were no signs of wound infection or severance distress postoperatively. The recipient rats showed no signs of claudication and started to use their hind limbs to stand up for drinking.

Gross observations

This study consisted of 16 transplantation procedures. Among 16 recipients, two rats died postoperatively. The mortality rate was 12.5% (two of 16), and the overall success rate was 87.5% (14 of 16). The transplanted tissues were examined daily for signs of rejection such as erythema, edema, desquamation, hair loss, epidermolysis, exudation, or skin necrosis.

Recipients in Group I (syngeneic) survived over the experimental period and appeared viable for 7 days after transplantation, at which time all animals were sacrificed and the transplants studied histologically. None of the syngeneic group demonstrated rejection in the grafted skin. The wound between the graft and recipient healed well, and the skin on both sides could not be distinguished except for the existence of stitches and hairy skin or otherwise (Fig. 4). Animals in Group II (allogenic) showed signs of acute rejection after a mean period of 3 days, with the first appearance of skin edema, sloughing and even ulceration as have been reported in previous models [17–20].

Histologic examinations

Tissues were fixed overnight with 4% paraformaldehyde in phosphate buffered saline, dehydrated, embedded in

Table 1. Various models of composite tissue allotransplantation.

No.	Author, year	Model	Total operative time (minutes)	Harvesting time (minutes)	Warm ischemia time (minutes)
1	Liao, 2001 [37]	Osteo-myocutaneous transplantation model	145.3 ± 9.1	49.7 ± 5.8	65.4 ± 6.0
2	Yeh, 2000 [28]	Whole functional limb transplantation	4.5–6.5 h	*	75–90
3	Nazzal *et al.*, 2004 [27]	Heterotopic limb	90–120	30–45	45–60
4	Ulusal *et al.*, 2005 [12]	Heterotopic hind limb	60	*	35
5	Ulusal *et al.*, 2005 [12]	Orthotopic hind limb	105	*	85
6	Chang, 2007	Groin–thigh flap	80 (55–100)	30–65 (47)	20–50 (32)

Total operative time can be equal to the sum of donor harvesting time plus recipient operative time.
Donor harvesting time includes shaving, skin preparation, and donor graft detachment.
Warm ischemia time is the time from the detachment of the donor graft to re-attachment of the graft to the recipient.
Recipient operative time comprises the shaving, skin preparation, graft insertion, microvascular anastomosis, and skin closure.
*Not shown in authors' paper.

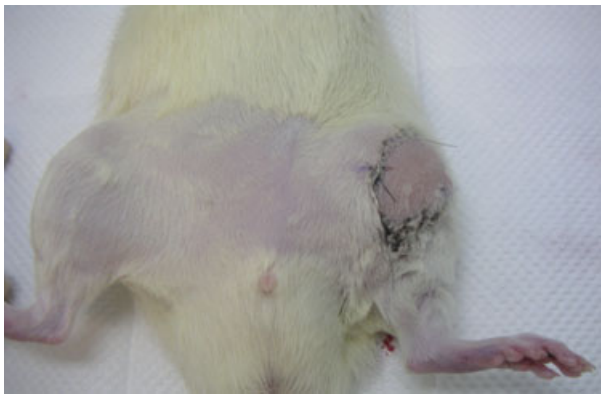

Figure 4 Seven days post-transplant.

paraffin, sectioned (6–8 μm), and stained with hematoxylin-eosin. The criteria used for evidence of rejection have been described in detail for the rat model [18,19,21,22]. Histologic examination of the isogeneic transplants revealed the normal appearance of the viable tissue (Fig. 5). Examination of the allogeneic transplants demonstrated diffuse lymphoid infiltration, microvascular thrombosis, and necrosis and was consistent with acute rejection (Fig. 6).

Discussion

In the current phase of research, the immunologic issues concerning rejection are the most challenging in the field of composite tissue allotransplantation, although functional result may be the final expectation [23–25]. Currently, the major obstacle to the progress of modern composite tissue allotransplantation is the need for developing new immunologic strategies for promoting transplantation tolerance. Unlike solid-organ transplantation, composite tissue allotransplantation consists of relatively larger amounts of highly antigenic tissues, such as skin and bone marrow [26]. This property requires higher levels of immunosuppressive agents than traditional solid-organ transplantation to prevent allograft rejection [18], so the recipient's morbidity and mortality rates may increase in such circumstances [27]. Therefore, theoretically, the experimental animal model needs to be designed to be simpler, taking shorter operative time and inducing as little morbidity and mortality as possible.

Although traditional orthotopic hind limb transplantation is the most standard model in composite-tissue allotransplantation studies as described by Doi [17], it is the nearly whole-limb transplants that involve extensive and time-consuming reattachments of vessels, muscles, nerves, and bone components. Because of the large amount of tissue replacement and nerve injury, claudication and other major morbidities are frequently encountered [28], and the animal invariably drags the transplanted limb postoperatively, which induces ulceration and frequently leads to self-mutilation. Even in the heterotopic model, the transplanted limb should somehow be protected as the animals still think of it as external attachment and it may also be autophagic.

Ideally, the animal model should be designed more simply, involving shorter time and less effort. At the same time, the recipients must demonstrate a decrease in morbidity and mortality. Varieties of studies on nonfunctional composite tissue transplantation have been developed; however, some were relatively inadequately humanized. A number of studies on nonfunctional heterotopic limb transplantation removed most of the skin portion and placed the transplant in subcutaneous tunnels [29–32]. In the paper by Nazzal *et al.* [27], the heterotopic limb model included limb tissue from between 5 mm above the knee joint (mid-femur) to the level of the ankle joint. There seemed to be a great deal of bulking, even though authors had removed the entire gastrocnemius muscle

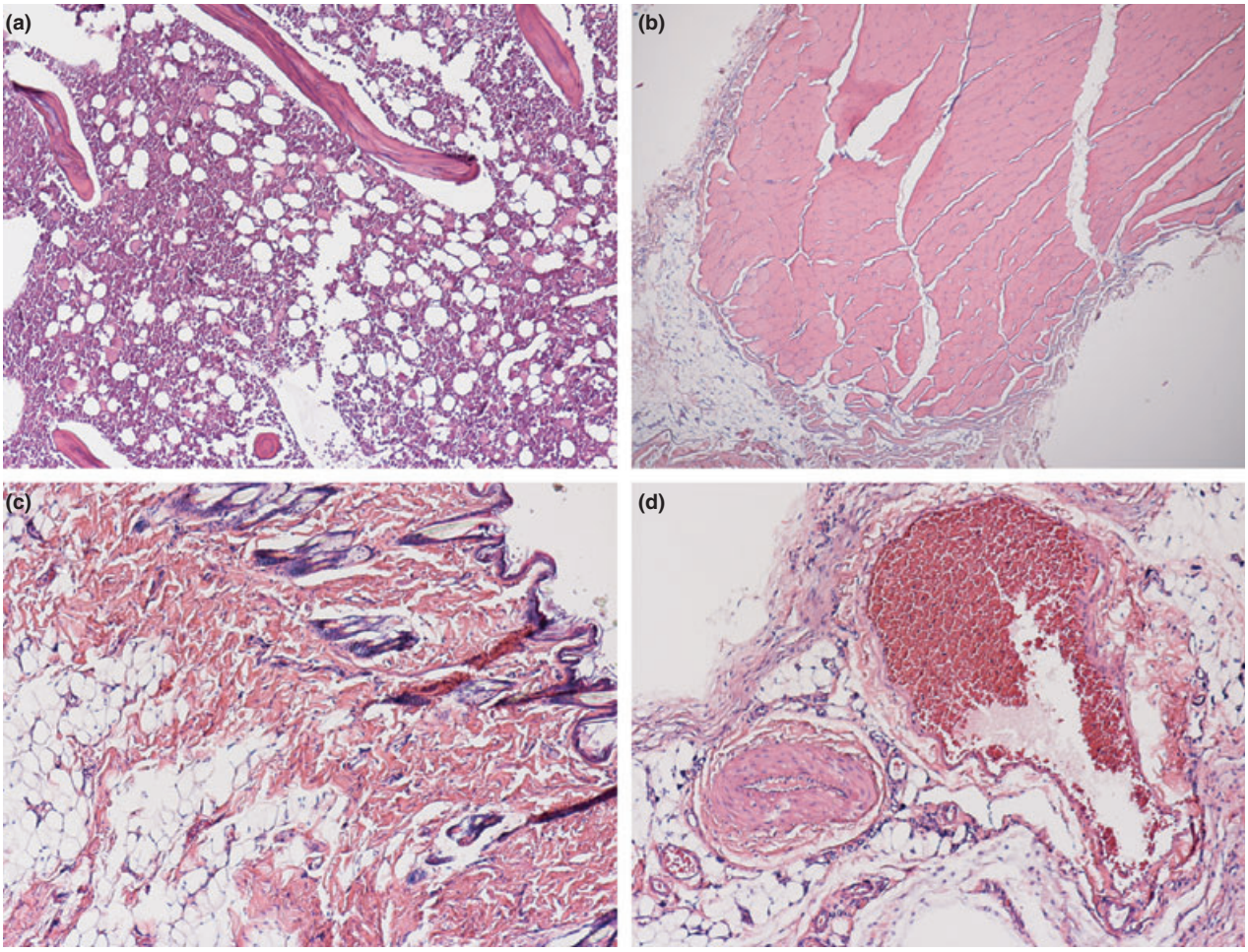

Figure 5 The specimen of bone marrow, muscle, skin and blood vessel reveal unremarkable changes except for minimal inflammation.

(this bulking could result in giving rats greater incentives to self-mutilate). On the other hand, they concluded that the inclusion of a skin island on the composite allotransplant has a negative effect on total graft survival. Although the size of the skin paddle could not be the only factor influencing the immune response, the skin is the most potent antigenic source. The immune reaction in composite tissue allotransplantation involves complicated interactions originating from various tissues, including skin, muscle, and bone marrow. In our groin–thigh flap model, an epigastric skin flap was able to serve as the immunologic effector organ, and the 3/4 femur bone offered the origin of bone marrow stem cells. Therefore, the groin–thigh flap added the least additional burden to the recipients and preserved an epigastric skin flap capable of serving as the immunologic effector organ.

The bone marrow inside the transplanted hind limb was shown to have an impact on prolongation of allograft survival through mixed chimerism [33,34]. It has been proposed that the percentage of donor (allogeneic) cells present within the chimera is critical for allograft acceptance to occur [25]. However, intramedullary fixation may insult the experimental animals. In our model, the change is less traumatic and a more fixed amount of the femur is preserved; the bone marrow cavity is less disturbed, so the total antigen load can be more constant. Ulusal *et al.* [12] stated that different osteotomy levels and insults during intramedullary fixation might alter the quantity of bone marrow. However, we believe that the rat hind limb model cannot be considered analogous with clinical human hand transplantation because of the percentage of bone marrow inside the graft. The bone marrow in a hind limb is equal to near-total femur plus tibia-fibula bone, whereas the clinical human hand transplant contains relatively small amounts of bone marrow in proportion to the whole body. Therefore, a standard hind limb allotransplantation cannot reasonably represent the classical hand allotransplantation in humans. Our model differs from other previous composite tissue allotransplantation models in this aspect, because our method

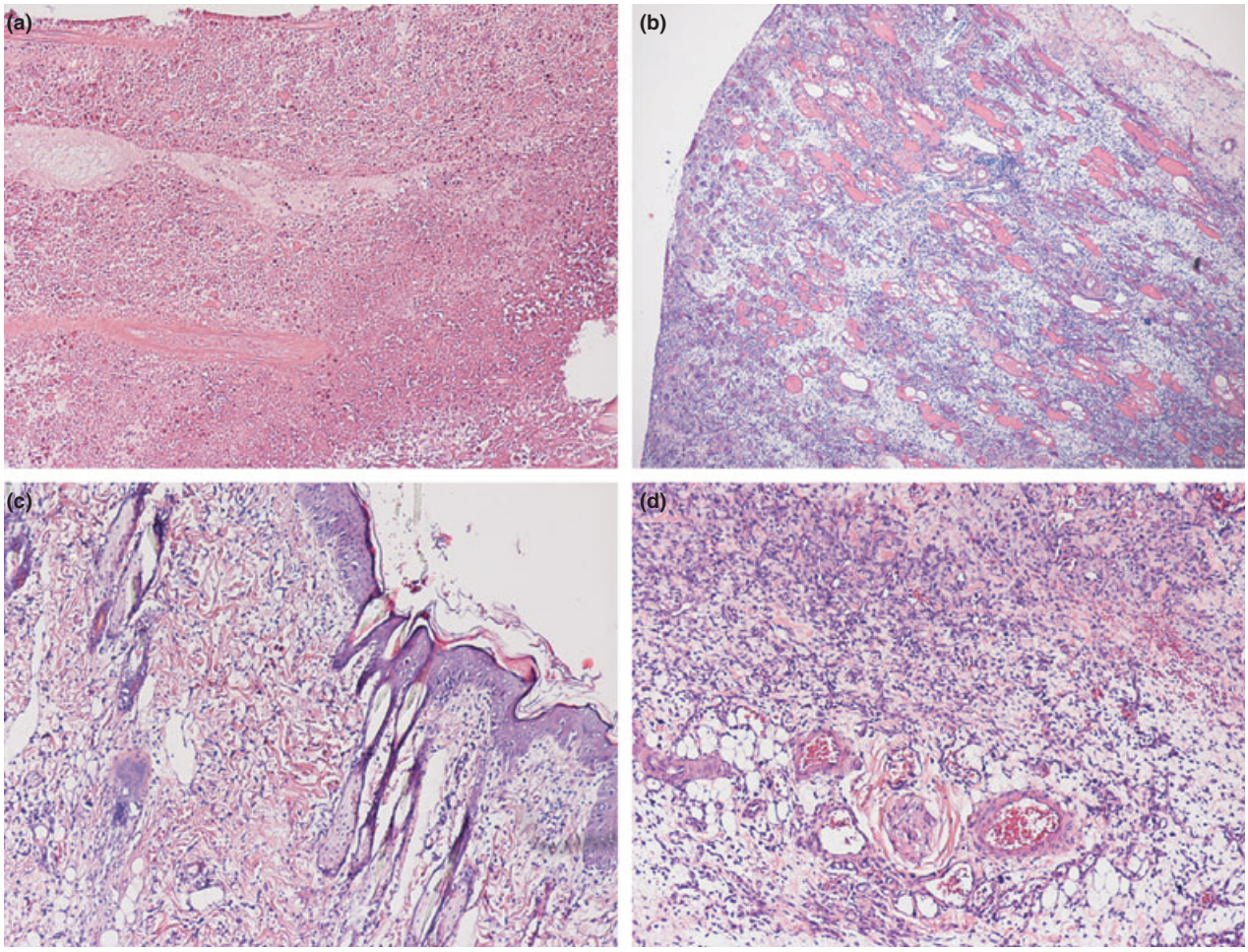

Figure 6 (a) The specimen of bone marrow reveals diffuse necrosis without conspicuous inflammation. (b) Myocyte shrinkage with vacuolation occurs in the transplant tissue. (c) The skin specimen shows chronic inflammation in the dermis. (d) Marked proliferation of capillaries in moderately inflamed and edematous stroma is noted in the transplant tissue.

deals with all the same tissues of the limb, not the amount with lesser bone marrow than other models. That is because there is only relatively little bone marrow transplanted regarding to bone marrow in the whole body in clinical cases, such as hand transplantations. Furthermore, as stated by Ulusal *et al.* [12], the level of osteotomy and weight-mismatches between donor and recipient animals dictates the tension of microvascular anastomosis, which may affect patency. While an overly long bone may result in excessive tension in the anastomosis, a short one can cause kinking. Our model does not have these drawbacks, and so transplantation between weight-mismatched animals could still be successful.

Although body size differences exist between rats and mouse, our rat model has some similarities with Tung *et al.*'s [35] mouse model. The first is that both are for the study of nonfunctional transplantation immunology. The second is that we both agree the mouse is a much more physiologically fragile model than the rat and the application of orthotopic limb transplantation was associated with a high mortality rate. The third is that the modified models of hind limb transplantation rather than whole hind limb more proportionately correlates with hand and distal forearm transplantation in regard to the amount of bone marrow that is present. However, our rat model is different from the mouse models in regard to ease of microsurgical anastomotic procedures due to larger size of vessels and to merely clamping femoral vessels due to no exposure of abdominal aorta [35].

In our study, the operative time and warm ischemia time were relatively shorter than for previous rat models using various kinds of composite tissue allotransplantation, and nearly the same as Ulusal *et al.*'s [12] heterotopic model (Table 1). That is because of the ease of insetting and because fixation of the bone segments was unnecessary. Therefore, the mortality rate of our model

(12.5%) was relatively less in comparison with other studies (20%). In addition, we believe that the groin–thigh flap model could be successful if histology at 7 days postoperatively revealed viable tissue and vessel patency, although some authors have shown such results 100 days postoperatively [36].

We advocate humanizing the experiments, which effectively means not adding extra limbs to the recipients. To ensure the survival of the femoral bone and retain the muscular portion, a small cuff of neighboring muscle should be preserved. Postoperative hematoma, which is a frequent cause for flap failure, should be avoided. There was no damage to the sciatic or femoral nerves of the recipients, so there was no interference with postoperative ambulation. On the other hand, the successful groin–thigh flap could be well-protected and well-hidden in the more lateral aspect of the lower abdomen, as the recipients can ambulate on their normal limbs and the allograft can easily be observed from their lateral flank.

In conclusion, the newly designed groin–thigh flap model also affords the researcher many benefits, while at the same time being less extensive and complicated than standard orthotopic limb transplant models. These advantages include shortened operative time, easy monitoring of graft rejection, decreased morbidity and mortality rates, and capability for earlier postoperative ambulation. However, there exist some limitations in our model from lack of sensory and motor innervation, less bone marrow in a vascularized microenvironment and more reduced antigenic load than traditional models. Although further investigations of long-term complications of limb transplantation, including chronic rejection, graft-versus-host-disease, and sensory and motor function are required, tolerance induction and maintenance are urgently required. Our results suggest that the model employed in this study provides relatively fast and reliable outcomes and is truly suitable for nonfunctional research projects in tolerance immunology, rather than nerve regeneration or sensory and motor analysis.

Authorship

S-DL and C-SL: designed research/study. K-PC and S-HH: performed research/study. L-LC: contributed important reagents. C-LL: collected data, analyzed data. K-PC: wrote the paper.

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