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Research Article

A Simplified Splint Tubing Technique of Heterotopic Heart Transplantation in Rat

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Abstract

Background: Heterotopic Heart Transplantation (HHT) in rats has been established for the study of transplantation immunology. Our goal was to describe a Splint Tubing Technique (STT) for cervical HHT in rats.

Methods: Wistar-Furth rats were performed in HHT using STT and Suture Technique (ST). With STT, the surgical procedure connected with the recipient external jugular vein (EJV), Common Carotid Artery (CCA) to the donor Pulmonary Artery (PA), Ascending Aorta (AA) using an 18G and 20G cuff, respectively. The surgical success rate and operation time was recorded. Graft function was assessed by pulse palpation and echocardiography. The cardiac pathology was analyzed by HE staining. TUNEL and western blotting were used to measure apoptosis in each rat.

Results: Averaged total operation time using ST was much longer than STT. The success rate of STT was 83.3%, which showed significantly higher than ST (55.0%). HE staining of STT group showed the same acute rejection compared to ST group. Same tendency of apoptosis in two groups was detected by TUNEL and western blotting.

Conclusions: Our simplified STT for HHT in rats is an easy, convenient, stable, and reliable method. It simplified the rat HHT procedure, shortened operation time, reduced surgical difficulty, improved technical success rate.

Keywords: Splint Tubing Technique, Heart Transplantation, Heterotopic Heart Transplantation, Rat, Simplified

Introduction

Heart Transplantation (HT) is the most effective treatment for end-stage heart failure. The main causes of allograft failure after HT are primary graft dysfunction, intractable acute rejection, and coronary graft disease [1,2]. In addition, HT is difficult in children than adults [3]. After pediatric and adult HT, there exist many challenges in allograft survival [3]. The battle of prolonging allograft survival is still being waged in research laboratories and in the clinic. Rat and mouse models of Heterotopic Heart Transplantation (HHT) are important for both basic and clinical research of HT [4,5].

Since the first intra-abdominal HHT rat model was described by Abbott et al. [6] in 1964, various subsequent modifications have contributed to the development of this animal model [7-9]. These micro suture techniques in HHT have been accepted worldwide. Unfortunately, these methods are associated with a significant rate of graft loss due to technical failure [10,11]. Therefore, this abdominal model requires investigators must have an ability of proficient microsurgical skills, and several months of training.

Heron first reported a rat HHT model by using a suture less cuff technique [12]. He transplanted the donor heart to the neck of the recipient. Numerous subsequent experimental researchers have continuously improved the above these models [13-15]. The main improves with HHT showed in different transplant sites or different vascular anastomosis method. Although abdominal and femoral techniques have their own advantages, cervical HHT still is the imperative model. The advantage of HHT in cervical is that evidence of functional graft viability, beating, is easily monitored by palpation or electrocardiography.

Based on Suture Technique (ST) and suture less cuff technique, we established a simplified HHT model in rat using STT, aimed to improve the HHT model for the study of HT.

Materials and Methods

Animals

Male/female Wistar-Furth (WF: RT1^u) rats with an average body weight of 280 g (250–300 g) were used in this experiment. Forty Wistar transplantations were performed using in ST (ST group) and sixty in our simplified STT (STT group). Both the ST and STT were applied in cervical HT. The animals were kept under standard conditions, and all animals were operated under anesthesia with chloral hydrate.

Methods

Heterotopic cervical heart transplantation

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Recipient preparation

Under deep anesthesia, the recipient's right anterolateral cervical region was prepared for transplantation. The following procedures from venous preparation to arterial separation were performed as previously described [15-17]. The Common Carotid Artery (CCA) and the External Jugular Vein (EJV) were isolated from the surrounding tissue. The distal sides of CCA and EJV were tightly ligated with a 7-0 silk suture, and cut off at distal sides of the ligation respectively.

F□or recipient using ST, the proximal sides of CCA and EJV were ligated with micro-clips to prepare for vessels suture (Figure 1A). However, for recipient using STT, the simplified surgical procedure connected with the recipient EJV to donor Pulmonary Artery (PA) using an 18G cuff and the recipient CCA to the donor Ascending Aorta (AA) using a 20G cuff (Figure 1B). The proximal sides of CCA and EJV were ligated with micro-clips. Then the CCA was traversed to one end of the 20G cuff with a cavity. Next, the vessel was lightly overturned with two micro-forceps and fixed on outer of the cuff tightly with 7-0 silk suture (Figure 1). Similarly, the EJV was traversed to one end of the 18G cuff with a cavity. Then, the vessel was lightly overturned with two micro-forceps and fixed on outer of the cuff tightly with 7-0 silk suture. The recipient operation time was recorded as well.

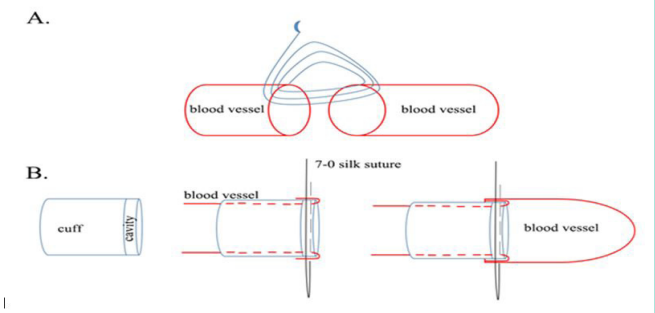

Figure 1: Mode chart of the surgical procedure. A, In ST group, the donor vessels were connected to recipient vessels by continuous suture. B, Method of vessel cuff preparation in STT group. Following clamping of proximal portion of vessel, the distal end of vessel was passed through the cuff with a cavity, everted, and fastened tied with a 7-0 silk suture.

Donor heart harvest

The donor heart harvest was performed as previously described [11,18]. A long midline incision was performed to enter the abdominal cavity after anesthesia with chloral hydrate. The abdominal aorta, Inferior Vena Cava (IVC), and Left Renal Vein (LRV) were exposed. One cc cold heparinized saline (100 U/ml) was injected into the LRV to prevent intravascular thrombosis. One minute later, abdominal aorta and IVC were cut to drain the blood. Then opened the thorax, cold normal saline was poured into the thoracic cavity to cool the heart. The heart was perfused through the super-hepatic IVC with cold 4 °C Ringer's solution to cool and arrest its beating. When perfusion was completed, PA and AA were isolated from the surrounding tissue. Next, the heart was pulled upward gently and transected below the first branch of AA and the branch of left and right pulmonalis. After that, the residual vascular was fastened together with a 7-0 silk surgical suture. By cutting distal from the ligature, the donor heart was harvested and subsequently stored in cool preservative solution at 4 °C. The time of donor heart harvest was recorded.

Heart transplantation

Well conjunction using STT is a key point, shown in Figure 1. The donor heart was placed on the right side of the recipient's cervical cavity. The free end of the arterial and venous cuff was inserted into the AA and PA of donor heart, respectively. The cuffs were tightly ligated with 7-0 suture, respectively. After completing the reconstruction of the graft's inflow and outflow tracts and inspecting the anastomotic sites whether it is bleeding, all micro-clips were released. The time of arterial anastomosis and venous anastomosis were recorded, respectively. After ensuring that the beating graft was well without congestion, the cervical incision was closed by a one-layer continuous suture. Finally, the animal was placed into a warming cage until it was completely recovered. Immediately after operation the animal was allowed free access to water and food.

Postoperative evaluation

Heart □palpation

Heartbeat of donor hearts in the two groups were checked by manual palpation every day. The scoring system of heartbeat intensity was graded as 'very strong contraction,' 'strong contraction,' 'weak contraction,' 'very weak contraction,' and 'no pulse.'

Echocardiography examine

A VisualsonicsVevo 2100 ultrasound system (VisualSonics, Canada) were used for graft monitoring. We periodically used echocardiography throughout the study to examine anastomotic patency, transplant viability via contractility, and acute graft rejection by way of absent contraction.

Histology evaluation of tissue sections

The transplanted hearts were harvested on Postoperative Days (POD) 1, 3, 5, and 7 (n = 3 for each time point). The cardiac tissues were immersed in 10% formalin, fastened in paraffin, sectioned at 7 μm, stained with HE for light microscopy, and images were collected directly. All slides were reviewed blindly by a certified histocytopathologist. Acute graft rejections were evaluated with the modified criteria of the International Society for Heart Transplantation (ISHT) [19,20].

TUNEL assay

The sections were deparaffinized by immersion in xylene, rehydrated, and incubated in phosphate-buffered saline with 3% H2O2 to inactivate endogenous peroxidases. They were incubated with proteinase K (20 mg/ml) for 10 minutes, then washed in phosphate-buffered saline, and incubated with terminal deoxynucleotidyl transferase and fluorescein isothiocyanate-dUTP for 60 minutes at 37ºC using an apoptosis detection kit. The mean number of TUNEL-positive cells was counted in at least five randomly chosen fields in each group. All counts were performed by two independent individuals.

Western Blot Analysis

Western blot was prepared from grafts at POD1 to POD 7 (n = 5 for each time point). To obtain samples for Western blot, the cardiac tissue was excised and snap frozen at −80 □ until use. Total protein was isolated from approximately 0.1 g of cardiac tissue at indicated time points. To prepare lysates, frozen cardiac samples were minced with eye scissors in ice. Total heart tissue protein was then homogenized in lysis buffer containing 1% NP-40, 50 mmol/l Tris, 1% SDS, pH 7.5, 5 mmol/ l EDTA, 1% Triton X-100, 1% sodium deoxycholate, 10 mg/ ml aprotinin, 1 mmol/ l PMSF, and 1 mg/ ml leupeptin), then micro centrifuge at 10,000 rpm and 4 □ for 20 min to collect the supernatant. After protein concentrations were determined with a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA), the resulting supernatant was subjected to with SDS-Polyacrylamide Gel Electrophoresis (PAGE). Proteins were transferred to Polyvinylidine Diflouride Filter (PVDF) membranes (Millipore) by a transfer apparatus at 300 mA for 2 h. The membranes were blocked with 5% nonfat milk in Tris-Buffered Saline with Tween (TBST) at room temperature for 2 h. The filters were immediately rinsed three times in TBST and then incubated overnight

with primary antibodies at 4 □. Finally, the horseradish peroxidase-conjugated secondary antibody was added to filters for an additional 2h, and the proteins were examined with an enhanced chemiluminescence detection system (ECL, Pierce Company, USA).

Statistical Analysis

The results were expressed as the mean ± SEM. Statistical analysis was performed by one way analysis of variance or by use of Student's unpaired t-test, as appropriate. P value of less than 0.05 was considered to be statistically significant. Each experiment consisted of at least three replicates per condition.

Results

Operative time and Surgical Procedure

Compared with the ST group, operation time of dissection CCA and EJV showed no significant difference in the STT group (8.32±1.24 vs 8.35±1.28, P> 0.05, Table 1). No significant difference was observed in operation time of donor preparation between the ST and STT groups (13.36±2.41 vs 13.34±2.38, P> 0.05, Table 1). However, surgical time of heart conjunction by STT, including arterial anastomosis and venous anastomosis, was significantly shortened compared with the ST group (Table 1). Therefore, averaged total operation time using ST was much longer than STT (70.32±5.57 vs 34.61±3.12, P< 0.05, Table 1). The detailed surgical procedure of STT group was recorded (Figure 2).

Table 1: Recorded Operative Time.

Events	Average time (min)	
	Suture technique (ST)	Splint tubing technique (STT)
Recipient Preparation		
Dissection of CCA and EJV Donor Preparation	8.32±1.24	8.35±1.28
Donor heart harvest	13.36±2.41	13.34±2.38
Heart Conjunction		
Arterial anastomosis	23.29±2.38	7.33±1.72*
Venous anastomosis	21.51±2.15	6.67±1.63*
Total operation time	70.32±5.57	34.61±3.12*

Note: Compared with the ST group, operation times of dissection CCA and EJV showed no significant difference in STT group. No significant difference was observed in operation time of donor preparation between the ST and STT group. However, STT shortened the arterial and venous anastomosis time obviously, and the total operative time of STT was shorter than that of ST.
CCA, Common carotid artery; EJV, External jugular vein.
*p < 0.05 vs ST group.

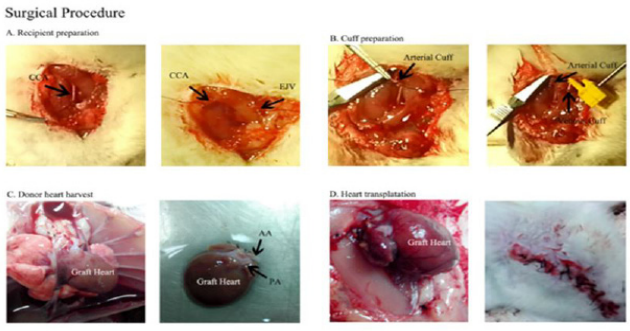

Figure 2: Surgical procedure of heart transplantation. A, Recipient preparation, the CCA and EJV were isolated from the surrounding tissue. B, Cuff preparation, CCA and EJV were traversed to one end of the 20G cuff and 18G cuff, respectively. And they were lightly overturned and fixed on outer of the cuff tightly, respectively. C, Donor heart harvest, the donor heart was harvested, and PA, AA were isolated from the surrounding tissue. D, Heart transplantation, Recipient EJV and CCA were in conjunction with donor PA and AA respectively.

Success Rates

Technically successful transplantation is defined as the forceful beat of the transplanted heart for 3 days. In ST group, forty heart transplantations were carried out with night graft losses: one rat died of anesthesia, one graft loss of congestion and weak heartbeat, two losses of excessive bleeding at the phase of heart conjunction, four losses of postoperative bleeding, one loss of thrombosis in the CCA (Table 2). The success rate of HT using ST was 55.0% (Table 3). In the STT group, sixty heart transplantations were performed with only five graft losses, including one losses of postoperative bleeding and four losses of thrombosis in the CCA and confirmed by necropsy (Table 2). The success rate of HT using STT was 83.33%, which showed significantly higher than ST (Table 3).

Table 2: Complications of two methods.

Complications	Suture technique (ST)	Mortality	Splint tubing technique (STT)	Mortality
Anesthesia	1	0.05	0	0
Congestion	1	0.05	0	0
Bleeding	2	0.1	0	0
Postoperative bleeding	4	0.2	1	0.033
Thrombosis	1	0.05	4	0.133
Total	9	0.45	5	0.166

Note: Complications of heterotopic heart transplantation in Suture Technique (ST) and Splint Tubing Technique (STT) groups. The mortality in STT group was significantly lower than ST group.

Table 3: Graft survival time and Success rate

Groups	n	Success rate (%)	Average survival time (days)
Suture technique (ST)	40	55.00%	8.27±0.62
Splint tubing technique (STT)	60	83.3 %*	8.48±0.65

Note: Heterotopic heart transplantation average survival time and success rate. Technically successful transplantation is defined as the forceful beat of the transplanted heart for 3 days. The success rate of heart transplantation using STT were 83.3%, which showed significantly higher than ST 55.0 %. But averaged total survival time was no differences between the two groups.
*p < 0.05 vs ST group.

Graft Survival Time

The donor heart survival time was summarized in Table 3. The average survival time of HT using STT is about 8.48±0.65 days, and 8.27±0.62 days in the ST group (Table 3). No statistical difference of average survival time of HT was found between the two groups, which indicated our simplified STT is useful for HT.

Heartbeat Score

□Heartbeat score of donor hearts in two groups was checked daily. The heartbeat of donor hearts was strongly palpable immediately post-surgery and gradually weakened after POD 3 in two groups. However, the comparison of the function evaluated with the palpation score demonstrated no statistical differences between the ST group and STT group.

Echocardiographic Findings

We periodically used echocardiography throughout the study to examine anastomotic patency, transplant viability via contractility, and acute graft rejection by way of absent contraction. Compared with the normal mouse heart, graft on POD 7 endpoint showed rhythmic ventricular contraction, full graft flow, and anastomotic patency. In addition, biopsy confirmed that graft was in the set of severe acute rejection (Figure 3).

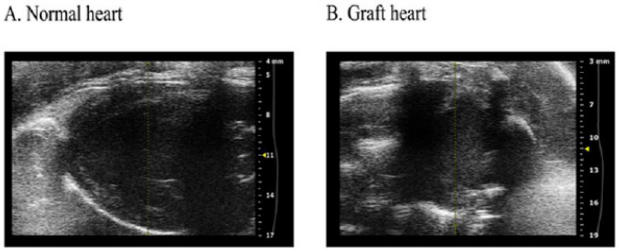

Figure 3: Echocardiography of rat heart. A, Normal heart echocardiography; B, Echocardiography of graft at POD 7 endpoint in STT group.

Graft Histology Evaluation

Cardiac grafts in the ST group and STT group were harvested and stained with HE. In the STT group, grafts on □□POD 1 showed as grade 0 rejection (Table 4) and cardiomyocytes were arranged regularly with no significant lymphocytes inflammation and myocyte necrosis (Fig. 4A and B). At POD 3, there were local inflammation and medium inflammatory infiltration and edema in myocardial cell, which indicated mild acute rejection (grade I, Table 4). When the graft at POD 5 suggested that the moderate acute rejection (grade III - IV) and moderate lymphocytic infiltration and faint myocardial cell necrosis in vascular and myocardial cell (Figure 4A). By day 7, cardiomyocytes necrosis and multifocal aggressive interstitial lymphocytic infiltration in vessels were seen in grafts (Figure 4A).

Table 4: Rejection grade in heart graft, according to ISHT.

POD	Heart allograft
1	0
3	I
5	III-IV
7	IV

Note: Three samples at each time point. ISHT, international society for heart transplantation; POD, postoperative days.

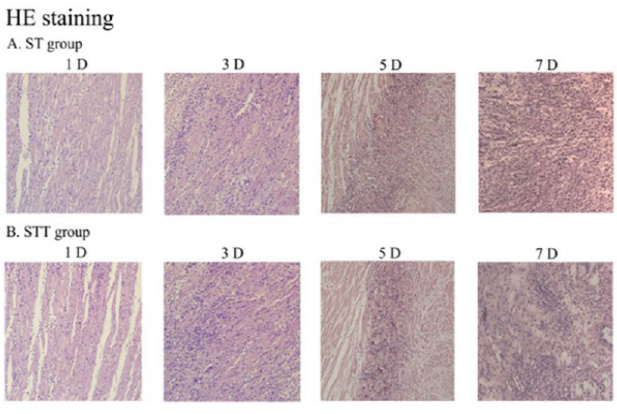

Figure 4: Light microscopic photographs of graft slice (original magnification ×20). Hematoxylin and eosin staining of sections of grafts in ST and STT group. A, ST group, POD 1, no significant lymphocytes inflammation and myocyte necrosis. At POD 3, local inflammation and medium inflammatory infiltration and edema. At POD 5, moderate lymphocytic infiltration and faint myocardial cell necrosis. By day 7, cardiomyocytes necrosis and multifocal aggressive interstitial lymphocytic infiltration. B, Similarly, the HE staining of cardiac grafts in the STT group showed the same acute rejection compared to the ST group.

Similarly, the HE staining of cardiac grafts in the ST group showed the same acute rejection compared to the STT group (Figure 4). □There was no significant difference in rejection grade in the same time points between ST and STT (Figure 4). Thus, these results indicated our simplified HHT model in rat using STT is profitable for HT. (Original magnification ×20)

TUNEL Analysis

Cardiomyocytes of grafts were stained using the TUNEL assay and observed using light microscopy. Positive TUNEL reactivity was observed in the left ventricular of STT rats (Figure 5A) and in ST rats (Figure 5B) on POD 1, and those observations become more obvious on POD 5. TUNEL assay showed the number of TUNEL-positive cells from the ST group, was no significant difference than in the STT group (P >0.05). In addition, the tendency of TUNEL-positive cells was similarly in two groups.

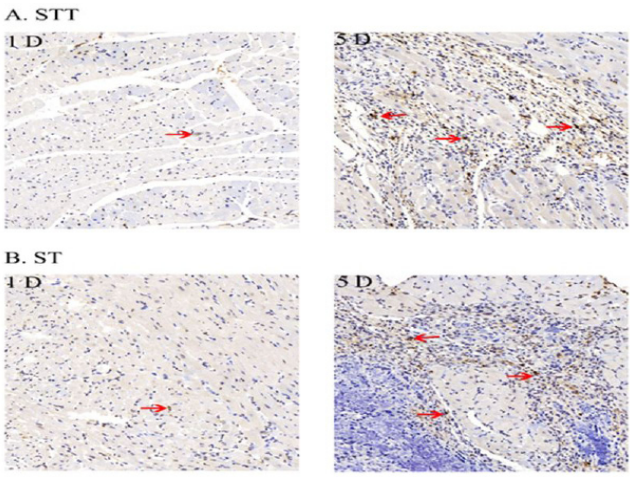

Figure 5: The representative stain apoptotic cells of cardiac sections from left ventricles in the STT (A) and ST (B) groups were measured by TUNEL assay. Positive TUNEL reactivity was observed in the left ventricular of STT rats and in ST rats after heart transplantation (red arrows). (original magnification ×20).

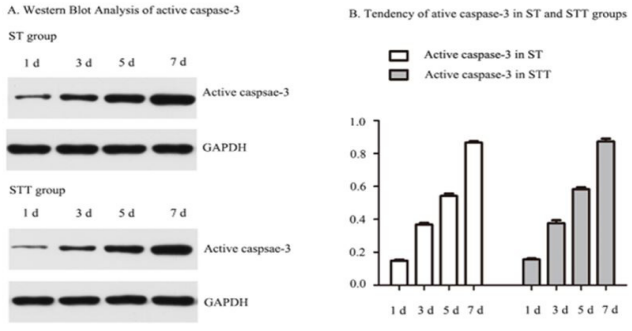

Figure 6: Association of active caspase-3 with apoptosis after heart transplantation in both ST and STT groups. (A) Western blot analysis of active caspase-3 in the cardiocytes at various survival times after operation in two groups. GAPDH was used as an internal control. (B) Tendency of active caspase-3 protein expression in two groups. The bar chart below demonstrates the ratio of active-caspase3 relative to GAPDH expression for each time point. The data are represented as the mean ± SEM (No significantly different in two groups).

Increased Expression of active caspase-3 in ST and STT groups by Western Blot Analysis

To examine the expression pattern of active caspase-3 (a marker of apoptosis), western blot analysis was performed on the two groups at various time points. The active caspase-3 expression had stepwise increased after heart transplantation in ST and STT groups (Figure 6, A, B). Although there was no significant difference of active caspase-3 expression in two groups, their expression indicated that apoptosis of cardiomyocytes had stepwise serious by time-dependent after heart transplantation.

Discussion

According to the different anatomical location, HT can divide into heterotopic and orthotopic transplantation [21]. Orthotopic HT requires complex operations and special equipment, and it is mainly performed for large animal models [21]. Therefore, it is difficult to be realized in the laboratory. However, HHT is mainly in small animal models, and it is easy to be achieved in the laboratory setting. Experimental models of heterotopic transplantation were more advantages as compared with orthotopic transplantation. In addition, HHT has been a fundamental animal model, and commonly used in immunology to study the effect of immunotolerance, new immunosuppressive drugs, and investigation of the mechanism of rejection [22-24].

The animal models used today for HHT consist of femoral, abdominal, and cervical technologies. Although abdominal and femoral techniques have their own advantages, cervical HT still is the crucial model. Cervical HHT is advantageous due to the graft location, which is convenient for investigators to directly observe the beat of the donor heart and to early predict the rejection of graft. Furthermore, cervical HHT prevents complications such as paraplegia, urinary retention, and anastomotic stricture caused by intraperitoneal transplantation [21]. Thus, cervical HHT is appropriate for the study of graft rejection in allogeneic HT.

Up to now, there are two vascular anastomosis methods in HHT: ST and SCT. Using ST method, there exists a problem that the operation is time-consuming, especially for beginner [10,25]. It is reported that the success of intervention is associated with operation time [26,27]. Long ischemia and long operation time have long been thought to be risk factors for low graft survival rates [25,28]. On the contrary, short ischemia and operation time are beneficial to better graft and recipient survival [11,29]. The complexity of the surgical procedure has prevented its widespread use. Therefore, establishing a reliable animal model is an essential prerequisite for in-depth experimental studies.

In this study, we established a simplified HHT model in rat using STT, aimed to improve the HHT model for the study of HT. This model is relatively easy to perform, and postoperative monitoring is simple. Briefly, CCA and EJV were traversed to one end of the 20G and 18g cuff, respectively. They were cut a mouth, lightly overturned, and fixed on outer of the cuff tightly with 7-0 silk suture. The free end of the arterial and venous cuff was inserted into the AA and PA of donor heart, respectively. After the operation, the blood of the donor heart circulated as follows: recipient CCA → donor aorta → coronary artery → myocardium → coronary veins → right atrium → right ventricle → pulmonary artery → recipient EJV. In addition, echocardiography showed compensatory hypertrophy of the right ventricle, which demonstrated that the left ventricle of the heterotopic heart is not involved in normal ejection events.

As compared with ST method, our histopathologic findings in STT confirmed the presence of acute graft rejection when heart beat was absent. There was no significant difference in rejection grade in the same time points between ST and STT. In addition, TUNEL assay and western blot analysis also showed apoptotic cells from the ST group, was no significant difference than in the STT group. Although there was no significant difference of active caspase-3 expression and TUNEL-positive cells in two groups, their expression indicated that apoptosis of cardiomyocytes had stepwise serious by time-dependent after heart transplantation. Thus, our simplified cervical HHT model is equally efficacious in terms of graft study. In addition, our previous study has reported the reliability under this model for subsequent experiments [3,30-34].

Study Limitations

One of the most worth noticed things was whether surface structure of splint tubing would increase thrombosis formation. Due to hemodynamic changes of donor heart, the left ventricle and left atrium of the donor heart are not involved in blood circulation, which increasing the risk of thrombosis. To avoid thrombosis formation, first, the donor animals were injected with heparin via the inferior vena cava to heparinize the heart. Second, we chose the tube which is routinely used in human vessels, and the tube was soaked in heparin sodium before it was utilized. Although there exists some thrombosis formation, it is not easy to form in the tube. No significant difference was observed in thrombosis formation of donor heart between the ST and STT groups.

Like all animal models of human disease, the model we developed here is not a perfect counterpart of any human condition. However, HT induces more complex immune rejections that limit the clinical application. Therefore, establishing a reliable animal model of HT is an important prerequisite for in-depth experimental studies.

Conclusion

In conclusion, the simplified STT is an easy, convenient, stable, and reliable method for HT models. It simplified the rat HHT procedure, shortened operation time, reduced surgical difficulty, improved technical success rate. With these advantages of easier technique, superficial location, and reproducibility, cervical STT model is suitable for beginner and ideal for studying the effect of immunotolerance, new immunosuppressive drugs, and investigation of the mechanism of rejection.

Author Statements

Conflict of Interest

The authors confirm that this article content has no conflict of interest.

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