

Microsurgical techniques for experimental kidney transplantation and general guidelines to establish studies about transplantation immunology¹

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ABSTRACT – Experimental models of organ transplantation played a crucial role to establish the principles of transplantation immunology. The renal transplantation in rodents became the most used model to study the mechanisms of allograft rejection. To perform it, it is necessary to master the microsurgery techniques and the research group should cooperate with other specialists in the field. In this article we review the surgical techniques employed in rats, and we draw guidelines to establish studies about transplantation immunology.

KEY WORDS – Kidney transplantation. Microsurgery. Transplantation immunology. Rat.

Introduction

The principles of transplantation immunology have developed fast after the establishment of experimental animal models. They were critical to discover the mechanisms of tolerance and the genetic basis of cell-mediated allograft rejection. Initially, skin grafts were used to study immune responses; but then, experiments with rat kidney grafts showed that immune response to vascularized organ allografts was different from skin grafts. Since then, the renal transplantation in rodents became the basic model to study the mechanisms of allograft rejection. Experimental transplantation in rats and mice, using microsurgical techniques, opened a new avenue for the conduction of researches about physiology and immunology of transplants, such as the screening of new immunosuppressive agents¹. Among the advantages of this experimental model are: low cost, practicality, more disponibility of technology (transgenic and knock-out strains, monoclonal antibodies, gene mapping, cytokines, etc.), which make it more adequate for the immunological studies².

Methods

Since the rejection reaction will depend on the mismatch of the MHC antigens, the animals should be inbred to standardize the experiments results. In order to search for statistically significance, they should ideally be blindly distributed in groups of at least six animals, and the operations performed by the same investigator.

We prefer to use male rats because of the ease and rapid access to the dorsal penis vein, and 3-months-old animals, since old ones have more fat, what makes dissection more difficult. The age of the animals used is also important for immunological studies, since cell and cytokine patterns change with age.

There are several models of experimental transplantation in rats. The most used ones are:

Model of hyperacute rejection: Guinea-pig to rat (rejection within minutes to hours)³.

Model of acute rejection: DA rats to Lewis rats (acute rejection in 5-8 days).

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Model of chronic rejection: In the model F-344 rats as donors, and Lewis rats as recipients (+ Cyclosporine 1,5mg/kg, i.m, for the first 10 days) allografts develop chronic rejection with 24 weeks⁴. The model where DA rats as donors, and BN rats as recipients (+ metilprednisolone 2mg/kg, azathioprine 2mg/kg, cyclosporine 5mg/kg, i.m, daily) allografts develop chronic rejection with 40-60 days⁵.

Instruments and suture

In order to perform anastomosis as small as 3mm of diameter, it is used surgical microscope with zoom ranging from 8 to 40 times, microsurgery instruments (micro needleholder, n°5 extra-fine jeweller forceps, self-retaining tissue retractor, mini Satinsky, mini bulldogs, aneurism clamps, 7.0 silk thread, 8.0 and 10.0 prolene sutures), catheters, cotton swabs, warm operating board (Temp 38°C), and micro bipolar cautery.

Anesthesia

We use an anesthesia vaporizer with a mixture of isoflurane 1,2%, nitric oxide 2l/min and oxygen 2l/min. Although very expensive, it has advantages such as: rapid induction, safety, control of anesthesia depth, and rapid recovery. Ether, chloral hydrate, xylozin + fentamin, and pentobarbital can also be used⁶.

Pre-operative management

Depending on which protocol of immunosuppression or pre-treatment is to be done, donors or/and recipients are treated before operation. Fasting before operation and sterilized instruments are not necessary.

Surgical techniques

Basically there are two techniques of renal transplantation: orthotopic, and heterotopic. The method of choice depends on the preference of the investigator. But the end-to-size anastomosis is the method of choice for double kidney transplantation, retransplantation, and when the graft has two or more renal arteries.

Since the structures are very fragile, handling during operation is critical. The risk of thrombosis in small caliber vessels is very high, and proportional to the inflammatory reaction on the vessel walls caused by poor surgical technique. The more careful and gentle the manipulation, the better⁷⁻¹⁰. The operator must master the microsurgical techniques in order to standardize the results. Before starting the experiment, it is advised that the microanastomosis success rate during the training period should be higher than 80%, and the time needed to perform the vessels anastomosis less than 30min.

Donor operation

After induction of anesthesia the rat is shaved, placed in a supine position and fixed on the operating board with its tail towards the investigator. To assure normal volemia at the time of harvesting 1ml of saline is given through the penis vein using a fine needle (27G) before starting the operation, and saline is instilled in the abdominal cavity when it becomes dry. A midline incision from the xiphoid appendix to pubis and exposure achieved using small retractors to keep liver and bowels away from the kidney. The bowels can also be wrapped with saline moist gauze and left outside the abdominal cavity, on the contralateral side. Both kidneys can be used, but if just one need to be harvested, it is preferable to use the left one, since the left renal vein is longer. If during inspection we notice that the kidney has two arteries arising at different points on the aorta, it either should be discarded or explanted with an aorta patch and transplanted using end to side technique. Anastomosis of two renal arteries would increase too much the warm ischemia time that the kidney is submitted.

The kidneys can be perfused and harvested isolated or en bloc. We prefer to perfuse through the aorta because it avoids spasm of the small calibre renal artery and consequent perfusion defects during dissection of the renal pedicle. So, the first step is to localise and dissect the inferior abdominal aorta, and the aorta just beneath the diaphragm. Before perfusion it is given 200 units of heparin diluted in 0,5ml of saline through the penis vein. Next, a 20 gauge plastic catheter is inserted just above the aorta bifurcation, the aorta clamped with hemostat below the diaphragm, and the vena cava cut. Immediately after that, perfusion is begun, and cold water instilled in the abdominal cavity. To perfuse the kidney we use 20ml saline dispensed from a bottle 50cm above its the level. If the kidney has to be stored before transplantation it is additionally perfused with 10ml University of Wisconsin solution. It is preferred not to perfuse the kidneys with syringe because it is difficult to standardise the perfusion pressure throughout the experiment, what can influence the ischemia-reperfusion injury and the end results. After this, we separate the kidney from the perinephric fat and adrenal gland. Then, we begin to carefully and bluntly dissect the renal pedicle, avoiding direct manipulation of the vessels. The gonadal and adrenal vessels are ligated with 7.0 silk and divided. With the end-to-end technique the renal vessels are cut close to aorta and vena cava. If the end-to-side technique is to be carried out the renal vessels should have an elliptical patch of the aorta and vena cava, or the renal vessels should be opened up 3mm along its length. The ureter should be cut about the level of the inferior renal pole, and it should not be

stripped of its fat, otherwise its vascular supply will be compromised and necrosis occurs.

Recipient operation

After induction of anesthesia the rat is placed in a supine position and fixed on the operating warming board. To assure normal volemia throughout the operation 1ml of saline is given through the penis vein using a fine needle (27G) before starting the operation. A midline incision from the xyphoid appendix to pubis is done, and exposure is achieved using small retractors to keep liver and bowels away from the kidney.

Before dissecting the pedicle, it is dropped on it a spasmolytic solution to avoid vasospasm. Vasospasm is a common problem in microvascular surgery and it reduces organ perfusion, and predisposes thrombosis at the anastomotic site. It is induced by: direct manipulation of the vessel wall, endothelial damage, hypoxia, cold, bleeding, dryness of tissues, and use of the bipolar close to the vessel. Lidocaine 20% or Papaverine 30mg/ml are the most effective agents¹¹.

The renal pedicle is bluntly dissected and the vessels skeletonized, without stripping the adventitia. Stripping the adventitia is associated with necrosis at the site of anastomosis, vasospasm, and false aneurysm. First, we suture the artery, next the vein, and at last the ureter. Spacing and tension of the sutures are of critical importance.

The warm ischemia time should preferably be below 30 min, in order to avoid irreversible ischemic lesion. Before 30 minutes of warm ischemia kidney transplants function immediately, after 30 min it takes days or weeks to regain function and after 1,5 hour it never functions. The rat kidney is very sensitive and can not be cold stored over 48 hours¹².

Usually we give saline intravenously proportional to the bleeding (one completely moist swab has about 0,1ml of blood) and 5ml saline is instilled into the peritoneal cavity to keep normovolemia or even hyperhydration, since it is important for the renal function to have a high initial filtration rate. Gentle pressure with a cotton swab over the vessel anastomosis for 1 minute usually stops bleeding. We use also hemostatic products around the anastomosis (Gel foamTM or TabotampTM) to reduce bleeding. Fibrin glue is also used to provide hemostasis.

To evaluate patency of the vessel anastomosis we should check⁷:

1. Colour of the kidney
2. Pulsation of the renal artery distal the anastomosis

3. Urine production
4. Up-lifting test
5. Empty-and-refill test

After opening the clamps the kidney should have a bright red colour. When the artery is stenotic or thrombotic, the kidney is pale. If the renal vein is stenotic or thrombotic, the kidney is dark red or purple, the vein segment proximal the kidney dark and dilated, and the urine is hematuric (because of elevated vein pressure). If we still not sure about patency, the "empty-and-refill test" is performed clamping the vessel proximal the anastomosis, then the blood inside the vessel is milked away sliding a closed forceps along the vessel. The clamp is opened, and refilling distal to the anastomosis should occur immediately, if it is patent. The patency rate of anastomosis performed by experienced microsurgeons is more than 90%. Complete endothelial covering of the area of anastomosis takes 8-11 days for the artery and 4-8 weeks for the vein¹⁰.

Urinary reconstruction can be established with ureter-to-ureter anastomosis with or without stent (plastic catheter), ureter-to-bladder anastomosis, and bladder-to-bladder anastomosis. We prefer ureter-to-ureter anastomosis without stent, since it may predispose stone formation and can dislodge and obstruct. Bladder-to-bladder anastomosis is associated with necrosis of the donor bladder segment and neurogenic bladder.

The ureter is sutured with 8.0 or 10.0 sutures. After carrying out the stay sutures, one or two stitches are done on the anterior and posterior side. Care must be taken to avoid suturing it under tension, what can result in ischemia, and then in necrosis and leaking.

The abdominal wall and skin are closed with a continuous absorbable suture (VycrilTM 4.0), which avoids the need of thread removal.

Orthotopic transplantation (end-to-end anastomosis)

Before dissecting, lidocaine 20% is dropped on the renal pedicle to avoid vasospasm. The gonadal and adrenal vessels are ligated and divided, and the kidney is freed from its perinephric fat. Next, the renal vessels are bluntly dissected and skeletonized. If there are two renal arteries arising separately from aorta, one should be ligated and cut. Vascular clamps are applied across the renal artery and vein close to aorta and vena cava, the vessels are cut between their medial and middle thirds (about 3mm away from the clamp) and the cut ends flushed with saline to remove blood and clots. The artery can be dilated introducing the tips of the forceps and opening them carefully. The ureter is cut

at the level of inferior renal pole, and the kidney discarded, leaving place for the donor kidney.

To anastomose the renal artery, two stay sutures (10.0 prolene suture armed with a 3mm needle) are placed at opposite ends of the artery (inferiorly and superiorly), leaving a long piece of thread. During suturing, these angle sutures will help positioning of the next single stitches. The needle should enter the vessel wall at 90°, and take all vessel layers, taking care not to suture the back wall to the front wall, and pulling it following its curvature. Between these stay sutures, two or three stitches should be done, making sure that the distance among them are the same. To tie the suture, two knots are enough and should not to be pulled too tight, because it can damage the media exposing the subendothelium, and cause necrosis. To suture the back of the renal artery, the stay sutures are inverted, pulling the threads of the stay sutures over and under the artery. This twisting of the artery makes what was on the back to be on the front. Again, two to three sutures are performed, depending on the diameter of the vessel. If there is size discrepancy between donor and recipient arteries we can: 1. Dilate the smaller artery, 2. Take larger bites on the bigger artery, 3. Oblique cut the smaller vessel, 4. Perform telescoping suture.

The vein is extremely thin and collapses easily after cutting, so it is advised to flush with saline while suturing to provide better visualization and separate the anterior and posterior walls. Before suturing the vein we should make sure it is not twisted. After performing the second stay sutures, suturing is carried out in a continuous way with 10.0 prolene around the circumference of the vein. One can first begin to suture the back wall of the vein transluminally, or the back wall can be sutured after the front wall, by twisting the vein as described for the artery. Depending on the size of the vein, four to seven times the vein should be stitched on each side, avoiding to catch the opposite wall. Care should be taken to avoid pulling too much the thread, and before tying the sutures, the vein should be gently stretched to avoid stenosis. Before tying the last stitch, the vein should be flushed with saline to avoid air embolism and to remove possible clots.

After both anastomosis are done, the clip over the renal vessels should be promptly removed. First the clip over the renal vein is removed. Blood will flow back into the kidney and bleeding should be minimal and stops soon. The clip over the renal artery should be loosened and replaced successively, with a pause between each to allow pre-clotting along the line of anastomosis. If bleeding persists, the vessel can be clamped again and an addition sticht can be done. If the anastomosis was technically well done, the kidney

should become bright red every time the clip is loosened; if not, the animal can be hypotense and saline reposition should be tried; or the artery is spastic and papaverine can be tried; or the anastomosis is stenotic or trombosed, what nothing can be done.

Heterotopical transplantation (end-to-side anastomosis)

The transplant vessels should have a patch or should be opened along their long axis to increase the diameter of anastomosis. The preparation of the recipient is the same as for orthotopic transplantation, except that the infra renal aorta and inferior vena cava need to be dissected, and after ligating lumbar branches, clamped above the bifurcation with a mini Satinsky clamp, or using two minibulldog clamps. The aorta and vena cava are slit longitudinally with a sharp needle or a small elliptical opening is made with curved scissors, and the openings flushed with saline to remove blood. The elliptical aortotomy opening is associated with lower incidence of trombosis. The size of the openings should match that the size of the patches of the donor vessels. Two stay sutures of prolene 8.0 are placed 180° to each other, and both of them armed with a needle. The continuous suturing can be carried out first at the back wall transluminally; or first the anterior wall and then the posterior wall after flipping the kidney over to the opposite side. After suturing, the distal clamp is opened first to allow preclotting, and after 30 sec the proximal clamp. This should be loosened and applied successively until the bleeding stops, when it should be removed. This technique is faster, reducing warm ischemia time; and has also the advantage of greater anastomosis diameter, what reduces the risks of stenosis and trombosis. On the other hand, it is associated with more bleeding.

Postoperative management and follow-up

Antibiotics are usually not necessary, unless a high dosis immunosuppression need to be given. In this case a single i.m injection of Benzyl penicillin 5000 U/200g is given.

After the operation, the animal is placed under a heating lamp for about one hour and kept alone in a cage for 24h. When immunosuppression is used, it is started just after the operation, and maintained according the experiment protocol. The contralateral nephrectomy is done after 10 days; or it can be done during the transplantation, if the kidney was not stored and the warm ischemia time too short. The nephrectomy is done double ligating “en bloc” renal vessels and ureter with silk 7.0.

The renal graft function is assessed periodically with seric creatinine and urea levels, creatinine clearance; and proteinuria. To collect blood we take 0,5ml from the tail vein, and to measure 24h proteinuria the animals are left for 24h in metabolic cages without food, and weighted thereafter. If a chronic rejection model is used, we take urine and blood after 10 days, and then at monthly intervals. Normal values: creatinine (42,5 μ mol/l), urea (6,9mmol/l), proteinuria (<20 mg/24h)¹³. Biopsies, if desired, can be performed with true cut needle (core biopsy) or with ultrasound-directed fine needle (aspiration biopsy). Death of the animals is generally regarded as renal failure; but we carry out necropsy, and if the organ is not necrotic, it is analysed.

Complications

Early:

1. Mechanical: vasospasm, arterial thrombosis, venous thrombosis, ureteral obstruction by blood clot, urinary leak

2. Non-mechanical: Hypotermia, hypovolemic shock, acute tubular necrosis, infection.

Late: ureter stenosis and hydronephrosis, and chronic pyelonephritis.

By far, the most common complications are bleeding leading to shock, and arterial thrombosis.

Graft harvesting

After the observation time animals are prepared, as described for donor operation, and the kidney grafts perfused in situ (through the plastic cateter at the aorta) with saline for 3 min, to flush out the blood and allow better microscopic visualization of the tissues morphological details. The organ is sectioned coronally and samples snap frozen for PCR and immunohistology; and fixed in formaline 4% for histology.

Assessment

For each operation a report should be filled, where: Type of preconditioning, quality of perfusion, animal weight, immunosuppressive regime, cold and warm ischemia times, quality of anastomosis (vasospasm, stenosis, kinking, twisting, high tension, and bleeding) and quality of reperfusion, are informed.

After the observation time, grafts are taken for histology, immunohistology, and PCR. Histological changes are classified according to Banff criteria (Table 1) or chronic allograft damage index, CADI^{14,15}. Subsets of infiltrating leucocytes, adhesion molecules and cytokines expression are quantified, and statistical differences determined by Student's t test.

TABLE 1 – Banff classification of kidney transplant pathology. It is very important that the research project

1.	Normal
2.	Borderline No intimal arteritis Mild to moderate mononuclear infiltration (>25%) Mild tubulitis (1-4 mononuclear cells/ tubular cross section)
3.	Acute rejection
I	(Mild): Mild to moderate mononuclear infiltration (>25%) Moderate tubulitis (>4 mononuclear cells/ tubular cross section)
II	(Moderate): Severe tubulitis (>10 mononuclear cells/ tubular cross section) And/or mild or moderate intimal arteritis
III	(Severe): Severe intimal arteritis And/or transmural arteritis with fibrinoid change and necrosis Focal infarction and interstitial hemorrhage
4.	Chronic rejection
I	(Mild): Mild interstitial fibrosis and tubular atrophy
II	(Moderate): Moderate interstitial fibrosis and tubular atrophy
III	(Severe): Severe interstitial fibrosis, tubular atrophy, and tubular loss.

involves cooperation among surgeons, immunologists, pathologists and clinicians to perform the work-up and to draw conclusion from the experiments.

Conclusion

The renal transplantation in rodents became the basic model to study the mechanisms of allograft rejection. Mastering the microsurgery skills is essential to perform the delicate anastomosis of this model. There are many variations of the technique to carry out kidney transplantation. To establish a project about transplantation immunology a series of guidelines should be followed, since the work-up of the experiments is complex and need interdisciplinary cooperation.

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RESUMO – Os princípios da imunologia do transplante estabeleceram-se após o surgimento de modelos experimentais. Esses modelos foram fundamentais para descoberta de mecanismos de tolerância imunológica e as bases genéticas da reação de rejeição. Transplante renal em roedores utilizando técnicas de microcirurgia tornou-se o modelo ideal, e abriu um novo horizonte para condução de pesquisas sobre imunologia e fisiologia de transplantes. Neste artigo revisamos as técnicas de transplante renal, e esboçamos diretrizes para elaboração de estudos imunológicos em modelos de rejeição.

DESCRITORES – Transplante renal. Microcirurgia. Imunologia do transplante. Ratos.

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