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Difficulties, guidelines and review of developing an acute rejection model after rat intestinal transplantation☆

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ABSTRACT

Experimental small bowel transplantation (SBT) in rats has been proven to be a useful tool for the study of ischemia-reperfusion and immunological aspects related to solid organ transplantation. However, the model is not completely refined, specialized literature is scarce and complex technical details are typically omitted or confusing. Most studies related to acute rejection (AR) use the orthotopic standard, with small sample sizes due to its high mortality, whereas those studying chronic rejection (CR) use the heterotopic standard, which allows longer term survival but does not exactly reflect the human clinical scenario. Various animal strains have been used, and the type of rejection and the timing of its analysis differ among authors. The double purpose of this study was to develop an improved unusual AR model of SBT using the heterotopic technique, and to elaborate a guide useful to implement experimental models for studying AR. We analyzed the model's technical details and expected difficulties in overcoming the learning curve for such a complex microsurgical model, identifying the potential problem areas and providing a step-by-step protocol and reference guide for future surgeons interested in the topic. We also discuss the historic and more recent options in the literature.

1. Background

Small bowel transplantation (SBT) continues to be an immunological enigma with a high mortality rate [1]. The mechanisms of rejection are not completely understood, and treatment is frequently empiric. Thus, animal research models are still necessary to study immunological pathways and therapeutic alternatives to those currently used.

Experimental SBT in rats has been the most commonly used model due to ethical and economic advantages [2]. However, this technique requires excellent microsurgical skills to overcome a steep learning curve before achieving survival, and worldwide only a few groups of surgeons perform it. Furthermore, mortality during the early postoperative days is high, particularly in the orthotopic model if there is no close monitoring similar to that performed on humans [3]. These complications appear to diminish in the heterotopic model [3–6], although this will never provide the same information as an orthotopic model, which is similar to that experienced in human clinical practice [3,7–9].

An ideal acute rejection (AR) model is difficult to find in the literature for several reasons: only a few groups have published their experience in rat SBT, thus sample sizes are limited; most do not provide many details about the model itself and there are no data regarding the time consumption and cost-effectiveness of the procedure, particularly when starting to reproduce it; most groups use the orthotopic model for AR whereas the heterotopic is more frequently used for chronic rejection (CR); each group uses different strains according to the availability in their respective countries—therefore histoincompatibility and the timing of rejection varies depending on each strain; and the euthanasia day varies among authors. For these reasons, it is difficult to compare the various publications and to establish conclusions before starting as a novice in the field [4–6,8–18].

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In our hospital, where rat SBT has been performed by various surgeons for two decades, the surgical technique has been previously described [6,16,18–20]. When studying rejection, however, there are still important questions about the ideal strain, type of transplant and anastomosis, which was the reason we searched for the ideal AR model in the literature. We also previously published the major complications that occurred during the procedure, which led to finding some significant prognostic factors for success, such as the total transplant duration—particularly the warm ischemia time—and lack of postoperative bleeding, as has been described by others [7]. However, the results were limited by the surgeons who participated with varying skill levels, experience and dedication, and the procedure was not always performed for studying rejection, but also for ischemia preconditioning, bacterial translocation or technical details [17,19–21].

2. Objective

Therefore, the goal of this study is threefold. First, we aim to describe an unusual AR model using the heterotopic technique, providing a step-by-step protocol and guidelines to answer the questions that could help the beginner to make the right decisions. Second, we report the difficulties in developing such a complex microsurgical model, with the aim of shortening the learning curve. Third, we summarize and discuss the historic and more recent options in the literature.

3. Materials and methods

An AR model was developed after SBT. We initially began with the orthotopic model, but we switched to a heterotopic model, which resulted in a higher success rate and longer-term survival. All the experiments were approved by La Paz University Hospital's animal welfare ethics committee.

3.1. Animals/preoperative care

A total of 320 male inbred rats weighing 250–300 g were purchased from Janvier Labs (France): 160 Brown Norway (BN) rats served as donors and 160 Lewis rats as recipients. All the procedures were performed in accordance with the principles of the federal law regarding the protection of animals (RD 56/2013). All the rodents were housed individually in standard animal facilities at La Paz University Hospital until transplantation, at a room temperature of 21 ± 2 °C, relative humidity of 45 ± 15%, maintained at a 12-hr light/dark cycle, and fed commercially available chow (Safe A04, Panlab) and tap water ad libitum. Food was withheld from the donor for 24 h prior to surgery.

3.2. Surgical procedures

Allogeneic SBT was performed using standard microvascular techniques as previously described [22,23].

3.2.1. Anesthesia

General anesthesia was used, with sevoflurane 5% during the induction and laparotomy and 2% for the rest of the procedure, as maintenance.

3.2.2. Donor operation

The procedure was clean but not sterile. Five milliliters of physiological saline was perfused subcutaneously just before the incision. A median laparotomy was performed and the entire small bowel from the ligament of Treitz to 3 cm from the ileocecal valve was prepared on a vascular pedicle consisting of the superior mesenteric artery (SMA) on an aortic cuff and the portal vein (PV). Just before removal, heparin was intravenously administered (0.2 ml 5%), the infrarenal aorta was cannulated, the infradiaphragmatic aorta was clamped and the graft was perfused with Ringer's lactate (RL) until the exiting effluent was clear through the PV. At this point the graft was removed, and the intestinal lumen was flushed with RL (4 °C). The graft was cooled with ice, as is performed in humans, and stored at 4 °C in the same solution until implantation into the recipient after 30–45 min.

3.2.3. Recipient operation (SBT)

We initially began by placing a catheter in the tail vein to keep the animal hydrated during the procedure, particularly just after unclamping. This was useful to keep the animal alive in the first transplants, which had significant bleeding. Once the transplant success rate increased, with very little bleeding, a total of 5 ml of physiological saline perfused subcutaneously at the beginning of the procedure was sufficient, thus minimizing the risks of pulmonary emboli. After mobilization of the cava vein and the aorta from the surrounding connective tissue, transplantation was performed by anastomosing the graft SMA on an aortic cuff to the recipient infrarenal aorta, and anastomosing the PV to the recipient infrarenal cava vein in an end-to-side fashion with 9–0 absorbable suture (Dafilon®). Blood flow was restored after unclamping and the absence of significant bleeding was checked.

In the orthotopic model, the entire native small bowel was resected, leaving only 5 cm of jejunum and 5 cm of terminal ileum. After unclamping and restoring blood flow, both bowel ends of the graft were anastomosed with the corresponding ends of the recipient with interrupted sutures (Prolene 7/0). In the heterotopic model, the native intestine was not removed. After restoring blood flow, the bowel ends were exteriorized as ostomies on the right abdominal wall (Prolene 7/0). Finally, the wound was closed with 3/0 running sutures in two planes.

3.3. Postoperative care

After the procedure, the animals were resuscitated, heated with thermal blankets and placed in individual cages. During the intervention, they were subcutaneously administered tramadol 25 mg/Kg (Adolonta®) to reduce postoperative pain, and again in the following days if necessary. They were immediately offered water ad libitum and food after 24 h.

The animals were observed and weighed daily until euthanization. Their clinical status was assessed daily: appearance, posture, feeding, activity and body weight. Allograft rejection was determined clinically by palpation of induration of the abdomen and by gross examination of the exteriorized stomas. For those with significant weight loss due to low food intake, the water was replaced with 5% dextrose until they began to gain weight. Ceftriaxone 75 mg/Kg/day (saline carrier) and an extra 4–5 ml of physiological saline was subcutaneously administered daily to prevent infection and maintain hydration. Tacrolimus (TAC) (Astellas Pharma S.A. Spain) 0.5 mg/Kg/day (saline carrier) was also subcutaneously administered when indicated. Those animals with poor health, showing graft failure symptoms (e.g., antalgic posture, general discomfort, anorexia) before the scheduled day were euthanized immediately, and all the data were recorded.

3.4. Data collection

With the aim of describing the setup as well as the learning curve, we measured the survival of the animals after the procedure, at 24 h after the procedure and at the time of euthanasia. All the data concerning transplantation were recorded (*learning curve database*, $n = 160$ *SBT*): donor and recipient weight, data regarding the donor surgery, recipient surgery, administration of TAC, duration of anastomosis, duration of warm and cold ischemia and surgery recovery. We registered all intra- and postoperative complications, incidents and survival, as well as evolutive data in the survivors until euthanasia (e.g., daily weight, welfare and treatment toxicity). All problems and difficulties during the study were also recorded, as well as the modifications and strategies employed at each moment to improve results.

Finally, a new database was created including only those surviving >6 days under the heterotopic model (*rejection study group*). All the results provided by the pathologist were recorded.

3.5. Experimental groups

Euthanasia was initially scheduled for the survivors on the 7th postoperative day (POD), the time at which some groups report that animals develop severe AR. However, what we observed at that time was only a mild or indeterminate histologically confirmed AR; thus, based on the findings described by others and our own experience, we euthanized on the 14th POD, when we observed severe AR.

Thus, the recipients were divided into 5 experimental groups (all under the heterotopic model), according to the immunosuppression and observation time:

1. ALLO Control _7: euthanize at 7th POD ($n = 9$)
2. ALLO Control _14: euthanize at 14th POD ($n = 17$)
3. ALLO + TAC_7: TAC group (daily); euthanize at 7 POD ($n = 3$)
4. ALLO + TAC_14: TAC group (daily); euthanize at 14 POD ($n = 6$)
5. ALLO + TAC5_14: TAC short-course group (5 days, from POD 0 to POD 4); euthanize at 14 POD ($n = 15$)

Groups 1 and 2 would allow us to establish the AR model at different times and to analyze the mechanisms involved in rejection; groups 3, 4 and 5 would allow us to study the effects of TAC on AR.

3.6. Euthanasia, graft evaluation and sample collection

The animals were again anesthetized with isoflurane and a new laparotomy was performed at the end of the observation period. At the time of necropsy, a general inspection of various organs was made and rejection was evaluated macroscopically according to the appearance of the graft surface, Peyer's patches, mesenteric lymph nodes and mesentery thickness. The recipient's abdominal cavity was inspected for the presence of ascites, adhesions and macroscopic signs of peritonitis or abscesses. Samples from allografts and various tissues were obtained for histological and immunohistochemical analysis, just after exsanguination. The bowel lumen (native and graft) was flushed and washed with saline.

3.7. Sample studies/histopathological analysis

Grafts harvested from the rats, including the mesentery with lymph nodes, were fixed in 10% formalin, embedded in paraffin and cut into 5 μm sections, which were stained with hematoxylin-eosin following standard methods for routine morphological analysis. The slides were blindly reviewed by an expert pathologist for histological signs of AR, and were graded as described by Wu et al., according to the criteria established at the workshop at the XIII International Small Bowel Symposium (2003) (Table 1) [24]. Tissues were also frozen in liquid nitrogen and placed directly into a 80 °C freezer for future biochemical studies.

3.8. Statistical analysis

The statistical analysis was performed with IBM SPSS Statistics 19. The quantitative data were expressed as mean values ± standard deviation. The chi-squared test was selected for comparison between groups. A *P*-value of ≤0.05 was considered statistically significant. The validity of the acute rejection model was analyzed.

Table 1
Histologic grading for small intestine allograft acute rejection: Established criteria at the workshop at the XIII International Small Bowel Symposium.

	Indeterminate*	Mild ACR	Moderate ACR	Severe ACR
Grade	Ind	1	2	3
Inflammatory infiltrate	Minimal and localized	Mild and localized, with activate lymphocytes	Widely dispersed, in lamina propria	Widely dispersed, in lamina propria
Crypt epithelial injury	Minimal	Mild	Diffuse	Diffuse
Crypt epithelial apoptosis	Increased (usually with <6 apoptotic bodies/ 10 crypts)	Increased (usually with >6 apoptotic bodies/ 10 crypts)	Increased with focal confluent apoptosis	Increased with focal confluent apoptosis
Architectural distortion	None to minimal	Mild	More prominent. Possible mild to moderate intimal arteritis	More prominent. Possible mild to moderate intimal arteritis
Mucosal ulceration	No	No	No	Yes

*Changes insufficient for the diagnosis of mild acute rejection.

4. Results

4.1. Learning curve

a) *Average surgical times* (Table 2): Curves were significantly descending throughout the study (Fig. 1). The success rate for the time or "learning curve" is shown in Fig. 2. From a total of 160 SBT performed by the same surgeon (March 2013–November 2014), 135 recipients recovered after the surgery: 73% from the first half of the procedures ($n = 68$), 96% from the second ($n = 77$); 52% were alive 24 h later ($n = 83$): 17% from the first half ($n = 14$), 86% from the second ($n = 69$).

b) *Type of transplant*: The first 95 transplants were orthotopic; 11 of them survived between 4 and 6 days with no treatment, although none of them could be sacrificed alive. Due to high mortality at the 3rd or 4th postoperative day when most of the rats had already survived >48 h after the transplant, we switched to the heterotopic model. Survival drastically improved: Thus, the last 65 SBTs were heterotopic, 50 of them (77%) survived ≥6 days and 46 (92%) were successfully euthanized at the scheduled day and included in the rejection study. Only 7 of the 15 not included were due to technical defaults (2 thrombosis, 5 bleeding).

c) *Factors influencing survival*: Reasons for premature death in the orthotopic transplants were searched for: first, the accumulated expertise probably provoked a sudden change in survival at that moment; secondly, early underdiagnosed anastomotic stenosis or leaks could lead to sepsis and death; however, the autopsy of the 11 recipients who survived between 4 and 6 days after orthotopic

Table 2
Average surgical times.

	Learning curve ($n = 160$)	Rejection study group ($n = 50$)
Donor surgery	38 ± 8 min	32 ± 6 min
Venous anastomosis	18 ± 6 min	15 ± 6 min
Arterial anastomosis	16 ± 4 min	14 ± 3 min
Warm ischemia	38 ± 9 min	35 ± 7 min
Recipient surgery	98 ± 20 min	86 ± 15 min
Total transplantation	152 ± 23 min	138 ± 20 min

SBT did not show leaks, but dilated or normal bowel, probably due to stenosis, although rejection could be beginning at that point. Third, other technical hurdles could be also responsible and should be taken account for future studies: to prevent paralytic ileus not flushing the bowel lumen too strongly, use better preservation solutions, such as UW, prophylactic antibiotics in order to prevent sepsis and starvation, …These technical details seem to be crutial in the orthotopic model, although the microvilli damage, bacterial translocation… could also influence results in the heterotopic model and should also be taken into account

d) *Other considerations*: Other several factors were detected during the experiments and consequently modified:

I. *TAC treatment*: The first animals treated with TAC showed clinical sepsis and ongoing decline leading to death between the 5th and 7th POD after heterotopic SBT, which had not occurred with the first controls; this was attributed to immunosuppression, thus daily ceftriaxone was included in the protocol to prevent infection, with great success, given no sepsis recurred from this time forward.

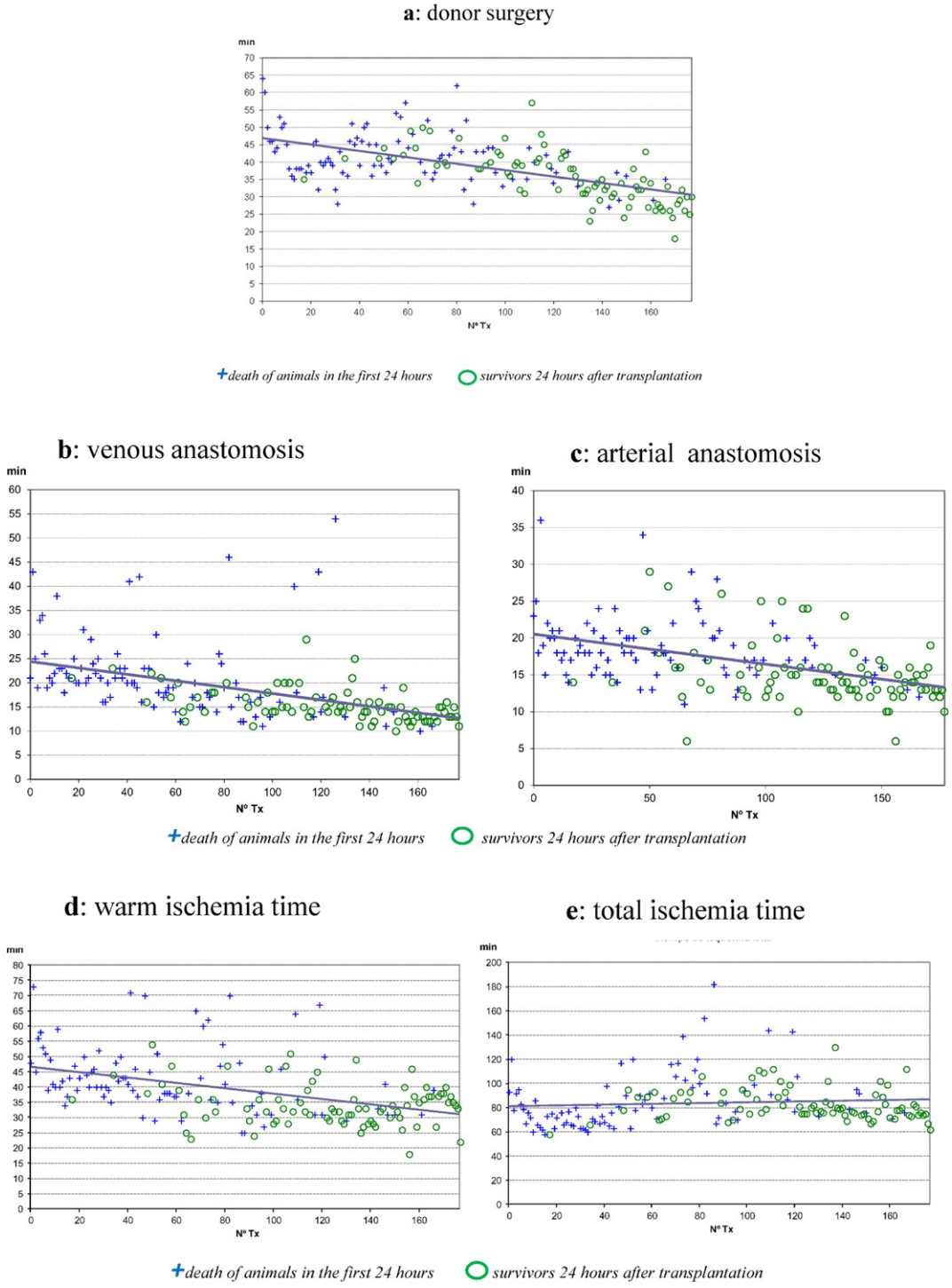

Fig. 1. Learning curves related to the duration of the procedures: 1a: donor surgery; 1b: venous anastomosis; 1c: arterial anastomosis; 1d: warm ischemia time; 1e: total ischemia time; 1f: recipient surgery; 1g: total transplant surgery.

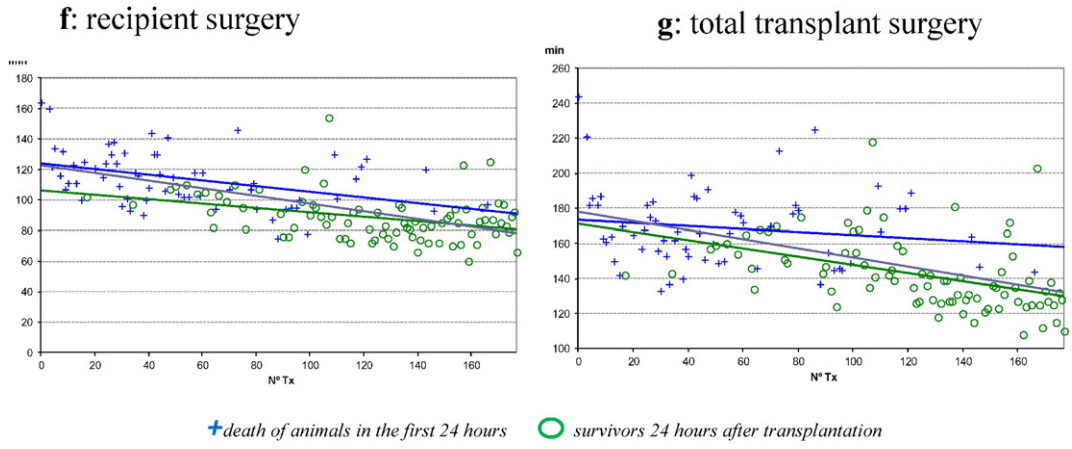

Fig. 1 (*continued*).

II. *Intraoperative complications* are described in Table 3; these include anesthetic problems, such as emboli, hypovolemic shock and central apnea. These complications were overcome by infusing physiological saline subcutaneously (5 ml) before beginning the procedure, employing exhaustive hemorrhage control by pressing gently with a cotton swab at the anastomosis site, removing sevoflurane for several seconds and increasing oxygen flow to recover the respiratory reflex. Regarding the surgical technique, 13 donors were scored as "poor" because of bleeding or poor perfusion. Venous complications (bleeding, stenosis) predominated over arterial complications (bleeding, thrombosis), all leading to death.

III. *Late complications* (*survivors* > 24 h): These included arterial thrombosis, intestinal obstruction (in the orthotopic model), stoma problems with evisceration requiring surgery (in the heterotopic model) and clinical sepsis, confirmed in the autopsy (Table 3). The reason for death remained unknown in 18 cases, although it was most probably multifactorial, including bleeding, particularly during the first half of the procedures. Ostomy care prolonged survival in these long-term survivors when they began to develop rejection, with mucus obstruction and abdominal distension. A simple abdominal massage or opening the stoma with microforceps was effective to empty the mucus, preventing premature death secondary to abdominal distress and consequently respiratory distress.

4.2. Rejection study group

4.2.1. Small bowel transplantation

Fifty transplants were successfully performed with the heterotopic technique and included in the Rejection Study Group, obtaining an homogenous sample. Average surgical times were shorter than that of the entire database group (Table 2).

4.2.2. Postoperative care and observation

Donor and recipient average weight just prior to transplant was 253 ± 23 g and 301 ± 39 g, respectively. All the recipients gained weight at 24 h (average 3 g) due to the saline injection during the surgery and to the additional graft placed in the abdomen. After the second day, the animals recovered their previous weight before transplant and began to lose weight (average 10 g/day) until the 5th or 6th day. After that, the animals stabilized and began to regain weight (average 3 g/day), and food intake was confirmed by the presence of well-formed feces in their cages. However, the recipients that started to develop rejection stopped regaining weight, lost fat and muscle mass and the abdominal circumference increased (Fig. 2). The stomas had a good appearance in most of the animals, except for 2 recipients, requiring new surgery due to stoma prolapse. Most of the animals expelled mucus through the stoma during the first week, during which those who developed rejection began to present abdominal distension and induration, which increased progressively throughout the following days

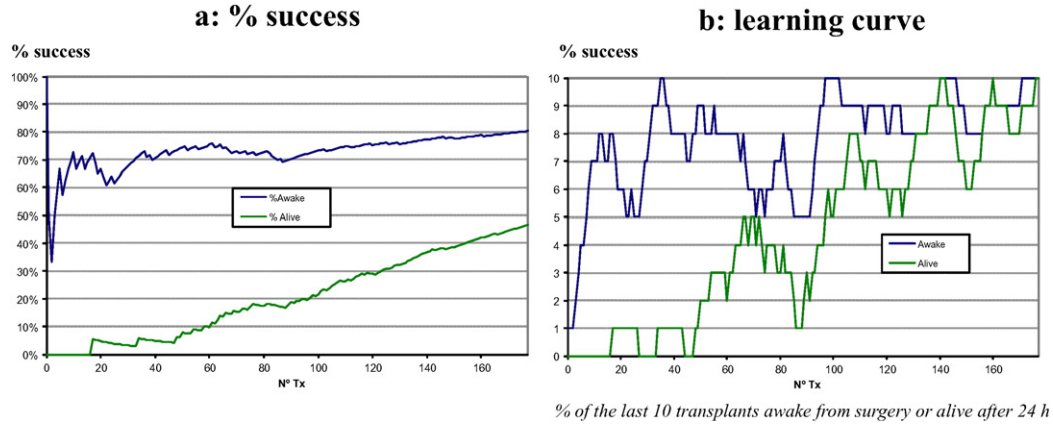

Fig. 2. Learning curves related to survival: 1a: success rate until the time of measurement; 1b: % of the last 10 transplants awake from surgery or alive after 24 h.

Table 3
Surgical complications (intraoperative and after transplantation).

Intraoperative complications	
• Anesthetic: emboli, hypovolemic shock, central apnea	16
• Venous:	26
o bleeding	13
o stenosis, congestion, thrombosis	13
• Arterial:	19
o bleeding	13
o acute thrombosis	2
• Poor donor/poor reperfusion	13
Late complications	
• Arterial thrombosis	4
• Intestinal obstruction	4
• Stoma prolapse/evisceration	6
• Sepsis	9

until euthanasia, and it became necessary to empty the stoma by abdominal massage. This need was particularly evident in those groups without tacrolimus treatment, followed by those with tacrolimus treatment for only 5 days, and compared with those without tacrolimus. This last group had a normal abdomen throughout the observation time until euthanasia.

4.2.3. Euthanasia and macroscopic graft evaluation

All the animals were alive until euthanasia except 3 rats, which had just died a few minutes or hours before. Those with severe rejection had important abdominal distension preventing them from moving easily. No macroscopic abnormalities were observed in the remaining organs in any case, except for the graft, which showed varying degrees of rejection depending on each group, with important mesenteric involvement in the most severe cases. In group 1, the graft was dilated and full of mucus, with a normal or mildly thickened mesentery, strikingly different from a normal native intestine. In group 2, the graft presented severe fibrosis not allowing separation of the bowel loops, with a thickened mesentery, making it difficult to differentiate both tissues. The mucus appeared extraluminal in one case, secondary to a perforation in the setting of rejection. The spleen size of those animals with severe rejection was bigger than in those with normal grafts. The native bowel was normal in all cases. All the animals from groups 3 and 4 appeared to be healthier than the rest, with a macroscopically normal graft (Fig. 3).

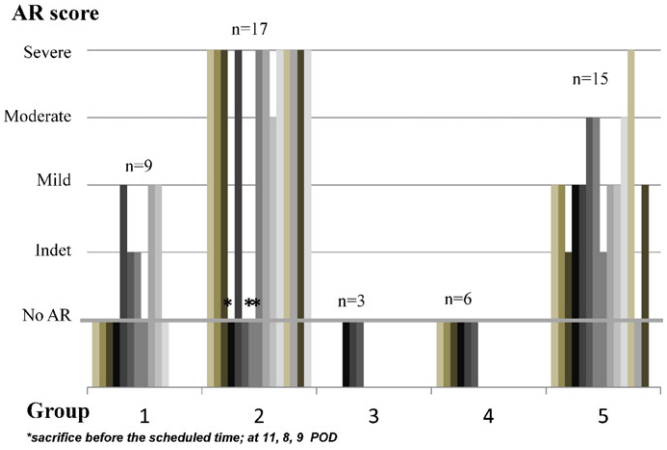

Fig. 4. Acute rejection score according to the Miami classification ($n = 50$).

4.2.4. Histological study of the graft

a) *Small bowel*

Macroscopic appearance of the rejection correlated well with the histological score (Figs. 3 and 4). All the controls from group 1 showed indeterminate or mild AR—even no rejection in some cases—most with diffuse lymphocyte infiltration and minimal apoptosis, despite the severe dilation and the large amount of mucus in the bowel lumen. However, all the controls from group 2 showed severe AR with mucosal ulceration and distorted architecture, except for one recipient that developed only moderate rejection ($p < 0.05$). Interestingly, 3 animals from this group were euthanized before time (POD 8, 9 and 11 after transplant), and none showed signs of acute rejection. Also note that the control animal that was sacrificed at 11 POD due to poor clinical status showed moderate rejection, so this might indicate when rejection progresses from moderate to severe.

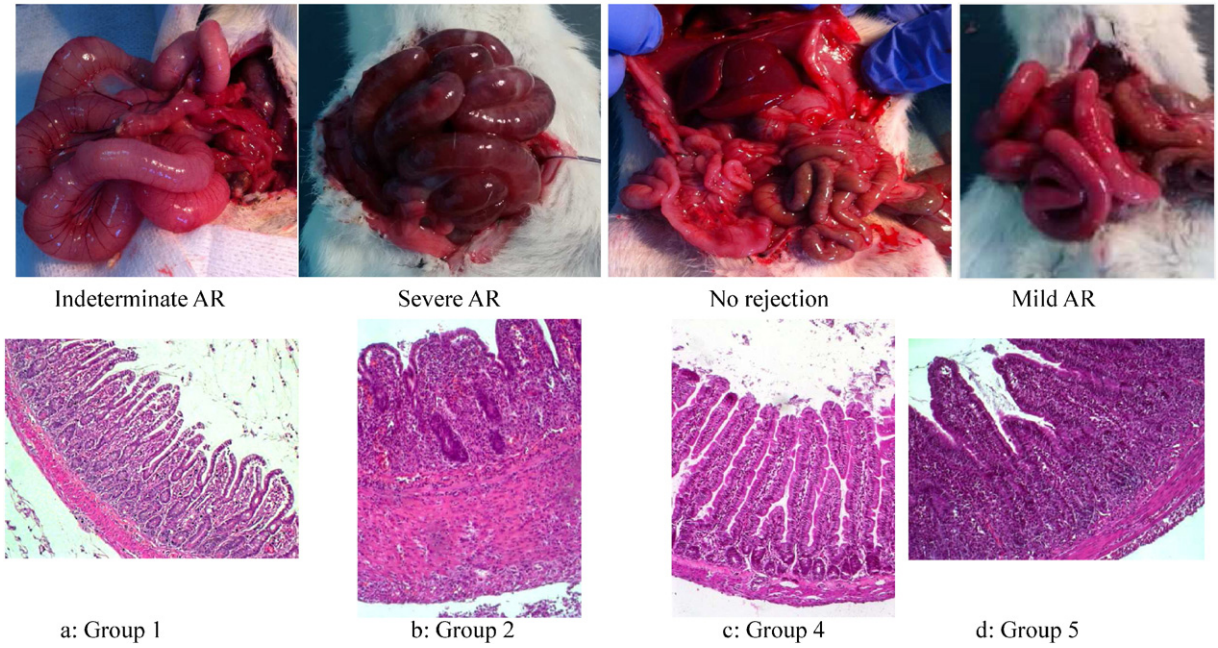

Fig. 3. Macroscopic and histologic graft appearance at euthanasia. 3a: ALLO Control_7 (group 1); 3b: ALLO Control_14 (group 2); 3c:Tacrolimus group (group 3 and 4); 3d: Tacrolimus short-course group (group 5), only 5 days.

All the animals from groups 3 and 4 showed a histologically normal graft. However, all those treated with TAC for a short time (group 5) developed mild rejection with >6 apoptotic bodies per 10 crypts. The degree of rejection ranged from indeterminate to moderate ($p < 0.05$); one animal did not develop rejection at all. These small differences could be explained by variations among individuals with respect to the absorption of TAC. Animals were weighed daily to calculate the dose, and 1 ml syringes were used to minimize errors by making the dilution. Only one animal showed severe rejection and this was probably due to the venous congestion during the procedure, given the rat was bleeding through the stoma during the first postoperative days. The animal also required a new surgery, so the rejection score could have been overestimated.

b) *Mesentery*

Animals from groups 3 and 4 did not develop rejection and had normal nonreactive lymph nodes. Those groups with mild or indeterminate rejection showed mild inflammation and little fibrosis at the mesentery. With the advancing degree of rejection, these lymph nodes appeared microscopically more lymphocyte-depleted and were replaced by macrophages. Those with severe rejection were lymphocyte depleted, showing empty lymph nodes, with severe necrotizing enterocolitis and fibrosis.

5. Discussion

Small bowel transplantation has been one of the last organ transplants to be used in humans because of the risk of developing rejection due to its immunogenic characteristics. Schraut et al. were the first to publish the transition from the experimental to the clinical field [25]. Since the first successful clinical transplant in 1989, >5000 SBTs have been performed around the world [1,26,27].

Results improved with the introduction of TAC in the early 1990s, with more centers beginning to perform SBT, although still far from the success achieved with other solid organs [1,28–30]. Once the surgical technique and the patient selection improved, problems with SBTs are primarily immunological, such as rejection or graft-versus-host disease, among others [31,32]. When these complications appear, treatment is frequently empiric, beginning with steroids, but second and third therapeutic lines are often necessary, along with powerful immunosuppressants that sometimes do not achieve the necessary tolerance between graft and host [31,33–35]. This rejection leads to retransplantation and sometimes to death, with survival approximately 60% after 5–10 years [1,27,36]. Research studies in the clinical as well as in the experimental field are therefore crucial to understanding the mechanisms involved and for designing therapeutic strategies.

Experimental SBT was described several decades ago, but few surgeons have achieved prolonged survival [10]; thus, sample sizes are usually reduced, infrequently applicable, and conclusions are difficult to establish. Historic experimental SBT series were searched in this study, examining the technical aspects, postoperative care and strains; few studies were found explaining the multiple problem areas to enable successful SBT and shorten the learning curve.

In 1971, Monchick et al. were the first to describe the heterotopic SBT technique (HSBT) in rats [22]. Prior to this report and throughout the 1970s, we also find experimental studies on dogs [37–43]; however, the rat soon become the ideal animal for research due to obvious ethical and economic reasons [41,44,45]. Later in 1973, Kort et al. published the orthotopic model (OSBT) [44], which was later modified and simplified by Harmel et al. in 1984 [46]. From the 1980s, we found numerous papers contributing to advances in model development, primarily focused on diminishing the complexity of the procedure, improving surgical times and achieving higher success rates. Sonnino et al. described a simplified and shorter technique of HSBT in 1986, working only on a 15 cm jejunal segment with no dissection of the other donor branches. Although they mentioned some of the surgical complications and tips such as slowly washing the bowel lumen, no further details are provided in the paper, including the rat strains. Theirs was also a small sample, with only 8 survivors [14].

Two years later, Wallander et al. described the cuff method to simplify the heterotopic model, in which warm ischemia time was reduced [47]. Several articles from the Kobayashi group subsequently supported this technique for various models such as rejection [48]. Later, Nakao et al. made a comparison between the cuff method and the traditional method (hand-suture), in effect confirming a reduction in operation time, but also observing two remarkable limitations: the required recipient nephrectomy and the limited use for short segment SBT, given only 40 cm of the graft could be supported [49]. They also concluded that theirs was not the ideal technique for someone trying to develop microsurgical skills; however, use of this technique by others has resulted in useful liver and multivisceral transplantation, as observed in the orthotopic model. More recent publications have reported improved surgical times by performing the anastomoses to the renal vessels instead of the aorta and cava, with subtle modifications [4].

During the 1980s and 1990s, several studies emerged comparing portoportal with portacaval anastomosis, but no relevant differences were found; thus, experiments in rats using portoportal anastomosis, which is a more complex technique, have been reserved for specific objectives [19,50]. In our center, we traditionally opted for the complete and orthotopic hand-sutured SBT. The use of bipolar and improved microsurgical devices has significantly reduced surgical time compared with 20 years ago.

Regarding surgical and anesthetic complications, articles are scarce and would be useful for the beginner. In our study, we observed how most of the recipients began to survive from one point in the number of procedures performed, which should motivate researchers to persevere. We previously reported that the first transplant successfully performed in our center occurred after a median of 46 transplant procedures [7]. In 2007, Galvao also reported that only 5% of students were able to achieve survival, since it required the surgeon to have a considerable amount of training and perseverance [10]. Improvements in small details will likely be necessary before achieving a homogeneous model, which will require repeated and meticulously studied surgical and anesthetic maneuvers.

5.1. Heterotopic vs. orthotopic model

Some studies emerged in the 1990s comparing OSBT with HSBT [2]. Each has its advantages and disadvantages, and it is important to choose appropriately according to the purpose of the study [3,51]. In expert hands, there are no significant differences regarding transplant duration [52,53]; however, differences regarding timing and evolution of rejection must be kept in mind. In syngeneic combinations, all the recipients survived >60 days with intact grafts. The HSBT recipients survived longer after a fully allogeneic combination, despite developing severe rejection due to graft encapsulation. This evolution is different from OSBT, in which conditions such as intestinal obstruction and weight loss due to rejection led the controls to death.

Another advantage of HSBT over OSBT is the lack of intestinal anastomoses, in which complications are commonly observed. However, refinement of the surgical technique and other maneuvers, such as gently washing the lumen to prevent paralytic ileus, as well as the appropriate use of antibiotics can minimize these complications. The maintenance of the stomas, however, requires the recipients to be housed individually to prevent stoma cannibalism [52], as well as the removal of the mucous secretion from the transplanted intestinal lumen by irrigation to prevent excessive dilatation.

On the other hand, a disadvantage of HSBT is that the heterotopically transplanted graft is disconnected from the normal gastrointestinal transit displaying mucosal atrophy and severe changes in the microbiome

that can impact on the degree of intestinal wall inflammation and, also, rejection. It is known from IBD and transplant research that the mucosal immunity and inflammation is closely regulated by nutrients, commensals, enteric glia cells and other intraluminal trophic factors, and all these regulative mechanisms are bypassed in the heterotopically situation. A higher overgrowth of pathogens with bacterial translocations and sepsis in the heterotopic model has also been described [54]. However, intestinal permeability was described to be higher after HSBT than after OSBT by others, which would allow use of the model for several research purposes [51].

To date, HSBT has not been used to study early graft changes secondary to rejection [14]. In our experience, HSBT resulted in a valid and technically easier model, since long-term survival improved dramatically once we switched to HSBT at a time when most of the animals were already living at least 3 or 4 days after OSBT. Therefore, although the orthotopic model would be the ideal since it is more similar to human clinical practice, and should be attempted, the heterotopic one has also its advantages and at least the two methods can give complementing results about the molecular mechanisms of AR after SBT.

5.2. Preservation solution

Cold normal saline or Ringer Lactate (RL) have been the most tipically used preservation solutions, probably due to economical advantages. Orloff et al., Li et al., Nakao et al. use normal saline in their models. On the contrary, studies from Pittsburgh have historically used RL often combined with neomicyn sulphate solution. As RL seems to be the most widely accepted choice, we have traditionally used it in our center, with good results. However, Pech et al, and more studies from Germany, used UW satisfactorily in the orthotopic model, and it would be a good model to imitate, since this solution or similar is used in the human clinical setting, minimizing reperfusion injury and it will definitely impact results, particularly after OSBT.

5.3. Acute rejection

Many rejection studies emerged, coinciding with the first SBT in the clinical setting. During the era before TAC use, studies focused on the ideal cyclosporine (CsA) dose were performed by Lee at al. in isolated SBT [55] and later by Murase et al., who studied multivisceral grafts including the liver [56]. An interesting histological description of rejection can be found in the Murase study, particularly in the controls with no immunosuppression, as well as many references from the 1980s related to bowel rejection. Fishbein also described apoptosis in rat SBT [57].

The first evidence for the superiority of TAC over CsA dates back to the early 1990s; Lee et al. developed a rejection OSBT model with low-dose TAC [58–60], although results differ with later publications. Several studies regarding the ideal dose appeared at this time [61]. Murase at al. observed the ability of TAC to prevent rejection in liver transplantation, which developed spontaneous tolerance after a transient rejection crisis [62]; this was also described later by Thiede et al. using the same rat strains [63]. This tolerance was not as strong in heart transplants and was even less in SBT, independently of the rat strain, particularly when using low doses to reduce immunosuppression [62]. Meyer et al. described absence of rejection in liver/small bowel transplant using 1 and 2 mg/Kg/d of TAC, and 80% survival when using 0.5 mg/Kg for only the first 5 postoperative days; thus—and similarly to our research—this is the dose that was used for rejection studies [63].

Pech et al. from Germany published 3 studies between 2011 and 2012 using a BN-Lewis combination to develop AR after OSBT, similarly to studies from Pittsburgh, with the technique described previously by Schaefer. This group performed studies related to the intestinal muscularis and the relationship between dysmotility and immunological events [64–66]. We used the same score (0–3) to determine rejection [24]. First, Pech et al. studied the effects of the immunosuppressive agents when AR appeared; they then studied the specific combination of TAC and infliximab to reduce the inflammatory response and dysmotility; finally, they studied the regeneration, residual function and immunological status after treating rejection [8,9,13]. We initially attempted to reproduce the Pech model in our experiments. Interestingly, they euthanized the control group at the 7th day to prevent extreme suffering of the rats after developing severe AR. Pech and Schaefer described the onset of AR from the 4th postoperative day, mild at that time, and severe from the 7th day. When we began to euthanize the recipients at the 7th day, however, we did not find relevant histological signs of AR, but minimal or insignificant changes. In addition, when they administered TAC 1 mg/kg/d for 14 days until euthanasia, with or without sirolimus, the autopsy showed indeterminate rejection; however, this finding was not observed by our pathologist using an even lower dose for 14 days, showing an absolutely normal bowel.

These differences might be explained by the fact that these studies used the orthotopic model compared with the heterotopic one that we ultimately used. However, this approach differs with prior articles from Nakao et al. [3], in which rejection appeared to occur earlier in the heterotopic model. Finally, in 2012, Hu et al. published the characteristics of chronic rejection (CR) using Lewis-BN rats as well, and they were similar to our findings [67]. Although this was a study related to donor-derived bone marrow transfusion and CR, the description of the findings in the controls helped us to develop our model because they also described mild AR observed in those controls that were euthanized at the 7th day, which became severe AR by the time they were euthanized at the 14th day and before starting to develop CR, as in our findings. However, it is remarkable in this study that they could keep the rats alive for months. In our experience, we observed that the animals with severe AR at the 14th day had poor clinical status, malnourishment, severe abdominal distension and induration, which did not allow them to breathe properly and makes it difficult to imagine that these animals could survive longer. Despite the results, the sample size in this study is again small and the conclusions are not significant.

5.4. Rat strain

It was difficult to determine which was the best strain to use to establish a useful AR model and the ideal moment to determine the typical histological features [67–72]. We initially used Wistar rats as recipients based on previous publications from our center (not directly related to rejection) [17,19–21]. Because we did not find signs of rejection at the 6th day, we changed to Lewis rats, based on the previously described models [8,9,13,73,74], and given the fully allogenic BN-to-Lewis combination appears to be histoincompatible and rejection has been described as the dominant immunologic response either without or with immunosuppression [56,75]. BN rats have been reported to have "universal donor" qualities for unknown reasons. The most solid clue was evidence of a defect in the invariant chain of MHC class II molecules in this strain, which could lead to inefficient processing of donor antigens without necessarily implying immune abnormalities [76].

In 1994, Tanabe et al. studied the influence on intestinal transplant outcome of 12 donor-recipient combinations of 4 fully allogeneic rats (LEW, BN, August Copenhagen Irish [ACI] and Piebald Viro Glaxo [PVG]) [73,74]. Differences were only found in long-term survival depending on the strain and the duration and dose of FK-506. According to this study, if we had maintained the short-dose TAC longer in our study, we would have found CR, which is interesting for future studies. In all combinations, the untreated recipients died of rejection between 5 and 14 days, meaning that all could be useful for developing an AR model. In another study, Sheng Sun found graft loss between days 4 and 6 in controls when using a heterotopic model with ACI-Lewis rats to study the role of immature dendritic cells in preventing rejection; this again differs with our results, in which rejection at the 7th day was indeterminate [77].

Interestingly, several studies found that the Lewis-to-BN combination resulted in a useful graft-versus-host-disease (GVHD) model; here, Murase et al. explained the different immunologic reaction, describing the variations in lymphocyte populations for each strain, although they did not provide much more information. It is also important to consider the higher weight and strength of Lewis rats compared with BN rats of the same age, which are more labile and vulnerable, which is crucial to know when planning a difficult postoperative period. Based on previous studies, Ye et al. described a similar GVHD model with ACI-Lewis combination rats, although they also did not provide details [76,78–83].

5.5. Chronic rejection (CR)

Since the late 1960s, transplant models involving Lewis and Fisher 344 rats have been used to develop CR [84–87] because these strains differ at "weak histocompatibility loci" [5,12,88]. Further modification of these models by the use of short-term, low-dose CsA achieved the consistently reproducible vascular lesions typifying CR [89]. In 1990, Lee et al. developed a heterotopic model of CR, to compare TAC and CsA effects [59]. Orloff developed a reproducible HSBT model of CR in 1999 based on the work of White et al. [86], with excellent survival rates; she described the clinical features of AR in controls appearing after a median of 11 days after transplantation: progressive weight loss, decreased physical activity and graft induration; histologically, AR was characterized by apoptosis and mesenteric lymph node enlargement, probably representing lymphocyte expansion associated with mononuclear proliferation as part of the early immunological response in both AR and CR, similar to our findings despite the different rat strains [5,88]. However, differences with this F344-Lew combination were found in the long-term follow-up: although all the animals treated with low-dose CsA for 15 days showed histological CR (villous blunting, lamina propria fibrosis, mesenteric inflammation and fibrosis, epithelial cell apoptosis and lymphocyte depletion in the mesentery), only a few showed clinical and macroscopic signs of rejection, such as shrunken or scarred lymph nodes, whereas others gained weight and appeared healthy with no clinical signs of rejection at the predetermined endpoint of 120 days. In this study, Orloff emphasized the transplant vascular sclerosis, present in the majority of mesenteric vessels, as likely responsible for ultimate graft failure, and the importance of macrophages, CD4 and CD8 + cells in the development of CR.

6. Conclusion

In summary, numerous articles regarding rat SBT and rejection have been published in recent decades; however, most do not provide details about the model itself or the difficulties in achieving survival, which would be useful for the beginner. There are few studies to help make the learning curve shorter. The orthotopic model to study AR has been the rule, despite the lower sample size and higher technical difficulties, whereas the heterotopic model has been reserved to study CR. Various strains have been used, and the exact time when AR appears differs between authors. In this study, we developed a simpler unusual AR model for SBT using the heterotopic technique, with excellent survival rates, obtaining a wide spectrum of rejection scores depending on the dose and the sacrifice day. Therefore, although the orthotopic model would be the ideal since it is more similar to human clinical practice, and should be attempted, the heterotopic one has also its advantages and at least the two methods can provide complementing information about the molecular mechanisms of AR after SBT.

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