



The Effects of Vascular Tissue Transplantation and Anastomosis at Different Storage Time Periods on Tissue Repair in a Rat Ischemia Model

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Abstract

Vascular occlusion-induced ischemia can occur in any part of the body, causing oxygen deprivation and microvascular dysfunction, which restricts blood flow to muscles, tissues, and organs. The severity of ischemia depends on whether the vessel is partially or completely occluded, with symptoms varying based on the degree of blood flow restriction.

Objectives: To study the effects of different time periods after ex vivo vascular tissue transplantation and anastomosis on tissue repair in ischemic models.

Methods: Fifty male Wistar rats (*Rattus norvegicus*, body weight: 200-300 g, age: 8 weeks) were obtained from the Biomedical Institute and Experimental Animal Center of the National Institute of Medical Sciences. The rats were randomly divided into two groups: Control Group (n=10): Underwent direct end-to-end vascular grafting of a 0.3-0.5 mm diameter artery in the right femur. Experimental Group (n=40): Underwent end-to-end microvascular grafting using donor vessels preserved for 3 days and 7 days.

Results: In the limb ischemia model, in the end-to-end anastomosis of allogeneic vascular transplantation, the tissue cell ischemia-reperfusion injury recovery ability of the vascular anastomosis preserved for 3 days was better than that of the vascular anastomosis preserved for 7 days.

Conclusion: In the limb ischemia model, microvascular transplantation surgery showed different degrees of tissue edema, uneven cell distribution, tissue necrosis and other phenomena, which were more obvious in the 7 day ischemia group. When selecting vascular transplantation anastomosis, through our comparative study, it is best to choose blood vessels with a shelf life of less than 3 days.

Keywords: Donor artery; Vascular transplantation; Rats; Microsurgery; Postoperative complications.

Introduction

Ischemia caused by vascular occlusion can occur anywhere in the body, leading to hypoxia [1] and microvascular dysfunction, which restricts blood flow to muscles, tissues and organs. The severity of ischemia depends on whether the blood vessel is partially or completely blocked, and symptoms vary depending on the degree of blood flow restriction [2].

Peripheral artery disease (PAD) is prevalent in people over 50 years of age and usually presents with mild or no symptoms [3]. According to statistics, in 2015, approximately 236 million people worldwide had PAD, an increase of

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about 23.5% compared to 2000. This increase is attributed to the aging of the global population and the rising prevalence of risk factors such as diabetes mellitus (DM) [4]. A sudden reduction in blood supply to a specific limb area manifests as pain, pallor, loss of pulse, cold extremities, and paralysis. These symptoms can lead to serious complications, including acute multiple organ failure, hyperkalemia, and metabolic acidosis [5], and the annual risk of amputation is about 10%-15%, or 1.5 per 10,000 people, with a 30-day mortality rate of 15%-25% [6].

In 2016, more than 5 million reconstructive surgeries were performed in the United States, and allogeneic transplantation improved the quality of life for recipients. Despite the success of hand and face transplants, many transplant organs are not used in a timely manner due to transportation distances and limitations in the transplant network. According to the U.S. Department of Health and Human Services, only about 10% of patients' transplant needs are met, and many patients die while waiting for a suitable organ [7].

Ischemia-reperfusion injury is characterized by oxidative damage, inflammatory cell infiltration, and endothelial damage, all of which can lead to further vascular dysfunction and graft failure [8]. When blood flow is obstructed due to vascular occlusion or stenosis, the affected tissues become hypoxic and deprived of essential nutrients. The pathological consequences of ischemia include tissue necrosis, inflammation, and apoptosis. In cases of severe ischemia, these tissues may suffer irreversible damage, leading to limb loss, unless effective interventions are taken [9]. Among various treatment modalities, microvascular transplantation has become a key surgical intervention for patients with severe peripheral ischemia. This technique involves anastomosing a donor vessel to the ischemic area to restore blood flow, thereby preventing further tissue damage and promoting regeneration [10]. Despite the success of microvascular transplantation, it is often limited by ischemic injury, and prolonged ischemia can exacerbate tissue damage and reduce transplant success rates [11,12].

In recent years, innovative therapies such as microvascular transplantation, artificial vascular graft replacement, and endovascular surgery have been introduced to treat ischemia caused by vascular occlusion. However, despite these advances, amputation rates remain high, and research on the efficacy of microvascular transplantation and its impact on tissue regeneration remains limited. This study aims to evaluate the effects of different ischemia durations on microvascular transplantation outcomes in a rat limb ischemia model, focusing on graft patency, tissue regeneration, and complications associated with prolonged ischemia. By understanding the relationship between ischemia duration and vascular transplantation outcomes, and by evaluating the efficacy of microvascular transplantation in a controlled limb ischemia model, this research gap can be filled.

Materials and Methods

Animal grouping and study design

Fifty SPF-grade female Wistar rats, 8 weeks old and weighing 200-300 g, were purchased from the Laboratory Animal Science Center of the National Medical University of Mongolia. All procedures were performed in accordance with the 3R principle, approved by the Animal Ethics Review Committee of the National Medical University of Mongolia. To ensure consistency, the experimental environment ($23\pm 2^{\circ}\text{C}$, $45\pm 5\%$ relative humidity, 12-hour light/dark cycle), surgical instruments (all equipment and bedding were sterilized before use), food, and water were identical for all groups of rats.

Main Reagents and Instruments

- Hematoxylin-eosin: Purchased from Beijing Solarbio Science & Technology Co., Ltd.
- Histidine-tryptophan-ketoglutarate solution: Purchased from F. KOHLER CHEMIE GmbH, Germany
- SM-401TR desktop surgical microscope: Purchased from Shanghai Yuyan Scientific Instruments Co., Ltd., rat surgical microscope

Grouping and Model Building

Fifty female Wistar rats were randomly divided into two groups. The control group ($n=10$) underwent direct end-to-end anastomosis of the right femoral artery (diameter: 0.3-0.5 mm). The experimental group ($n=40$) established an ischemia model by ligating the femoral artery for 3 and 7 days, respectively, and then performed end-to-end transplantation using donor arteries preserved for 3 and 7 days, respectively ($n=10$ in each group). The efficacy of the two transplantation methods was compared 21 days postoperatively (Figure 1).

Anesthesia and femoral artery dissection: Prior to surgery, the weight of each rat was recorded. Anesthesia was induced by intraperitoneal injection of sodium pentobarbital solution (0.5 ml/100g body weight). A longitudinal incision was then made on the anterior right thigh from the greater trochanter to the lateral femoral condyle, and the subcutaneous tissue was carefully dissected to expose the femoral neurovascular bundle. To minimize vasoconstriction, 2% lidocaine was used, and the diameter of the femoral artery was measured using digital calipers.

Femoral artery ligation: In the experimental group, the separated femoral artery was ligated with 9-0 sutures to induce tissue hypoxia, thus establishing 3-day and 7-day ischemia models. In the control group, the femoral artery was dissected but not ligated. Finally, the vessel and surrounding soft tissue were repositioned to their normal anatomical location, and the wound was sutured.

Microvascular Transplantation: Vascular Dissection: The artery ends were carefully dissected to reduce vascular tension.

Adventitia Dissection and Anastomosis Adjustment: The adventitia was meticulously dissected for precise anastomosis. The vessel ends were aligned, ensuring accurate intimal (endothelial) connection and close adhering to the surrounding muscle layer. The damaged end was clamped with vascular clamps, and the adventitia was trimmed as needed to minimize the risk of thrombosis and protect the vessel wall.

Lumen Irrigation: After evenly trimming the vessel ends, the lumen was irrigated with 0.1% heparinized saline to prevent thrombosis during transplantation.

Transplantation Technique: After establishing the ischemic model 3 or 7 days after arterial ligation, end-to-end transplantation was performed using a donor artery. Ensure the transplanted blood vessel is of appropriate length and avoids twisting.

Specimen Collection and Processing

Three and seven days after establishing the two ischemia models in the experimental groups, rats were anesthetized by intraperitoneal injection of sodium pentobarbital solution (0.5 ml/100g body weight), euthanized by cervical dislocation, and appropriate amounts of surrounding tissues such as leg muscles and skin were removed to prepare tissue sections for observation of the effects of ischemia on tissue cells.

After establishing the ischemia model, end-to-end anastomosis of the transplanted blood vessel was performed. Twenty-one days post-surgery, rat tissue was collected using the same method, and sections were prepared to compare and observe the recovery of tissue cells after blood flow reperfusion.

HE staining to observe pathological changes in rat muscle tissue

Rat thigh muscle tissue was soaked in 10% formalin solution for 48 h, then dehydrated sequentially with ethanol at 90°C and 95°C (2 min each time) to prepare tissue blocks. Tissue sections (4 µm) were then prepared, and slides treated with poly-L-lysine were used for adhesion. After removing the water, the sections were dried overnight at 37°C and stored at 4°C. Rehydrated tissue sections were then immersed in hematoxylin staining solution for nuclear staining for 10 min; immersed in acidic ethanol differentiation solution (1%) for differentiation, rinsing with tap water for 3 min each time; and immersed in eosin staining solution for cytoplasmic staining for 10 min, followed by rinsing with deionized water. After dehydration with graded ethanol, clearing with xylene, and mounting with neutral resin, the tissues were air-dried in a ventilated area. Pathological changes in rat thigh

muscle tissue were observed and photographed under an optical microscope.

Transplantation status assessment

Following end-to-end anastomosis of allogeneic vascular grafts, the Acland test [13] was used to assess the patency and function of the grafted vessels. This assessment provides immediate feedback on the quality of the anastomosis and the recovery of blood flow. (Figure 1)

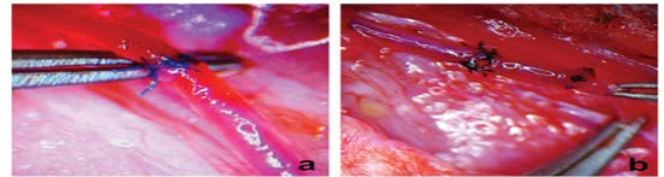


Figure 1: Acland test to assess vascular patency: a is the control group, b is the experimental group with end-to-end anastomosis.

Donor blood vessel preservation

Under microscope guidance, the femoral artery of the donor rat was dissected and cut into 1-2 cm segments. The vessels were thoroughly rinsed with 0.1% heparinized saline solution to remove residual blood and soft tissue. The cleaned vessel segments were stored in 50 ml of Custodial Cardioplegia solution at 4°C for 3 and 7 days, depending on experimental requirements.

Statistical methods

Data from 10 rats in the normal control group and 20 rats in the ischemia model group at 72 h and 168 h were statistically analyzed using SPSS 23.0 software. Data are expressed as mean ± standard error. Normality was tested for each group. Data conforming to a normal distribution were analyzed using a t-test, with $P < 0.05$ considered statistically significant. For non-normally distributed data, the Wilcoxon rank-sum test was used, with $P < 0.05$ considered statistically significant.

Results

Comparative analysis of body weight between the two groups before and 21 days after surgery

The average preoperative weight of mice in the control group was 230.09 g, which increased to 240.24 g 21 days post-surgery. A paired t-test showed a statistically significant difference ($t = -5.8419$, $P = 0.0002$).

In the 3-day ischemia group, the average preoperative weight was 243.39 g, which increased to 240.49 g after 3 days of ischemia, and to 256.73 g 21 days post-surgery. A paired t-test showed a statistically significant difference between preoperative and postoperative weight ($t = -11.2249$, $P = 0.0000$).

In the 7-day ischemia group, the average preoperative weight was 256.07 g, which increased to 253.86 g after 7 days of ischemia, and to 264.24 g 21 days post-surgery. A t-test showed a statistically significant difference between preoperative and postoperative weight ($t = -13.9642$) ($P = 0.0000$).

Comparative analysis of operation time between the two groups

The total surgical time, clamping time, vascular transplantation time, and single suture time for the two groups were 24.506, 11.624, 10.477, and 2.621 min for the control group, 39.826, 31.174, 29.759, and 3.451 min for the 3-day ischemia group, and 45.859, 36.70, 35.25, and 3.917 min for the 7-day ischemia group. The differences between the control group and the experimental group (3-day and 7-day ischemia groups) were statistically significant ($P = 0.000$) (Table 1).

Table 1: Comparative analysis of total operation time, vascular clamping time, vascular grafting time, and single suture time between the two groups.

Index	Group	Average value	Standard deviation	Quantity	P value	
					Control group	3-day ischemia group
Surgery time	Control group	24.506	2.2882463	10		
	3-day ischemia group	39.826	0.8839502	20	0	
	7-day ischemia group	45.859	4.0740585	20	0	0
Vascular clamping time	Control group	11.624	1.2227492	10		
	3-day ischemia group	31.174	0.9855802	20	0	
	7-day ischemia group	36.7	3.5495474	20	0	0
Vascular graft anastomosis time	Control group	10.477	1.2318736	10		
	3-day ischemia group	29.759	0.8541701	20	0	
	7-day ischemia group	35.25	3.4600987	20	0	0
Time required to suture one stitch	Control group	2.621	0.3076235	10		
	3-day ischemia group	3.451	0.2404124	20	0	
	7-day ischemia group	3.917	0.5078058	20	0	0.005

Comparative analysis of blood loss between the two groups

A comparison of intraoperative bleeding during vascular transplantation among the control group, the 3-day ischemia group, and the 7-day ischemia group revealed that the longer the femoral artery ischemia time in rats, the greater the intraoperative bleeding during vascular transplantation. This may be due to the prolonged ischemia time and changes in the tissue structure of the donor vessel (the average intraoperative bleeding during vascular anastomosis was 0.27 ml in the control group, 0.597 ml in the 3-day ischemia group, and 1.643 ml in the 7-day ischemia group).

Analysis of postoperative complications in the two groups

In the control group, 1 in 10 rats developed vascular stenosis after end-to-end anastomosis; in the experimental group, 3 rats developed stenosis after 3 days of ischemia and 5 rats after 7 days of ischemia, with rates of 10%, 15%, and

25%, respectively. Furthermore, wound healing was good in both groups, with no signs of inflammation observed.

Twenty-one days after vascular anastomosis, 1 in 10 rats in the control group developed thrombosis; 3/20 and 8/20 rats in the 3-day and 7-day ischemia groups, respectively, with incidence rates of 10%, 15%, and 40%. No postoperative complications such as death, infection, or hematoma were observed.

Analysis of thigh muscle tissue sections from two groups of rats 21 days post-surgery

In a limb ischemia model, high-power (X10) examination of the thigh muscle tissue of rats in the 3-day ischemia group revealed partial loss of connective tissue structures and slightly disordered arrangement of muscle bundles and cells, suggesting early morphological changes due to ischemia. Examination of the thigh muscle tissue of rats in the 7-day ischemia group revealed that most connective tissue, muscle bundles, and cells were disordered, with numerous fragmented

areas. These histological changes indicate that tissue damage intensifies and morphological responses become more severe with increasing ischemic duration. In the control group, tissue cells showed no ischemic damage, and the connective tissue, muscle bundles, and cells were neatly arranged.

Twenty-one days after end-to-end anastomosis of allogeneic vascular grafts, high-power field (X10) examination of the thigh muscle tissue of rats in each group revealed that in the 3-day ischemia group, most of the connective tissue, muscle bundles, and tissue boundaries of the thigh became clearer, and the torn and damaged areas gradually began to recover and approach normal. However, the degree of damage repair in the 7-day ischemia group was inferior to that in the 3-day group, or tissue repair was not obvious. Observational comparison showed that the recovery ability of thigh muscle tissue and cells after ischemia-reperfusion injury using 3-day-preserved vascular anastomoses was better than that using 7-day-preserved vascular anastomoses (Figure 2). Furthermore, in the experimental groups, the 3-day ischemia group showed the best postoperative tissue cell recovery after end-to-end anastomosis of transplanted allogeneic vessels stored for 3 days.

Analysis of stored allogeneic vascular tissue sections

Rat donor arteries were preserved for 3 and 7 days according to experimental requirements, then removed

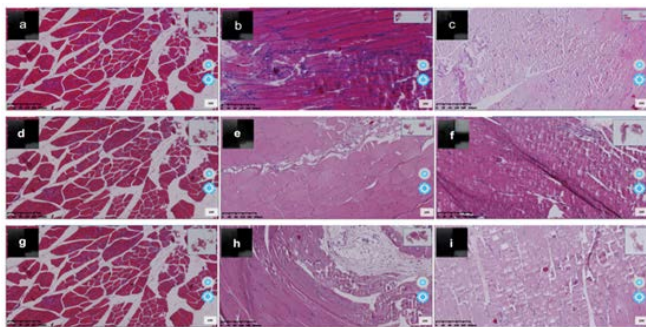


Figure 2: a shows the control group; b shows rat thigh tissue sections after 3 days of ischemia; c shows rat thigh tissue sections after 7 days of ischemia. d and g show rat thigh tissue sections 21 days after end-to-end vascular anastomosis in the control group. e shows rat thigh tissue sections 21 days after end-to-end vascular anastomosis using 3-day-preserved allogeneic vessels in the 3-day ischemia group; f shows rat thigh tissue sections 21 days after end-to-end vascular anastomosis using 3-day-preserved allogeneic vessels in the 7-day ischemia group; h shows rat thigh tissue sections 21 days after end-to-end vascular anastomosis using 3-day-preserved allogeneic vessels in the 7-day ischemia group; i shows rat thigh tissue sections 21 days after end-to-end vascular anastomosis using 7-day-preserved allogeneic vessels in the 7-day ischemia group. After 21 days of end-to-end vascular anastomosis using 3-day-preserved vessels, tissue recovery was good; however, after 21 days of end-to-end vascular anastomosis using 7-day-preserved vessels, recovery was poor, and tissue breakage was significant.

and dehydrated. Hematoxylin-eosin staining was used to assess pathological changes in the tissue structure. The specimens were examined under a digital optical microscope (Magscanner, KFBIO, KF-PRO-005) at 200x magnification. Histological examination revealed slight changes (close to normal) in the endothelial cells and smooth muscle of the donor blood vessels stored for 3 days; the surface was smooth and elastic, with slight rupture of the adventitia connective tissue. In contrast, the donor blood vessels stored for 7 days showed significant rupture of the intimal smooth muscle and adventitia connective tissue, with a rough surface and loss of elasticity (Figure 3).

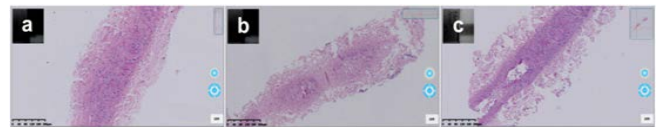


Figure 3: Vascular tissue analysis. A shows vascular tissue analysis of the control group rats. B shows donor vascular tissue analysis after 3 days of storage. C shows donor vascular tissue analysis after 7 days of storage.

Immunohistochemical evaluation of the morphology and structure of rat thigh skin

In the control group, the squamous epithelium, basement membrane, and loose connective tissue of the thigh skin were intact and structurally normal. On day 3 of ischemia, the squamous epithelium showed increased keratinization, and the collagen fibers in the loose connective tissue thinned. On day 7 of ischemia, the squamous epithelium tore, and hair follicle structure disappeared, indicating anemia. On day 21 after vascular transplantation, skin regeneration improved, similar to the control group.

To determine tissue regeneration, we investigated the expression of proliferating cell nuclear antigen (PCNA) protein using immunohistochemistry: On days 3 and 7 of ischemia, compared to the control group, the number of PCNA cells in squamous epithelial and hair follicle regeneration was significantly reduced (black arrows). On day 21 after vascular transplantation, the number of PCNA-positive cells increased, indicating enhanced skin regeneration (black arrows). The number of PCNA-positive cells increased, but the staining intensity was weaker than in the control group.

We detected the expression of keratinocyte growth factor (KGF), a protein that plays a crucial role in normal skin regeneration, using immunohistochemistry. KGF-positive staining was observed in keratinocytes of the control group rats. In the 3-day and 7-day ischemia groups, the number of KGF-positive cells in the squamous epithelium and hair follicles was significantly reduced (red arrows). At 21 days post-vascular transplantation, the number of KGF-positive cells increased, indicating enhanced regeneration (red arrows) (Figure 4).

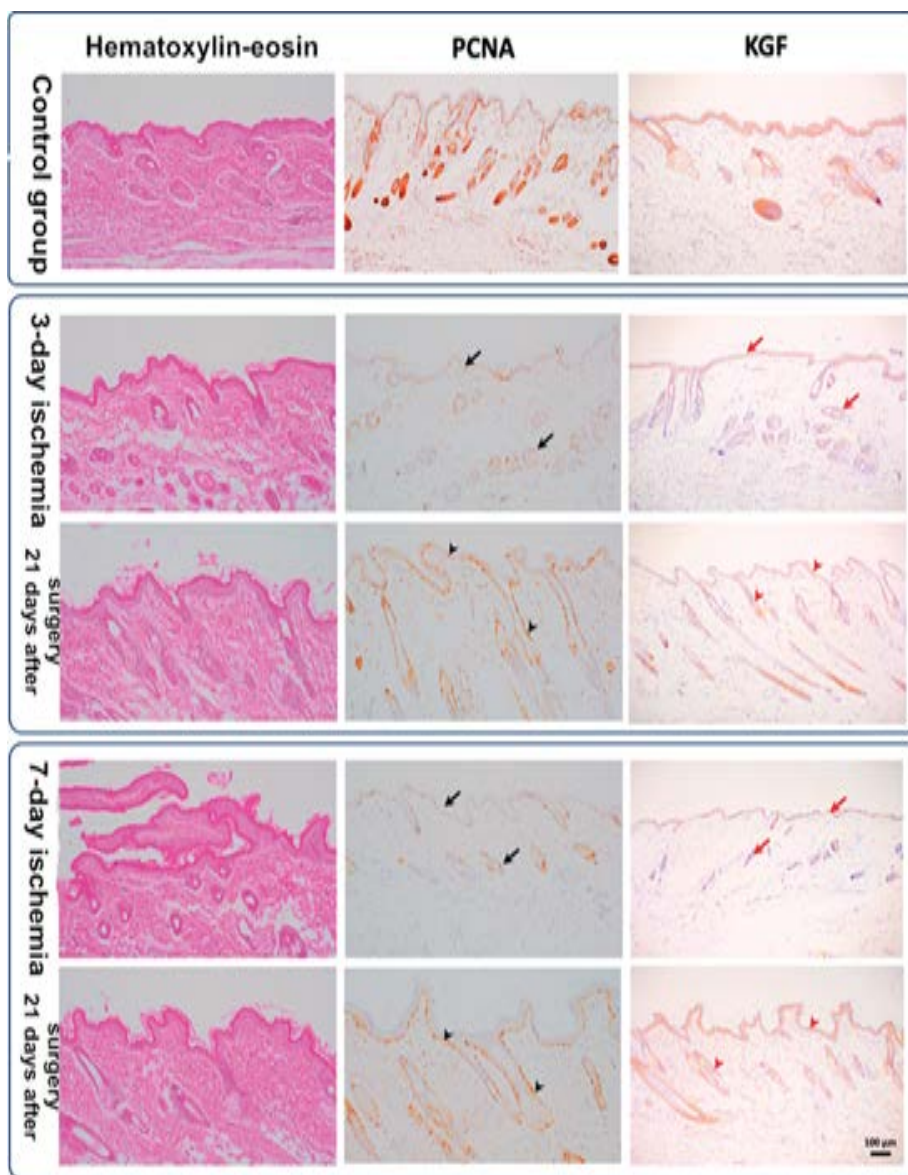


Figure 4: Immunohistochemical evaluation of the morphology and structure of rat thigh skin.

Table 2: Comparison of postoperative thrombosis and other complications between the two groups.

Index	Control group (10)	3-day ischemia group (20)	7-day ischemia group (20)
Bleeding			
Vascular stenosis	1 (10%)	3 (15%)	6 (30%)
Angioedema			
Thrombosis	1 (10%)	4 (20%)	9 (45%)
New blood vessel formation	-	+	+
Infect			
Vasculitis	-	3 (15%)	7 (35%)
Hematoma			
Die			

Postoperative complication analysis

Comparison of postoperative thrombosis and other complications between the two groups (Table 2)

In the control group, 1 in 10 rats (10%) developed thrombosis 21 days after vascular transplantation; in the 3-day ischemia group, 4 in 20 rats (20%) developed thrombosis; and in the 7-day ischemia group, 9 in 20 rats (45%) developed thrombosis. These results indicate that the risk of thrombosis during reperfusion increases with prolonged ischemia time. Furthermore, when observing the transplanted arterial vessels in the experimental groups (3-day and 7-day ischemia groups), neovascularization (angiogenesis) was observed 21 days post-transplantation.

Discussion

Cryopreservation of vascular tissue can lead to smooth muscle contraction and loss of endothelial function. During cryopreservation, the freeze-thaw cycle of tissues and cells and the formation of ice crystals are the main factors leading to loss of cell viability [14]. When cryopreserving donor blood vessels, vascular damage should be minimized as much as possible, and normal vascular structure or hemodynamic properties should be fully preserved.

Previous studies on rat limb ischemia and reperfusion injury models [15,16] have largely focused on comparing the effects of different ischemia times on tissue cells at 0, 2, 4, and 8 hours of acute ischemia. With an aging population, cardiovascular and cerebrovascular diseases, diabetes, peripheral artery disease, and trauma are increasingly leading to vascular thrombosis and vascular lesions. It is estimated that within one year of a diabetes diagnosis, approximately 40%-50% of patients undergo amputation, and 20%-25% face death due to ineffective treatment. This study simulates chronic limb ischemia (3 days and 7 days) as a starting point, observing changes in tissue cell damage at different ischemia times and using allogeneic vessels stored at different time points for end-to-end transplantation anastomosis. The aim is to compare the changes and regenerative capacity of tissue cells after reperfusion injury.

Comparing the body weight of rats in the control and experimental groups, no increase in body weight was observed in either group after establishing ischemia models for 3 and 7 days (except for a significant decrease in the 7-day ischemia group). Twenty-one days after vascular anastomosis, both groups showed a significant increase in body weight, but the increase was less pronounced in the 7-day ischemia group compared to the 3-day ischemia group. This result indicates that rat body weight decreases significantly with prolonged ischemia.

According to the research results of the researchers [17], the average time for vascular knot suturing was 11.97 min,

while the average time for vascular knot suturing in the control group in this study was 10.48 ± 1.23 min, the average time for vascular knot suturing in the 3-day ischemia group was 29.76 ± 0.85 min, and the average time for vascular knot suturing in the 7-day ischemia group was 35.25 ± 3.46 min. We have reason to believe that the risk of thrombosis increases with the extension of operation time, and shortening the operation time is an important factor in preventing thrombosis.

Shorter ischemic time (3 days) can lead to better graft patency, tissue regeneration capacity and overall surgical outcome, while longer ischemic time (7 days) can exacerbate tissue damage, leading to an increased risk of graft failure and postoperative complications. In 20 cases of end-to-end anastomosis using 3-day donor vessels, the success rate of transplantation was 100%. Two anastomoses failed 21 days postoperatively, with a success rate of 93.33%. This is similar to the results of Chiu YH et al. [18], whose donor tissue transplantation success rate was 90%-95%. Postoperative complications were related to technical errors during surgery and prolonged tissue bleeding. This is consistent with the results of that study [19], and we have reason to believe that even partial damage during reconstruction surgery can lead to reconstruction failure. In 20 cases of anastomosis using donor transplant vessels preserved for 7 days, 11 (53.33%) were unusable (the first suture failed, the second was successful), and the remaining 9 (46.67%) were anastomosed with transplant vessels in one go. In the study by Ramadan Jashari and Vanessa Bouzet [20], 7066 blood vessels were transplanted, of which 2407 (34.1%) were unusable and 4659 (65.9%) were used for treatment. This may be due to structural changes in the donor veins during long-term storage.

Postoperative complications can arise from insufficient surgical skill or experience, uneven suturing, prolonged tissue ischemia, and accidental damage to the vascular wall, all of which can lead to graft thrombosis and graft failure. Prolonged surgery time also increases the risk of vascular thrombosis.

Ensuring unobstructed blood flow within the graft is crucial for the success of microvascular surgery. Completing the anastomosis within a short intraoperative time, preserving tissue integrity, and preventing inflammation, vascular stenosis, and thrombosis are also essential. This study highlights the importance of ischemic time in determining the success rate of microvascular grafting. The results show that shorter ischemic time (3 days) leads to better graft patency, tissue regeneration capacity, and overall surgical outcomes, while longer ischemic time (7 days) exacerbates tissue damage, increasing the risk of graft failure and postoperative complications. These findings have significant clinical implications, particularly for patients with peripheral artery disease and those at risk of amputation. Timely intervention

and effective transplantation strategies are crucial for improving patient outcomes and reducing the incidence of limb loss due to chronic ischemia.

Our experimental sample size may be insufficient. In future research, we will further refine and address issues related to post-transplant complications, providing more comprehensive experimental evidence for lower limb ischemia-reperfusion injury and its tissue damage and repair in animal models.

Conflict of interest

All authors declare that they have no conflict of interest.

Author contribution statement

Yanbo Qi: Experimental design, paper writing; Sanchin Urjin: Data statistical analysis, graphing; Galindev Batnasan: Data and image data compilation; Baatarsuren Batmunkh: Research guidance, paper review, HE staining, immunohistochemical staining.

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