UNIVERSITY OF ALBERTA

THE IMPACT OF SURGICAL TECHNIQUE ON THE INCIDENCE AND SEVERITY OF GRAFT VERSUS HOST DISEASE (GVHD) IN A RAT SMALL BOWEL TRANSPLANT MODEL

BY

AYDIN TARIK KIZILISIK

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Aydin Tarik Kizilisik, M.D.

424 RH Misheuer Park

Edmonton, Alberta T6H 4M5

DATE: **January 1994**

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **THE IMPACT OF SURGICAL TECHNIQUE ON THE INCIDENCE AND SEVERITY OF GRAFT VERSUS HOST DISEASE (GVHD) IN A RAT SMALL BOWEL TRANSPLANT MODEL** submitted by Tarik A. Kizilisik in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE IN EXPERIMENTAL SURGERY.**

Norman M. Kneteman (Supervisor)

Ray V. Rajotte (Committee Member)

Alan B.R. Thomson (Committee Member)

Richard N. Fedorak (Committee Member)

January 12, 1994

DEDICATION

This text is dedicated to **Sema** and **Basak** for their continuous love and patience and to my parents **Suat** and **Gulen Kizilisik** who have always provided unfailing support and encouragement.

ABSTRACT

Small bowel and its mesentery contain a considerable amount of lymphoid tissue that can mediate graft versus host disease (GVHD) in small bowel transplant recipients. To prove that GVHD intensity is greater if a heterotopic procedure is performed, 12 adult Lewis rats received heterotopic and 12 received orthotopic small bowel transplants from Brown Norway (BN) donors. 12 Lewis->Lewis heterotopic small bowel transplanted animals served as the control group. All recipients were given cyclosporin A (CsA) (15mg/kg/day) subcutaneously. The parameters followed were: Weight gain and feed intake; Clinical signs of GVHD; Relative spleen weight; Popliteal lymph node enlargement assay; Histological evaluation of spleen, liver, skin, native and transplanted bowels.

According to the clinical scoring system, heterotopically transplanted animals were found to have a more severe GVHD than the orthotopic group. There were statistically significant differences between the relative spleen weights of the heterotopic transplant and control groups ($p=0.001$, 0.004, 0.047 in 7, 14 and 21 day groups respectively) and between heterotopic and orthotopic groups at 7 days ($p=0.037$). Lymph node enlargement assays were again statistically different between heterotopic and orthotopic groups ($p=0.019$, 0.020, 0.007 in 7, 14 and 21 day groups respectively). Histology results also supported the hypothesis that GVHD was indeed more severe in the heterotopic transplant group when compared with orthotopically transplanted animals.

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ABBREVIATIONS

(BN)	Brown Norway
(BrdU)	Bromodeoxyuridine
(CsA)	Cyclosporin A
(GALT)	Gut Associated Lymphatic Tissue
(GVHD)	Graft Versus Host Disease
(GVHR)	Graft Versus Host Reaction
(HBSS)	Hanks' Balanced Salt Solution
(HLA)	Human Leucocyte Antigen
(Lew)	Lewis
(MHC)	Major Histocompatibility Complex
(MLN)	Mesenteric Lymph Nodes
(RSW)	Relative Spleen Weight
(SBTx)	Small Bowel Transplantation
(SMA)	Superior Mesenteric Artery
(TPN)	Total Parenteral Nutrition

CHAPTER I

INTRODUCTION

The loss of function in most parenchymal organs, such as the kidneys, liver, heart, pancreas and lungs can be compensated for by successful organ transplantation. In contrast, grafting of small bowel, which is the only curative treatment after total or nearly complete loss of this organ, is not yet a routine clinical procedure.

Patients who have undergone massive intestinal resections are currently maintained on home parenteral nutrition (Home TPN). This treatment is an imperfect and expensive solution. It costs between $ 50,000 and 120,000 U.S per year (1). These patients are limited in their life style because of their dependence on TPN (2,3). In addition a number of serious complications are associated with TPN. Sepsis is an everpresent threat, bacterial contamination of the I.V catheter can lead to a catheter driven septicemia that may be fatal within hours. In addition, prolonged TPN has harmful metabolic results, such as liver impairment and altered bone metabolism (4,5). Long term TPN in children is even more problematic, with increased nutrient requirements, difficulty in patient compliance, and the risk of associated liver damage, especially in the very young infant (6). Their long term

outlook is less favourable (up to a 15% mortality rate) than that of adults because of the higher risk of liver impairment (7). Because of these consequences of treating the short gut syndrome with TPN, the implantation of a healthy small intestine is an important therapeutic possibility for treatment of short gut syndrome at any age.

However, small bowel transplantation (SBTx) also has problems:

1- Difficulties in preservation and operative techniques

2- Rejection

3- Infection

4- Graft versus host disease (GVHD)

Small bowel mucosa is very delicate and the luminal enzymes can cause rapid autolysis so that even short term preservation of the bowel before transplantation is difficult. The large lymphoid content of the small bowel distinguishes it from other forms of organ grafts. These graft cells are a massive target for rejection. Moreover near perfect function of the graft is essential for nutrient absorption and the maintenance of barrier function. Translocation of bacteria from bowel lumen to the blood stream is a common source of sepsis in patients with multiple organ failure (8). In small bowel transplantation even relatively mild rejection changes may be sufficient to compromise barrier function.

One of the most fascinating findings of the studies with small bowel transplantations has been the massive exchange migration of lymphocytes between

the graft and the host. Therefore in addition to recipient cells invading the graft to cause rejection, graft cells are capable of migrating to recipient tissues to cause GVH'D.

The following studies were undertaken to evaluate the impact of heterotopic and orthotopic small bowel transplant techniques on the incidence and severity of graft versus host disease in a rat small bowel transplant model. During the development of our small bowel transplant model we performed both heterotopic and orthotopic transplants and we observed that GVHD intensity was greater if the heterotopic procedure was performed. The results of various groups with clinical small bowel transplantation programs have not revealed GVHD as a major problem (9). Resection of the bowel with its mesentery and gut associated lymphatic tissues (GALT) which causes the short bowel syndrome and necessitates (SBTx) in the clinical scenario, may be one of the reasons for the lower incidence of GVHD in humans after SBTx. In addition to our own previous experience with rat SBTx models, these clinical findings in human SBTx have lead us to hypothesize that the native bowel and especially the GALT may provide a highly effective site for activation of the graft intestinal lymphocytes and can therefore set the stage for GVHD. In the orthotopic model where the native bowel is resected with its mesentery and lymphoid content, this site of lymphoid activation has been removed and the resulting GVHD may therefore be milder than in the heterotopic model.

CHAPTER II

LITERATURE REVIEW

CLINICAL SMALL BOWEL TRANSPLANTATION

The first unsuccessful attempts at clinical small bowel transplantation were carried out by Detterling in 1964. In 1967 Lillehei et al. successfully grafted the entire small bowel and right colon in a 46 year old patient, but the patient died some hours after the operation (10). Three subsequent attempts were as unsuccessful as was the case reported by Fortner and coworkers in 1972 (11). Nevertheless these discouraging results identified the problems that required resolution. While primary operations of organ harvesting and grafting were technically successful, the poor overall results were caused by unresolved immunological problems. The complex immunological reactions following a small bowel transplantation comprise a rejection reaction and an additional Graft Versus Host Reaction (GVHR), which is induced by the immunocompetent cells within the graft's mesenteric lymph nodes and the gut associated lymphatic tissue (GALT). Both reactions, which interfere with each other, are directly dependent on the length of the graft (12,13) and the grade of histoincompatibility between donor and recipient (14). The physiology and absorptive capacity of the graft appear to influence both. During the 1970's different research groups contributed to the understanding of these complex reactions (15). These

investigators elucidated the basic mechanism of immune reactions on a morphological and functional basis so that, together with the introduction of the immunosuppressant Cyclosporin A at the start of the 1980's, the foundations were laid for a new era of clinical small bowel transplantation.

The first case during this period was reported by Cohen and coworkers, who in 1986 performed a transplantation of the entire small bowel (16). This attempt was unfortunately unsuccessful like Goulet's patient who died after 211 days from inadequate graft function despite a technically successful transplantation and treatment of multiple rejections (17). Nevertheless the way to successful transplants had been opened. Extensive experimental work was done using several animal models on the surgical, immunological, and physiological questions. Based on this work, two European groups started clinical programs of small bowel transplantation in March 1987 (Paris, France) and in November 1987 (Kiel, Germany) which led to the first successful transplantations. The longest survival was achieved in London, Ontario, Canada by Grant and coworkers (18). A patient with combined liver and small bowel transplants is still alive after more than 5 years. A recent series of successful combined small bowel and liver transplants using FK 506 immunosuppression was reported by Starzl et al. in Pittsburgh (19).

EXPERIMENTAL SMALL BOWEL TRANSPLANTATION

Transplantation of vascularized organs, including the bowel, was first attempted by the French surgeon Alexis Carrell (20). His pioneering efforts at the turn of the century demonstrated the feasibility of small bowel transplantation. The problem of rejection of such transplants was soon recognized, and interest languished until the late 1950's, when Lillehie's group in Minnesota were investigating the effects of ischemia on gut organs. They found that cooling and perfusion with heparinized saline would reliably allow preservation of small bowel for 4 hours, and that this preserved bowel could be reimplanted and would function indefinitely as a homograft (21). Their model consisted of a one stage operation; the superior mesenteric vessels were isolated, clamped and divided, the bowel was flushed and then revascularized using the mesenteric vessels of a similarly prepared recipient, reestablishing bowel continuity using end to end anastomosis of the native duodenum and ileum to the graft. They also used isolated loops of bowel placed in the neck permitting the study of immunosuppressive agents and graft function in a controlled fashion, since rejection of the graft would not lead to the death of the animal (10). They had no success with allografts, and as with work by others, minimal survival advantage was found using azathioprine and steroids, the immunosuppressants available at that time (22,23,24).

The dog model was used to evaluate the rejection process in detail, and after the introduction of cyclosporin in the late 1970's, the orthotopic model of

transplantation in the dog was first used to assess its effect on small bowel transplants (25,26). The significant prolongation of graft survival demonstrated by Reznick et al. from Toronto (25) was encouraging but the overall rate of success was low. In this landmark study they improved the average survival in 11 dogs treated with CsA (25mg/kg/day i.m.) to 91 days, while untreated controls survived an average of 11.5 days.

The description of heterotopic bowel transplantation in the rat by Monchik and Russel in 1971 greatly facilitated the study in this field (27). The rat model has served as a standard for initial investigations of immunosuppression, function, and techniques since. In this model, the bowel is isolated using the aorta below the superior mesenteric artery and the portal vein as the vascular pedicle. It is then revascularized in the recipient using the infrarenal vena cava, and aorta. The bowel is left as a Thiry-Villa fistula with proximal and distal stomas (heterotopic graft). They also documented function of this bowel by resecting the native bowel and reestablishing gastrointestinal continuity using the transplanted segment (orthotopic graft). Kort described a one stage orthotopic procedure in 1973, wherein the native bowel was resected at the initial operation, with immediate reestablishment of gastrointestinal continuity (28). However this technique was plagued with a high rate of technical failures (40%). Deitz described a two stage procedure which had improved survival (80%) (29). At the same time, Lee and Schraut described their experience with the one stage procedure; they had improved the technique described

by Kort so that survival of isografts was greater than 80% (30). This became the standard model for investigation of small intestinal transplantation in the rat. The majority of these studies have been performed using a portocaval anastomosis for venous drainage of the graft. However, Kort demonstrated the possibility of using a porto-portal anastomosis to provide a more physiological state posttransplant (28). This has been thought to confer some immunological advantages to the graft (28,31,32) and to establish a more physiological route for venous drainage from the graft (33). However it is a more difficult procedure with a higher risk of technical failure. More recently, other variations have been introduced, including combined small bowel and colon transplantation (34), the use of the renal pedicle for the vascular connections (35), and the use of superior mesenteric artery as the arterial connection (36). However these models have not been shown to offer any specific advantages over the previously described techniques.

The pig is an excellent model of human bowel physiology, with a more defined genetic lineage than the dog (37,38). Although earlier attempts had been made (39,40), Ricour and colleagues were the first to successfully perform small intestinal transplants in the pig and achieve allograft survival (41). The techniques used parallel those used in the dog, with both portal and caval routes of venous drainage being described.

CLINICAL AND EXPERIMENTAL GRAFT VERSUS HOST DISEASE

An individual's immunologic identity is expressed in cell surface proteins encoded by the major histocompatibility complex (MHC). MHC proteins (termed HLA in humans) are critical to the proper function of the immune system as it identifies and destroys foreign invaders while preserving normal, healthy tissues. MHC antigens and non-MHC antigens (minor antigens) expressed on tissues that are transferred from one person to another normally are recognized by the recipient, leading to rejection of the foreign tissue in a host versus graft reaction. Immunologically competent cells contained in the graft can result in immunologic recognition in the opposite direction, initiating a graft versus host (GVH) reaction. This GVH phenomenon was first noted when irradiated mice were infused with normal spleen cells. Furthermore, mice given allogenic marrow although recovering from radiation injury and marrow aplasia, subsequently died of secondary disease, a syndrome consisting of diarrhea, weight loss, skin changes, and liver abnormalities (42). This phenomenon was subsequently recognized as GVHD, a syndrome analogous to runt disease in mice infused at birth with allogenic cells. These observations led Billingham to formulate in 1966 the requirements for the development of GVHD:

1- The graft must contain immunologically competent cells.

2- The recipient must express tissue antigens that are not present in the transplant donor.

3- The recipient must be incapable of mounting an effective response to destroy the transplanted cells (43).

According to these criteria, GVHD can develop in various clinical settings when tissues containing immunocompetent cells (blood products, bone marrow and solid organs) are transferred between persons. The immunocompetent cells responsible for GVHD were recognized by Gowans nearly 30 years ago as small lymphocytes (44) and Mc Gregor showed that they are derived from bone marrow stem cells (45). We now know that these immunocompetent cells are mature T cells present in the bone marrow (46). Recent clinical studies confirm experimental data demonstrating that the severity of GVHD correlates with the number of donor T cells transfused (47).

The second requirement, that the recipient must express tissue antigens not present in the donor, became the focus of intensive research after the discovery of the MHC. HLA antigens, the protein products of the MHC on the cell surfaces of all nucleated cells in the body, are essential for the activation of T cells and are potent stimuli of allogenic T cells (48). MHC antigenic differences between donor and recipient are the most important risk factors for the induction of GVHD. In addition, there are minor histocompatibility antigens encoded for by less well defined genetic loci that can also be recognized as foreign. These minor antigens are thought to be responsible for the development of GVHD in recipients of MHC identical transplants. Finally, in experimental models and under certain clinical conditions,

GVH reactions can occur between genetically identical persons (49).

Billingham's third requirement stipulates that the recipient of immunocompetent T cells must be immunocompromised. A patient with normal immune system function will reject T cells from a foreign donor and thus prevent GVHD. This requirement is most commonly met in allogenic bone marrow transplantation, where recipients have usually received immunosuppressive doses of chemotherapy and radiation before marrow infusion, but may also be met in other situations such as in recipients of small bowel transplants. Recipients of solid organ grafts are treated with immunosuppressive drugs to prevent rejection of the transplanted organ and thereby become susceptible to the attack of T cells present in the donor graft (e.g.,small bowel).

GRAFT VERSUS HOST DISEASE IN EXPERIMENTAL SMALL BOWEL TRANSPLANTATION

Because of its large quantity of lymphoid tissue, the small intestine is unique among solid organ allografts in its ability to induce GVHD. Early studies of small intestinal transplantation in dogs, in which untreated recipients died within nine days postoperatively with enlarged mesenteric lymph nodes but relatively normal bowel histology, suggested that GVHD may occur following small bowel transplantation (10,50). In order to further define the immunologic reactions with small bowel transplantation, Monchik and Russel (27) developed an auxiliary, heterotopic small

bowel allograft model in inbred rats in which GVHD and rejection could be examined independently. In this model, small bowel transplantation from a parenteral strain donor into an F1 hybrid recipient induced an unidirectional graft versus host reaction with death of the recipient in 12-20 days. Subsequent studies in the rat have shown that donor lymphocytes in the allograft precipitate GVHD. Kirkman et al. (51) demonstrated that immunocompetent cells in the donor are necessary for the GVH reaction to occur. Pomposelli et al. (52) showed that donor lymphocytes recovered from the spleen of small bowel allograft recipients with GVHD cause enlargement of the popliteal lymph node when injected into the footpads of animals isogeneic to the recipients. This so called popliteal lymph node assay has become the classic measure of GVHD. Others have shown that the intensity of GVHD varies directly with the length of small bowel or amount of lymphoid tissue transplanted (12,13).

CHAPTER III

MATERIALS AND METHODS

ANIMALS

Male Brown Norway (BN) and Lewis (Lew) rats (200-250 gr) were obtained from a commercial source (Charles River Canada, St.Constant,PQ), and were housed in individual plexiglass cages, with free access to food (Tekland Premium Lab Diet, Textron Corp., Madison,WI) and water. Feed intake and animal weight were monitored daily. Weight gain is described relative to the initial pretransplant weight for transplanted animals. The guidelines of the Canadian Council on Animal Care were followed. The experimental protocol was approved by the Animal Welfare Committee of the University of Alberta. (Protocol No: 91-356)

CYCLOSPORINE

CsA in a powder form was a gift of Sandoz Pharmaceuticals (Sandimmune, Sandoz Pharmaceutical Corp., Montreal, PQ). The powder was dissolved in medium chain triglyceride oil (Mead Johnson, Belleville, ON) at concentrations of 15 mg/ml and 10 mg/ml, and sterilized by microfiltration through a sterile 0,22 um filter (Millipore Products, Bedford, MA). Animals were injected subcutaneously in the nape of the neck with 15 mg/kg body weight (0.1 ml) of the CsA solution just prior to

transplantation, then daily for 6 days, and with 10 mg/kg body weight (0.1 ml) of the CsA on alternate days thereafter until sacrifice.

HETEROTOPIC INTESTINAL TRANSPLANTATION

DONOR OPERATION

The donor was fasted overnight with free access to water. This procedure was modified from the technique developed by Monchik and Russel (22). The donors were anaesthetized with 5% Halothane mixed with 5 L/min. of oxygen. Once the animal was fully anaesthetized the amount was tapered down to 1.5% and 2 L/min. of oxygen mixture. The abdomen was prepared by shaving the fur and application of 70% alcohol. 3 ml. Ringer's lactate was given via the penile vein before opening the abdomen. A midline incision was made, exposing the sternum. A JKH (model no: 039011) operating microscope was used to mobilize the graft. All the colonic vessels were tied and the distal colon was divided from the sigmoid. The peritoneum between the transverse colon and the pancreas was divided by ligating the vessels with 8-0 silk. Mosquito clamps were placed on the pylorus and the third part of the duodenum and the pancreatic tissue with small pancreatic vessels were exposed. These vessels were tied and then divided. The portal vein was then separated from the pancreas. The proximal jejunum was divided 2 cm. distal to the ligament of Treitz. The right side of the aorta was cleaned by ligating and dividing the right renal and small lumbar

arteries proximally to the coeliac artery and distally distal to the left renal artery. The coeliac artery was tied and the tissue between the coeliac artery and superior mesenteric artery (SMA) was cauterized. The portal vein was exposed totally and isolated proximally. The pyloric and splenic vessels were tied and divided separately. Next the small bowel was transferred and placed to the right side of the body and the left side of the aorta was cleaned up to the confluence of the coeliac artery and down to the left renal artery. A 6-0 silk tie was placed around the aorta proximal to the SMA but not tied. At this point 200 U heparin and 3 ml. Ringer's lactate was given via the penile vein. After waiting 2-3 minutes the aorta was clamped distally. A microvascular clamp was placed on the aorta between the SMA and left renal artery, the aorta was divided distally and a polyethylene catheter was inserted and secured. The proximal aorta was tied with the 6-0 silk, the microvascular clamp was taken off and flushing of the graft was started very slowly with 2 ml. of +4 C Ringer's lactate with 300 U Heparin in it. The portal vein was divided close to the liver to relieve intravascular pressure. Flushing was continued until the venous return was clear. The proximal aorta was divided proximal to the 6-0 tie. The cut end was then pulled proximally and the left renal artery was seen under the left renal vein. The distal aorta was then divided distally above the left renal artery. The graft was taken out and placed into the ice bucket. The vessel ends were cleaned on the dissecting table and correct orientation of the graft was confirmed (Figure 1).

RECIPIENT OPERATION

The recipient was fasted overnight with free access to water. Anaesthesia was started with 5% Halothane mixed with 5 L/min. oxygen and maintained with 1.5 % Halothane mixed with 2 L/min. oxygen. The abdomen was prepared by shaving the fur and applying 70% alcohol. 3 ml. Ringer's lactate was given via the penile vein before opening the abdomen. A midline incision was made and retractors were placed. The native bowel was covered with a wet, warm sponge and placed to the left side of the animal. The infrarenal aorta and vena cava were exposed thoroughly by dividing the peritoneum over these vessels. Two retraction ties were put around the aorta and cava each 1 cm. away from each other. 3 ml. Ringer's lactate was given via the penile vein and a modified Lee's clamp was placed across the aorta and cava (Figure 2). First the vena cava and then the aorta was punctured with a 30 gauge needle and the vessels were flushed with heparinized Ringer's lactate. A 1 cm.long aortotomy and venotomy were carried out with vessel scissors. 10-0 silk stay sutures were placed in the ends of the venotomy and tied with one knot. The graft was then brought into the operative field and placed on the right flank of the animal and covered with a sponge soaked in chilled saline. Correct orientation was achieved by positioning the portal vein of the graft so that the splenic and pyloric ties stay on the left side of the animal. First the anastomosis of the back wall was completed with continuous 10-0 silk sutures. The venous end to side anastomosis was completed by anastomosing the front wall continuously. The portal vein was stretched gently before

tying the knots. The arterial anastomosis was completed in like fashion (Figure 3). Avitene was put around the anastomosis and the clamp was taken off while applying pressure with a sponge. The graft should turn pink evenly within 30 seconds. 3 ml.of Ringer's lactate was given again via the penile vein. Next a distal stoma was created and secured with four 6-0 silk sutures between the skin and the everted mucosa of the graft. The proximal end of the transplanted bowel was tied and left inside the abdomen (Figure 4). The abdomen was closed with 3-0 chromic and the skin with 3-0 silk. 10 ml. Ringer's lactate and 0,1 mg/kg Buprenorphine was given subcutaneously. The animal was then placed in a cage over a heating pad and under a heating lamp. The transplanted animal was restrained from food or water for 24 hours.

ORTHOTOPIC INTESTINAL TRANSPLANTATION

DONOR OPERATION

The donor procedure in orthotopic transplantation is the same as for heterotopic transplantation.

RECIPIENT OPERATION

After completing the venous and arterial end to side anastomoses, which are the same as described for heterotopic transplantation, the recipient's jejunum and ileum are removed leaving only 2 cm. each of the native jejunum and ileum to be

anastomosed with the graft (Figure 5). The intestinal anastomoses were performed over a piece of dry macaroni (Kraft Dinner, Kraft Corporation, Montreal, PQ) using interrupted sutures of 6-0 silk (Figure 6). After anastomosis the macaroni stent was pushed distally beyond the anastomotic site. The animals received subcutaneous injections of Ringer's lactate (10 ml) two times daily for two days postoperatively with 0,1 mg/kg Buprenorphine for postoperative pain. Food and water was reintroduced after 24 hours.

FACTORS AFFECTING THE OUTCOME OF SMALL BOWEL TRANSPLANTS

Hypovolemic shock is the cause of most operative mortalities. It can occur even without excessive bleeding. It has been demonstrated that the animal's blood pressure dramatically decreases after revascularization of the intestinal graft. Moreover, the enormous quantity of isotonic fluid lost from the intraluminal space of the transplanted gut may worsen the case. Adequate fluid replacement is essential to counterbalance the profound hypovolemic shock after this procedure and it requires extensive postoperative care.

EXPERIMENTAL GROUPS

Three separate experimental groups were formed.

Group 1- Heterotopic small bowel transplantation was performed in 12 animals (BN>Lew). (Fully allogenic two way model)

Group 2- Orthotopic small bowel transplantation was performed in 12 animals (BN>Lew). (Fully allogenic two way model)

Group 3- Twelve heterotopic small bowel transplants were performed between Lewis donors and recipients (Lew->Lew) as the control group (Table 1).

Four animals each from group 1, 2 and 3 were sacrificed on days 7,14 and 21

EVALUATION OF GRAFT VERSUS HOST DISEASE

Five criteria were used to evaluate the recipients for the evidence and severity of GVHD.

1- Daily weight gain and feed intake

2- Clinical scoring for the severity of GVHD

-Dermatitis

-Redness of the eyes, snout, and paws

-Hunched posture

-Diarrhea

3-Relative spleen weight

4-Lymph node enlargement assay

5-Histology

-Skin

-Liver

-Spleen

-Native and transplanted bowel

-Mesenteric lymph nodes

All animals in the 3 groups were weighed daily and these changes recorded. Their feed intake was also recorded daily.

Daily physical examination of the animals included evaluation of dermatitis, redness of the nose, eyes, ears, and paws, presence of hunched posture and diarrhea. Severity of GVHD was estimated by clinical grading, as previously described by Saat et. al (53).

Grade 1- Light redness of ears, snout, and paws

Grade 2- Moderate redness of ears, snout, and paws, light hair loss and diarrhea

Grade 3- Severe redness of ears, snout, and paws, alopecia, generalized dermatitis, and profuse diarrhea.

Four animals in each of the groups 1, 2 and 3 were sacrificed at days 7, 14 and 21 and specimens from organs were collected for further study. The relative spleen weight was obtained using the following formula;

$$\text{Relative spleen weight} = \frac{\text{Actual spleen weight}}{\text{Total body weight}} \times 100$$

This number is a dimensionless indicator of splenomegaly, which is a universal finding in GVHD (52).

A popliteal lymph node enlargement assay was performed using splenic lymphocytes. This is perhaps the most sensitive indicator of GVHD (52). Spleens of transplanted animals and control group were passed through a tissue grinder, the splenic cells were washed in Hank's balanced salt solution (HBSS) three times and resuspended to a final concentration of 25 million cells/ml. Normal Lewis rats were then anaesthetized with halothane and 0,1 ml of the cell suspension (2,5 million cells/ml) was injected into the right footpad of this healthy rat. Another healthy Lew rat was anaesthetized and the same number of splenic cells obtained from this healthy rat were injected to the left footpad of the same study rat which had already received the cells of the transplanted animals spleen to his right food pad. After one week the animals were sacrificed and the popliteal lymph nodes were excised and weighed on a precision balance. A ratio was then established, defined as;

$$\text{Index of enlargement} = \frac{\text{Weight of right lymph node}}{\text{Weight of left lymph node}}$$

An index of enlargement greater than one is an indicator of the presence of GVHD (52).

Specimens of native and transplanted bowel, liver, spleen, skin and mesenteric

lymph nodes were obtained,fixed in formalin and prepared with hematoxylin and eosin stain for light microscopy. All histological sections were evaluated blindly by a pathologist (T.K.S.).

STATISTICAL ANALYSIS

Data is expressed as mean ± standard error of the mean. Significant differences were determined between the experimental groups using analysis of variance (ANOVA) comparison. Groups exhibiting statistical significance were further compared using student's t-test. $p < 0,05$ values were regarded as statistically significant.

CHAPTER IV

RESULTS

Twelve small bowel transplants from Brown Norway donors to Lewis recipients were performed in each of the orthotopic and heterotopic groups. Twelve heterotopic transplants between Lewis donors and recipients served as the control group. Animals were examined, weighed and feed intakes recorded daily. The survival rate was 97%. There was only one perioperative death due to venous thrombosis and this animal was replaced with another orthotopic transplant. All the remaining animals survived until sacrifice.

WEIGHT GAIN:

All the animals in all three groups have experienced an initial weight loss after the transplant. Average weight loss was 6.8, 10.76, and 9.7 percent for heterotopic, orthotopic and control groups respectively. After the first week, however these recipients started to gain weight again and reached their pretransplant weights between days 12 and 18. After that there was no difference between the growth rates of the heterotopic and orthotopic transplant groups (Figures 7,8,9).

No statistically significant difference was found in the feed intake of the animals in all 3 groups (Figure 10).

CLINICAL SCORING:

All animals in both heterotopic and orthotopic groups showed classical signs of GVHD with redness of the eyes, snout, and paws. While 4 of the orthotopic recipients (33%) had diarrhea lasting only one day, 5 (42%) recipients in the heterotopic group developed moderate (lasting more than 3 days) and the remaining 7 (58%) had mild diarrhea (lasting less than 3 days). According to the clinical grading system previously described, all of the orthotopically transplanted animals developed a grade I clinical manifestation of GVHD while 6 (50%) of the heterotopically transplanted animals developed grade I and the remaining 6 (50%) developed grade II clinical manifestation of GVHD. None of the animals in either group developed grade III (severe) GVHD and none of them died during the course of the study (Table 2).

RELATIVE SPLEEN WEIGHT ASSAY:

The third criteria used to assess for the presence of GVHD was an increase in the relative spleen weight (RSW). Comparison of the groups by ANOVA test revealed statistical significance ($p<0.05$). Subgroups were then compared by student's t-test which revealed a significant increase ($p=0.001$, 0.004, 0.047) in the relative spleen weights of the heterotopic transplanted animals (0.3465 ± 0.018, 0.2738 ± 0.22, and 0.2565 ± 0.016) when compared with controls (0.2164 ± 0.018, 0.2030 ± 0.009 and 0.2055 ± 0.06). The difference between relative spleen weights was again significant

in the heterotopic and orthotopic subgroups where the recipients were sacrificed at day 7 (0.3465±0.018 vs. 0.2622±0.031) (p=0.0037). There was no statistical significance between animals of these two groups which were sacrificed at days 14 and 21 (Table 3).

POPLITEAL LYMPH NODE ASSAY:

In the popliteal lymph node enlargement assay, the capacity of splenic cells to induce local GVH response was examined. The results of the popliteal lymph node assay were also clear. Cells used in this assay were obtained from unoperated Lewis rats and from animals in the two experimental groups (heterotopic and orthotopic) described above. Splenic lymphocytes from Lewis->Lewis syngeneic heterotransplants do not mount a GVH response in Lewis rats. Thus, no popliteal lymph node enlargement was induced when these cells were injected into the footpads of Lewis recipients. Spleen cells from Lewis recipients of BN bowel did show a statistically significant positive response between heterotopic and orthotopic groups (p=0.019, 0.020, 0.007 in 7, 14, 21 day groups respectively) (Table 4).

AUTOPSY FINDINGS AND HISTOLOGY:

Autopsy findings presented different features in heterotopic transplants when compared to orthotopic ones. The native small bowel is found to be dilated and thin in the heterotopic groups. The spleen and especially the mesenteric lymph nodes

were found to be markedly enlarged. These changes were not seen in the orthotopic groups. A constant finding noted in the graft after revascularization, and present at laparotomy on day 7 was the presence of a turbid, viscus fluid in the lumen.

Histopathologically all animals in both heterotopic and orthotopic transplant groups had normal appearance of the liver with normal lobular architecture and hepatocellular detail. There were no signs of steatosis, cholestasis or hepatitis (Figures 11/a-b).

Histological evaluation of the spleens in both groups revealed mild to moderate hyperplasia of white pulp areas due to an increase of the reticuloendothelial cells. The red pulp showed a spectrum of changes ranging from hyperplasia of the pulp to atrophy (Figures 12/a-b).

There were not any major differences between the heterotopically and orthotopically transplanted bowels. Transplanted bowel specimens in both of the groups had normal villus architecture. Enterocytes covering the tips of the villi and enteroblasts lining the crypts of Liberkuhn were of normal appearance. There was occasional minimal edema of the lamina propria with normal numbers of lymphocytes. There were no inflammatory changes, no vascular changes nor necrosis noted (Figures 13/a-b).

Microscopic evaluation of native mesenteric lymph nodes of the heterotopically transplanted animals showed lymphoid depletion and appearance of immunoblasts in the paracortical areas in all 12 of the animals (100%) (Figures 14/a-b).

Upon examination of the skin specimens, in 10 out of 12 (83%) orthotopically transplanted animals the skin appeared normal with no signs of hyperkeratosis of the epidermis and again negative for mono or polymorphonuclear infiltration of the dermis. In 2 cases there were mild focal dermatitis with one hyperkeratosis and mild focal infiltration of the dermis by mono and polymorphonuclear cells. However in the heterotopically transplanted animals there were epidermal hyperkeratosis and mild mono and polymorphonuclear cell infiltration in 5 cases (42%) and hyperkeratosis with severe mono and polymorphonuclear cell infiltration in 2 cases (17%) (Figures 15/a-b).

CHAPTER V

DISCUSSION

Graft versus host disease has been discussed as a frequent and major complication after bone marrow transplantation or massive blood transfusion but has not been frequent in solid organ transplantation (54). In experimental small bowel transplantation, where the graft includes a large amount of lymphocytes, GVHD can be a crucial problem. Lymphoid tissue within the intestine consists of both mesenteric lymph nodes (MLN) and gut associated lymphatic tissue (GALT) in the form of Peyer's patches, lamina propria and intraepithelial lymphocytes. These allogenic lymphocytes in the lymphoid tissue become stimulated and develop an immune response against the host (12,13,55). The number of lymphocytes transferred and the recipients immune competence status determine how severe this GVH reaction will be (56). Deltz et al. (12) utilizing the rat model of small bowel transplants (Parent->F1 hybrid) showed that the GVH reaction was less intense when half of the small bowel was transplanted, than when transplantation of the entire small bowel was carried out. Pirenne et al. (57), using the Lewis->LBNF1 model and injecting cells isolated from the MLN of donors into the peritoneal cavity of the recipients, showed 100% mortality from GVHD in an average of 14.75 + 0.6 days.

In another study Lear et al. (58) found that graft cells migrate into the gut lymphoid tissue of the host within 24 hours of transplantation. In the absence of

immunosuppression, the vigorous host response attacks all elements of the graft, including its lymphoid tissue. Thereafter within the first 2 to 3 days of initial migration, the Peyer's patches and mesenteric lymph nodes of the transplant are progressively destroyed. The isolated graft lymphocytes are unable to proliferate within the host lymphoid tissue, and their disappearance by day 6 suggests active destruction by host cells. However a 7 day course of CsA immunosuppression has a dramatic effect on graft cell migration. The prolonged survival of the small bowel and its lymphoid tissue is able to fuel a much greater movement of lymphocytes to the host. This response appears to peak at 14 days with no graft cells remaining visible in the host tissues at 21-28 days.

Yagi et al. (59) by using bromodeoxyuridine (BrdU) labelling index of the mesenteric lymph nodes traced the migration of lymphatic cells from the grafts and found that the mesenteric lymph node BrdU labelling index of the graft was higher than that of the native intestine in the rejection group. In the GVHD group the index of the native intestine was higher than that of the rejection group and there was a significant difference between the native and transplanted intestines in terms of the BrdU labelling index, which supports migration of the T-lymphocytes of the graft into the mesenteric lymph nodes of the native bowel. They also documented that the BrdU labelling index and DNA synthesis time give evidence that the lymphatic system of the graft is stimulated, but that of the native intestine is strongly suppressed in the GVHD group.

These studies support the concept of immunocompetent T-lymphocytes being responsible for GVHD and that the presence of the host MLN and GALT augments this reaction in the heterotopic small bowel transplant models.

It has also been shown that:

1- In vitro radiation of small bowel with 1000 rad prevents GVHD without inducing radiation injury (60).

2- Donor pretreatment with antilymphocyte serum on days -2 and -1 prevents GVHD indefinitely (61).

3- Surgical removal of mesenteric lymph nodes aborted GVHD in 70-90% of the transplanted animals (13).

In the current experimental series we examined four indicators of graft versus host disease; 1- Physical examination and observation of the classical GVHD related changes, 2- Relative spleen weights, 3- Popliteal lymph node assay, 4-Histologic examination.

There were no differences in feed intake and weight gain between the heterotopic and orthotopic transplanted groups (Figures 7-10). The recipients in both groups regained their initial weights between 12 and 18 days after the surgery after an initial weight loss of approximately 10% at day 7. They continued to gain weight at the same rate until sacrifice. When we evaluated the severity of GVHD between the heterotopic and orthotopic groups according to the clinical scoring system described above, we saw that GVHD in the heterotopic group is more severe than

the orthotopic group. All of the patients in the orthotopic group developed Grade I manifestations of GVHD while 50% (6/12) of the animals in the heterotopic group developed grade II clinical manifestations of the disease. Since the clinical signs of dermatitis, redness of the ears, eyes, and nose and hair loss are indicative only of GVHD (62) and none of the animals in which rejection alone would have been manifested developed any of these physical signs (63), these results suggest to us that GVHD is responsible for these changes; the changes were more severe in the heterotopic small bowel transplant group. The timing of these clinical manifestations correlates with the expected time of occurrence of GVHD. Our clinical findings parallel Schraut et al. (32) in which they concluded that GVHD is indeed present in the BN>Lew small bowel transplant model at 5 to 7 days after the initial transplantation. Although these results were derived from clinical observations carried out by the author and therefore there is potential for bias, they were supported by the relative spleen weight and popliteal lymph node assays. Histological evaluation of the tissues by a pathologist blinded to treatment groups also supported these findings.

The results of the relative spleen weight assay clearly demonstrate the ability of a small intestine allograft to cause GVHD in rats. Splenomegaly is thought to be one of the hallmarks of GVHD, but spleen size varies greatly in rats. This problem was solved by measuring spleen weight as a fraction of total body weight. Our results demonstrate that the increase in relative spleen weights was significantly greater in

the heterotopic group when compared to the orthotopic group. In a series of experiments done by Kiyoizumi (52) it was found that GVHD was induced only when LBNF1 rats received Lewis small bowel allografts. No signs of GVHD were seen in LBNF1 recipients of LBNF1 small bowel isografts or Lewis cardiac or renal allografts. These studies further support the hypothesis that donor lymphoid cells that were abundant in small bowel grafts are responsible for the GVHD.

This was also suggested by the popliteal lymph node enlargement assay in our studies. Injection of spleen cells obtained from Lewis recipients of BN grafts with clinical GVHD into the footpad of syngeneic Lewis rats resulted in enlargement of the ipsilateral lymph node, and this enlargement was greater in the heterotopic transplanted group when compared to the orthotopic transplanted group. In contrast injection of spleen cells obtained from Lewis rats transplanted with Lewis grafts or splenic cells of normal Lewis rats failed to induce lymph node enlargement in the same Lewis rats which manifested lymph node enlargement in their right footpads because of injection of splenic cells from BN->Lew recipient. These results strongly indicate GVHD and support our hypothesis that donor lymphoid cells were indeed present in the spleens of Lewis recipients of BN donors exhibiting GVHD and that the disease is more severe in the heterotopically transplanted group.

The histopathology results obtained from both heterotopic and orthotopic animals clearly showed that a non lethal GVHD developed in both groups. It has also been seen by other investigators (14) that in non lethal GVHD the livers are

unremarkable, however in rats dying of GVHD periportal lymphatic infiltrate was occasionally noted in the liver.

Histologic specimens of the spleen in both groups revealed hyperplasia of the white pulp with an infiltrate of reticuloendothelial cells. This is a constant finding in GVHD and is in parallel with the findings of Shaffer et al. (61). It has been documented by various investigators (14,61) that in the spleen the loss of lymphocytes was parallelled by the appearance of reticuloendothelial cells that seemed to be in the process of differentiating into histocytoid cells; this process has been attributed to GVHD.

Although flattening or patchy sloughing of intestinal villi and increased regenerative activity within the crypts is a constant finding in rats with severe GVHD (61), near normal histological appearance of both heterotopically and orthotopically transplanted animals has also been seen by other investigators (64,65) as well in our case. We demonstrated that during CsA therapy the bowel mucosa showed no alteration in the villous morphology nor necrosis of the mucosal crypt epithelium.

Immunoblast accumulation in the paracortical areas are known to be the result of invasion by T-cells (66) and lymphoid depletion and detection of immunoblasts in the paracortical areas of the native mesenteric lymph nodes of the heterotopically transplanted animals is another sign of GVHD which was also detected by other investigators (52,67) and parallels our results, strongly suggesting that the native MLN were indeed an activation site for GVHD.

Evaluation of the skin samples showed major differences in the heterotopically transplanted group when compared to the orthotopic animals. Microscopic appearance of thickened epidermis and infiltration of dermis by mono and polymorphonuclear cells is regarded as the most important differential diagnostic sign of GVHD by many authors (14,68). Epidermal hyperkeratosis with mono and polymorphonuclear cell infiltration of the dermis was seen in 7 out of 12 animals (58%) in our heterotopically transplanted group. This is a clear sign of GVHD and since these findings were far greater in frequency and severity in the heterotopic transplant group when compared to the orthotopic transplant group, we conclude that GVHD was more severe in the heterotopically transplanted animals. The balance of findings in these studies and previously published work suggest that these changes occur because of the remaining mesenteric lymph nodes and gut associated lymphatic tissues of the native small bowel, which may serve as a more efficient site of donor lymphocyte activation. Although the current results support our hypothesis that GVHD is more severe after a heterotopic small bowel transplantation when compared to the orthotopic model these results do not conclusively prove this hypothesis. Further studies especially with MHC typing of lymphocytes in the organs highly susceptible to GVHD are required (spleen, gastrointestinal tract, skin).

CHAPTER VI

CONCLUSIONS

There are certain differences between humans and rats that do not allow direct application of these data to human small bowel transplants. Besides the major genetic differences, the mesenteric lymph nodes and the lymphoid tissues of the rats are very well developed relevant to their body and other organs when compared to humans, which makes them more vulnerable to GVHD. Indeed all allogenic transplanted animals in our study showed some degree of GVHD without rejection in this two way BN->Lew model. In human small bowel transplantation the amount of immunosuppressants given to prevent rejection is usually sufficient to prevent GVHD. Although GVHD is more than a theoretical problem in human small bowel transplantation it has not usually been a major cause of morbidity or mortality in the clinical setting. Our data support the hypothesis that the resection of the native small bowel with its mesentery and GALT which causes the short bowel syndrome itself and necessitates small bowel transplantation may be an important reason for the lower incidence of GVHD in humans.

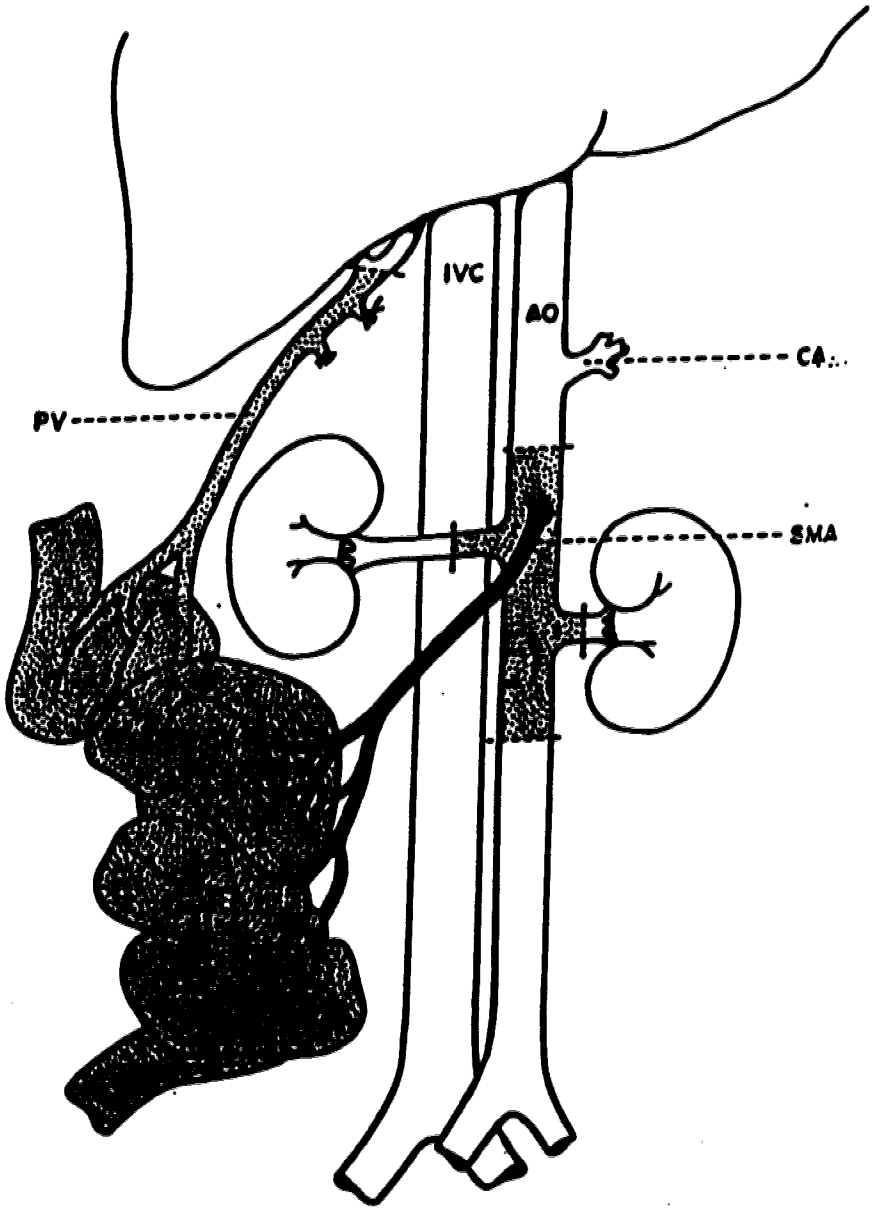

Figure 1. En-Block intestinal graft. PV: portal vein, IVC: inferior vena cava, AO: aorta, CA: celiac artery, SMA: superior mesenteric artery.

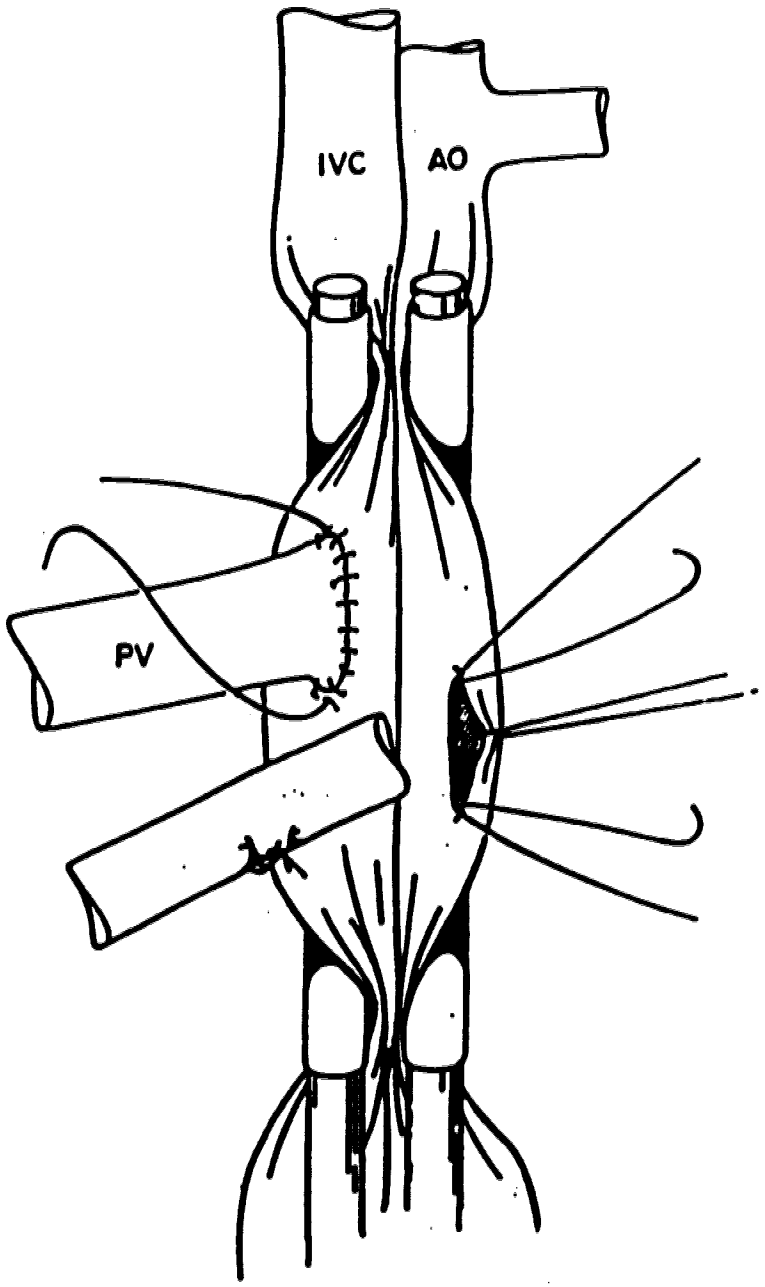

Figure 2. Cross-clamping aorta and inferior vena cava using a modified Lee's clamp.

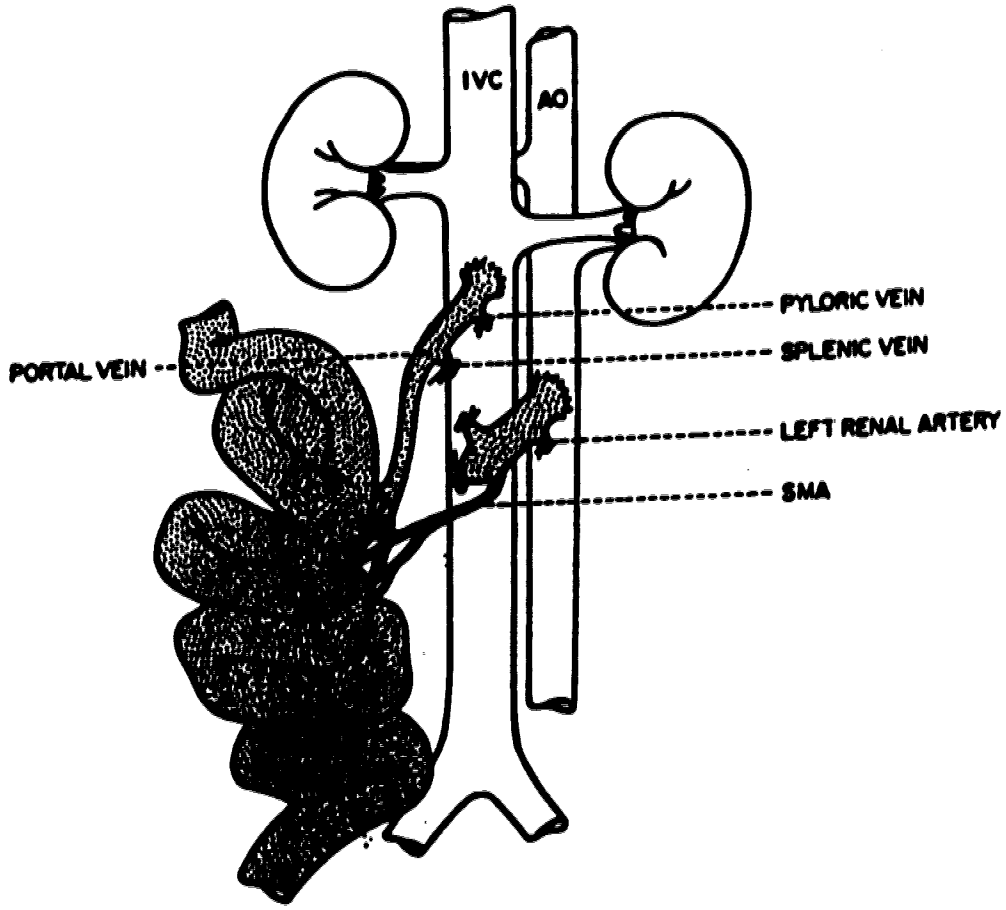

Figure 3. Vascular reconstruction. 1-The arterial and venous anastomoses are widely seperated to prevent torsion, 2-The correct portal vein orientation is ensured by placing pyloric and splenic vein ligatures on the left of the anastomosis, 3-Correct orientation of the aorta is ensured by placing the ligature of left renal artery on the left side of the aortic anastomosis.

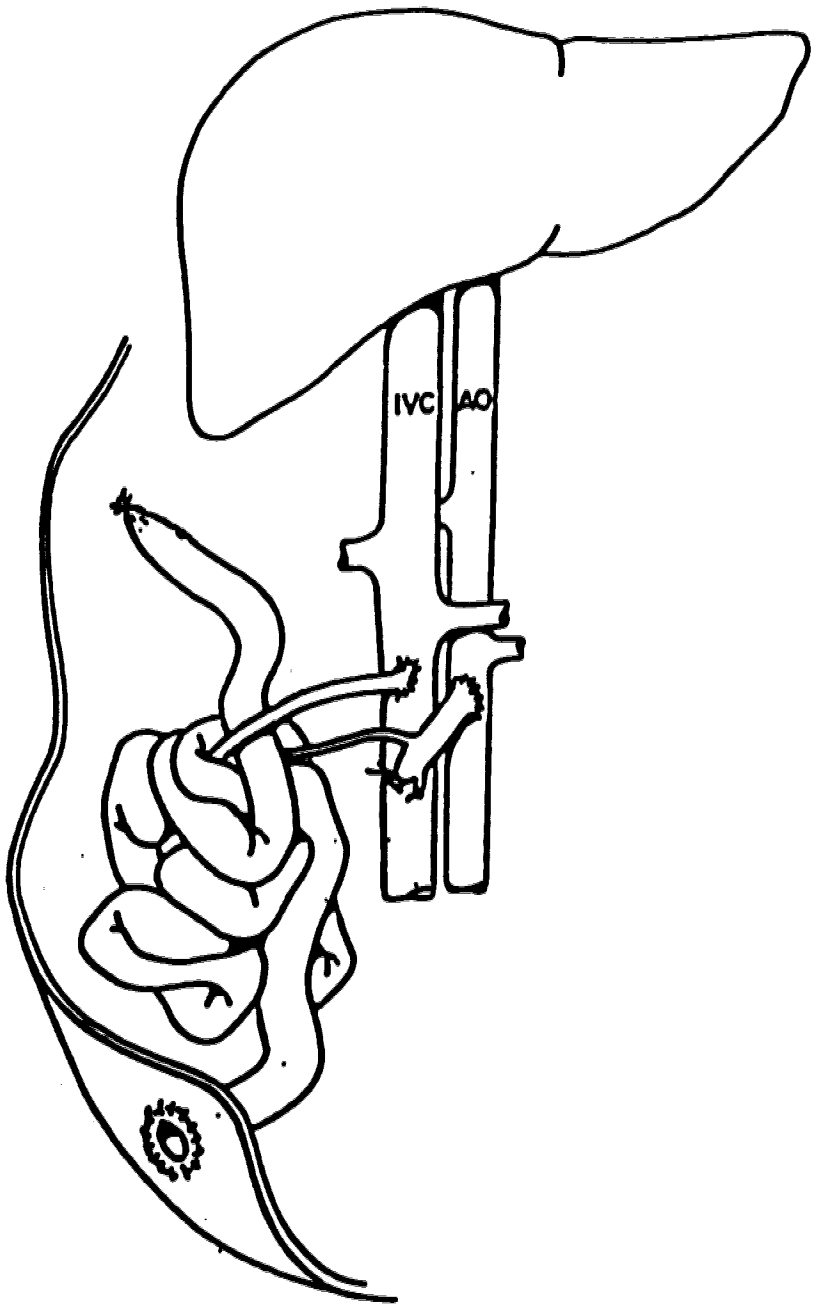

Figure 4. Heterotopic intestinal transplant model with distal end of the graft exteriorised as a stoma.

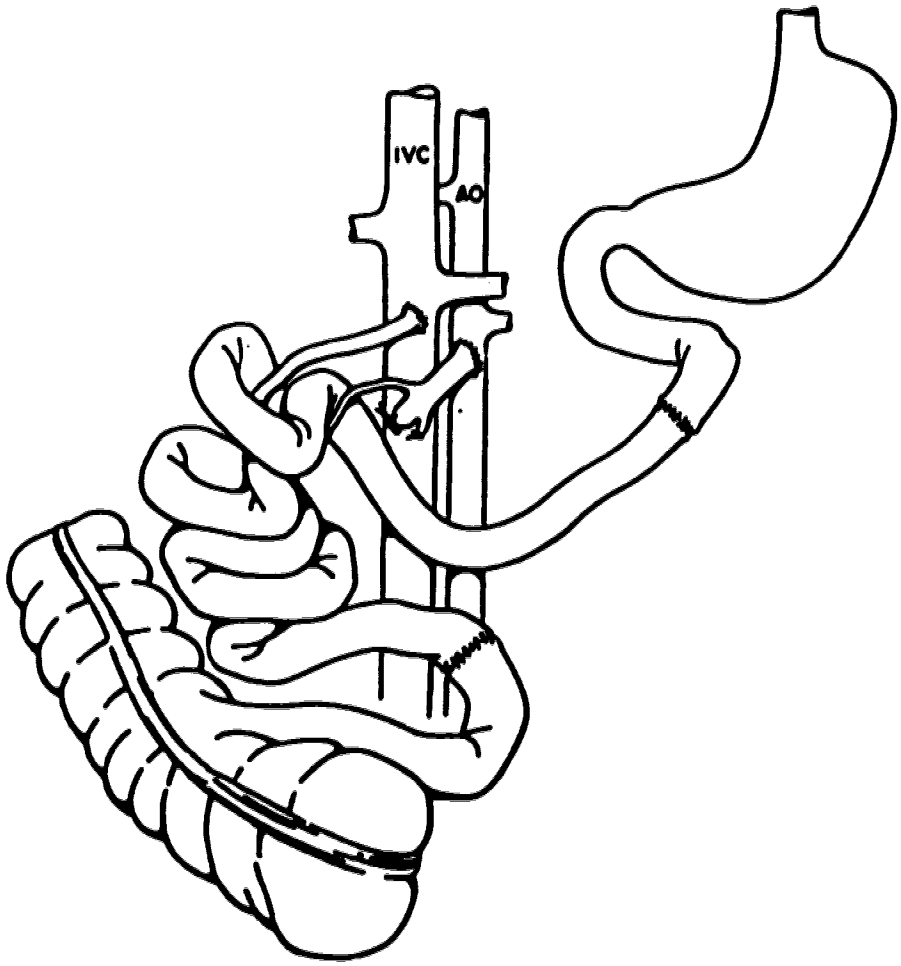

Figure 5. Orthotopic intestinal transplant model with both ends of the intestinal graft anastomosed to the proximal jejunum and distal ileum of the recipient.

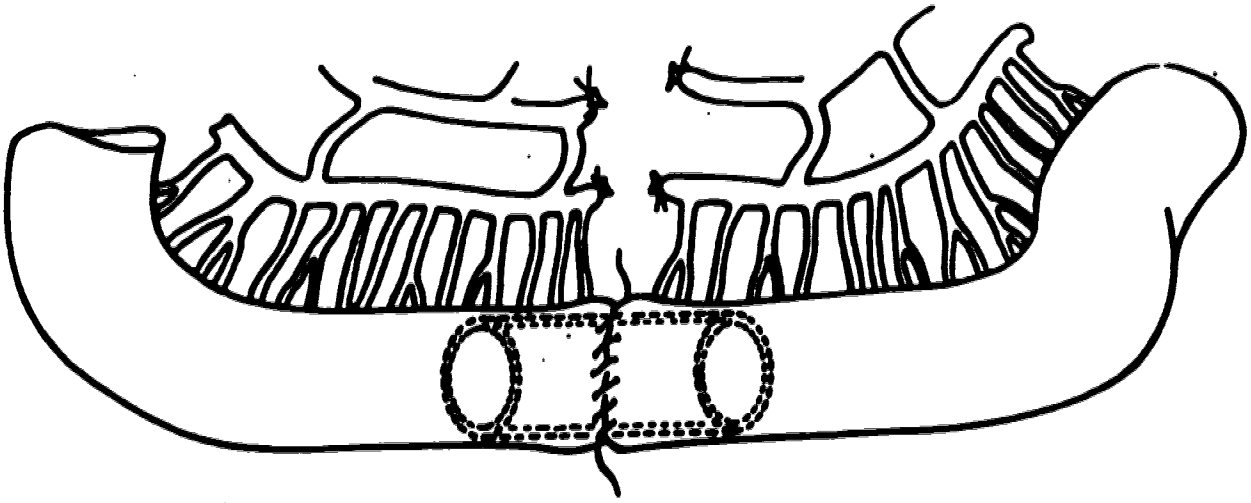

Figure 6. Macaroni noodle stent for the intestinal anastomosis.

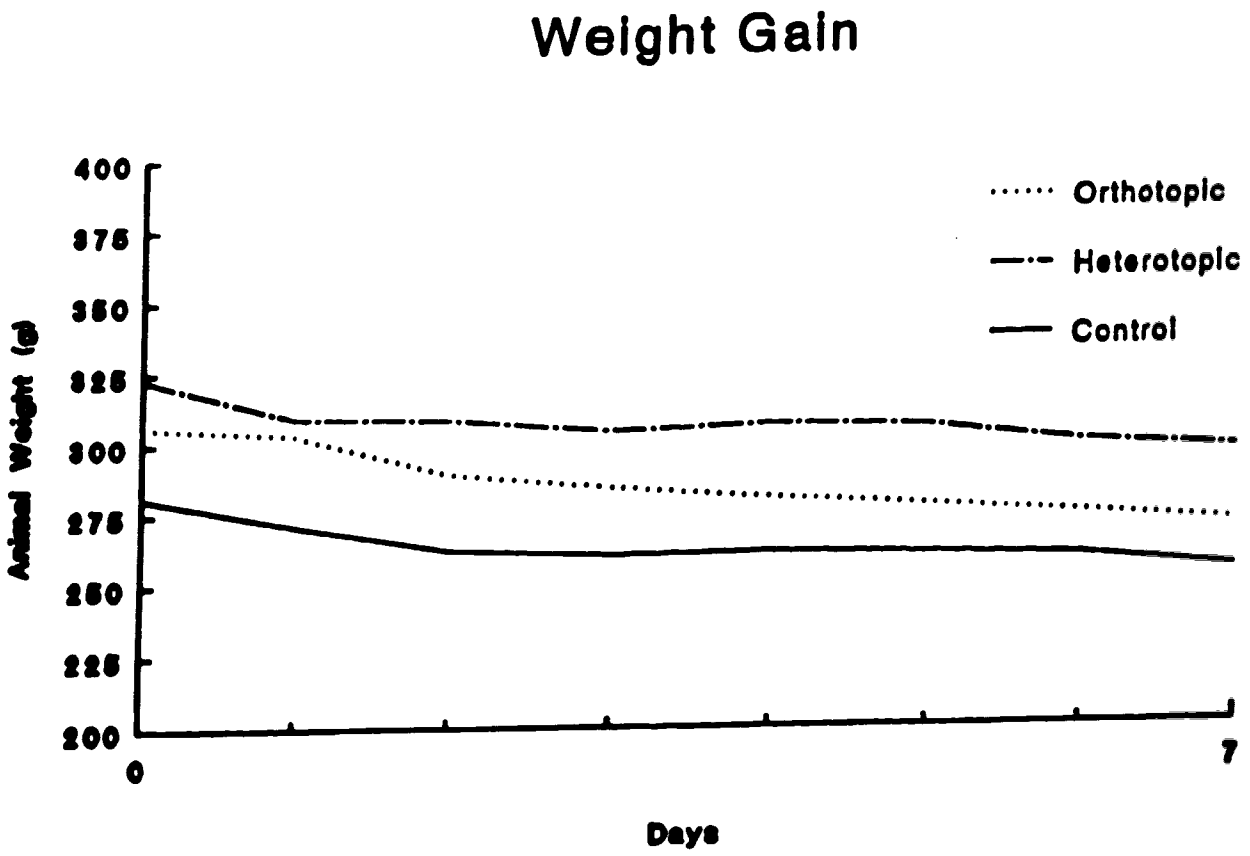

Figure 7. Weight gain in the 0-7 day group.

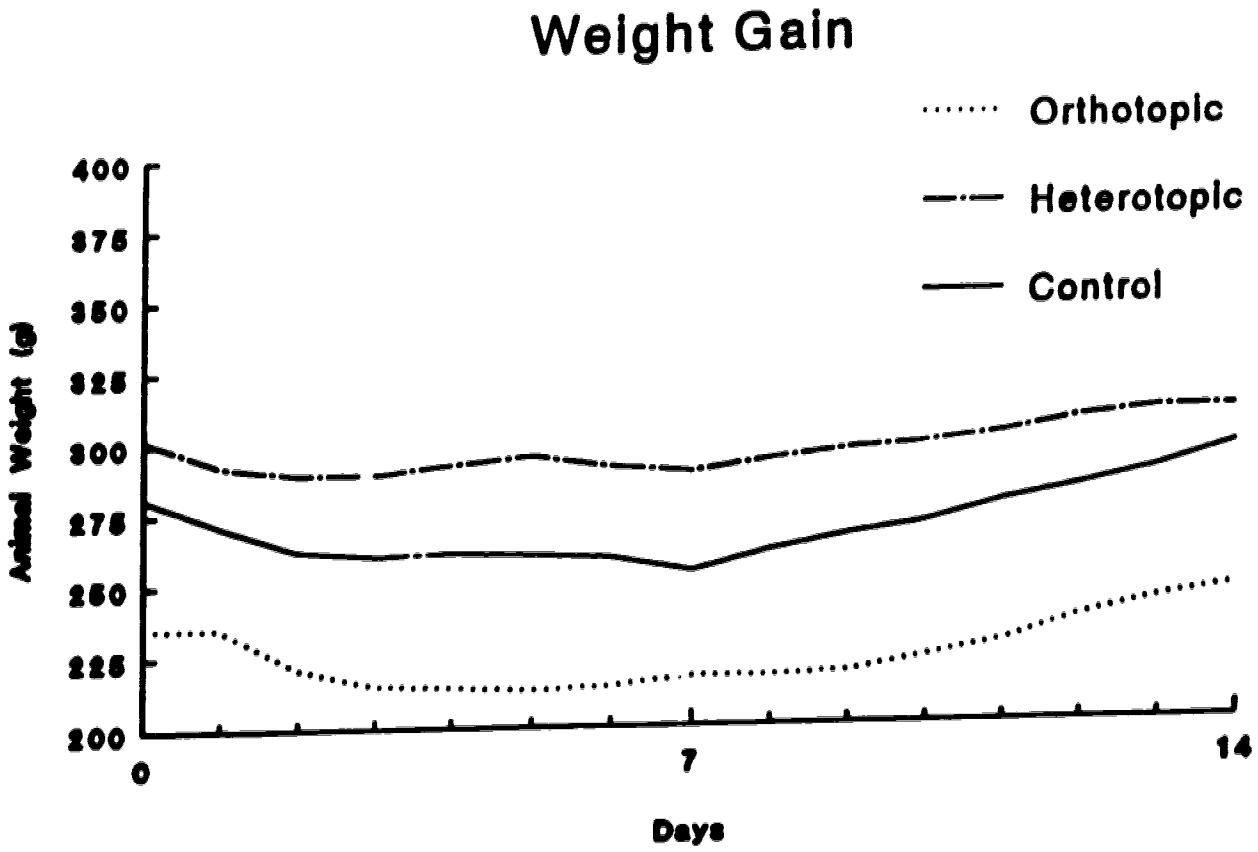

Figure 8. Weight gain in the 0-14 day group.

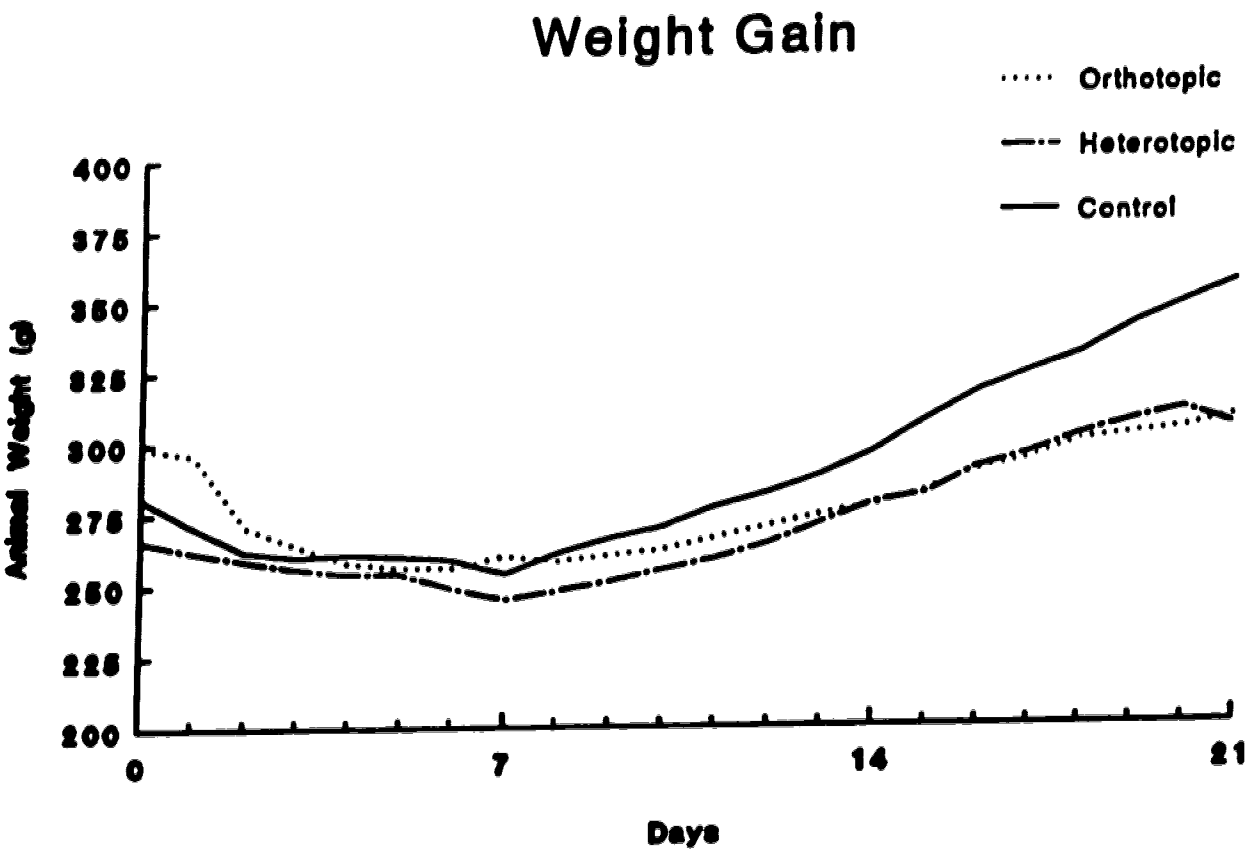

Figure 9. Weight gain in the 0-21 day group.

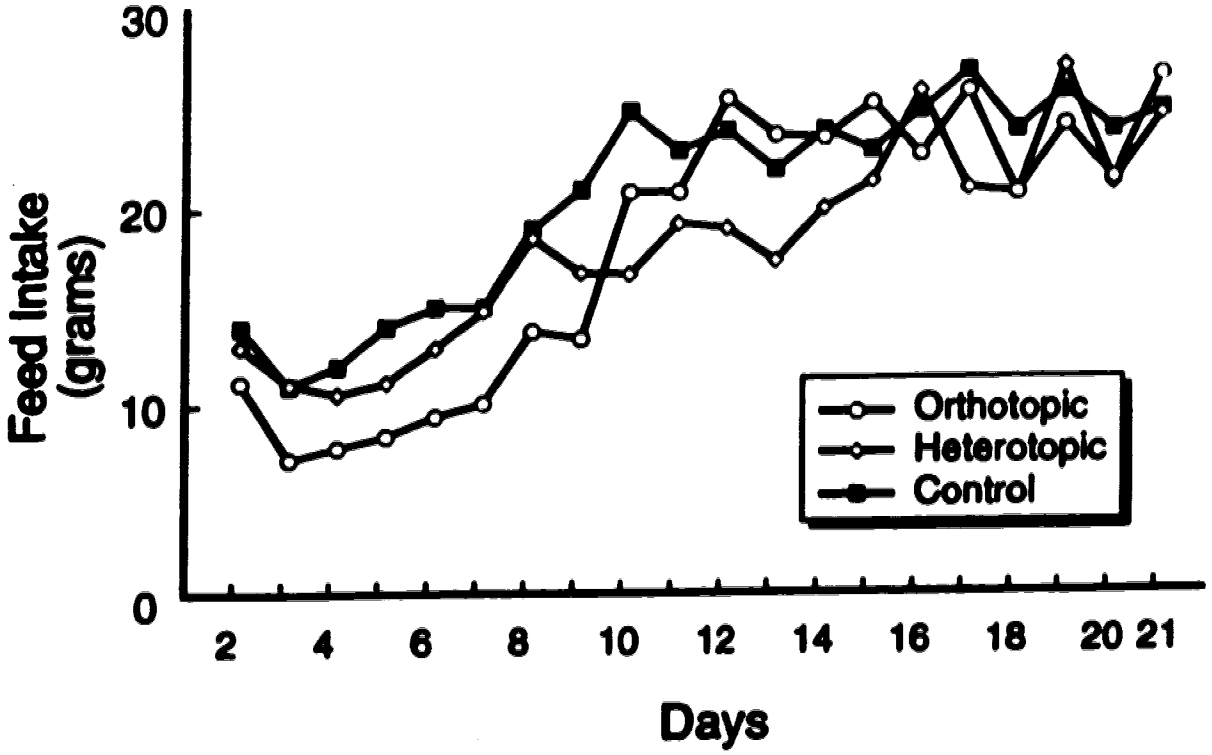

Figure 10. Feed intake in all 3 groups.

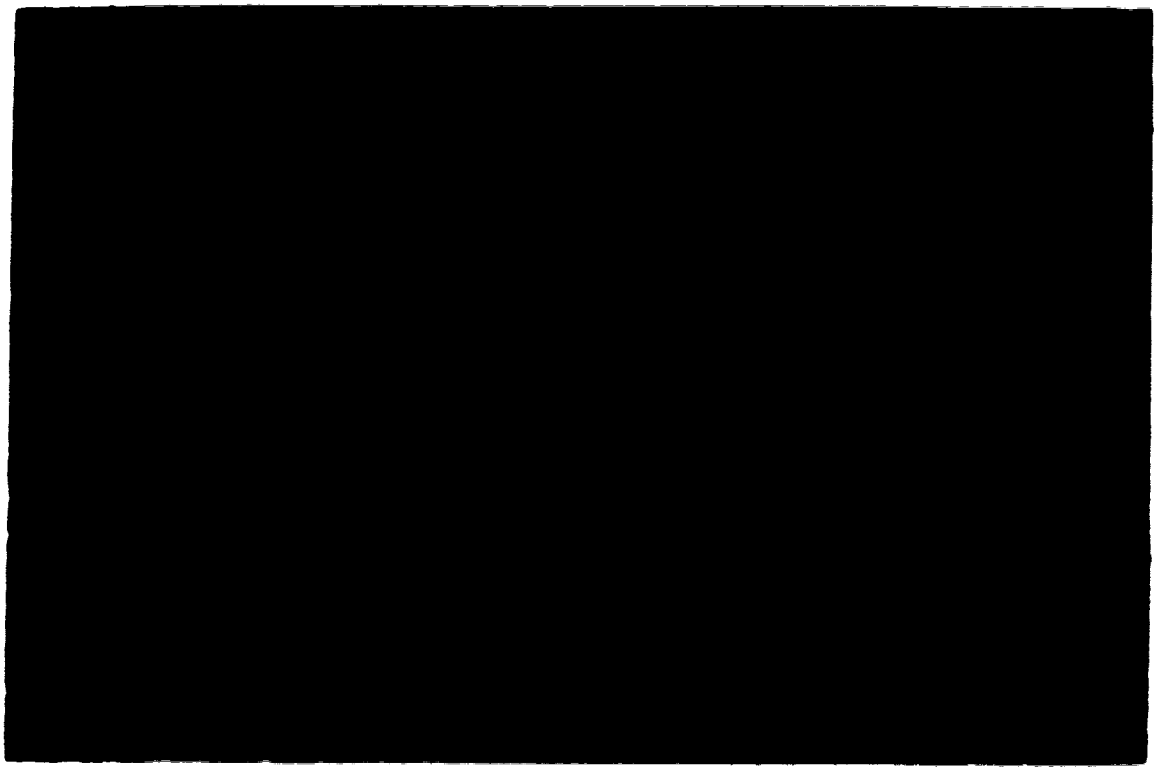

Figure 11A

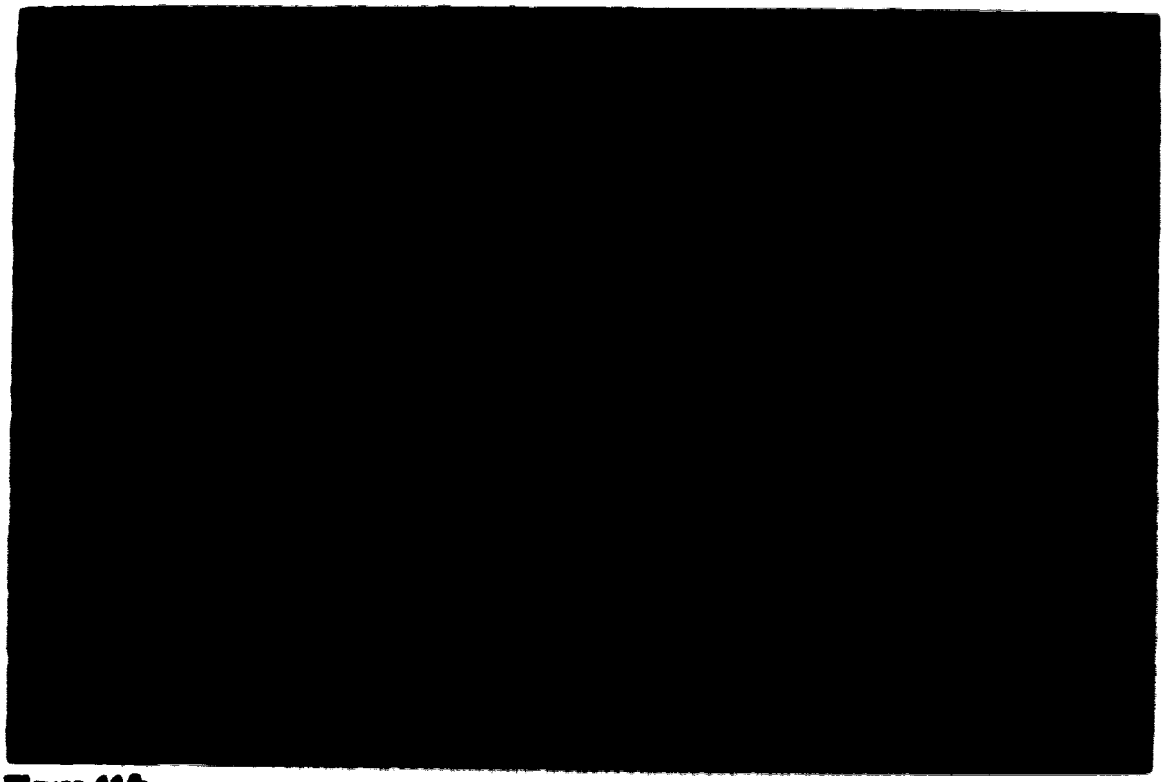

Figure 11B

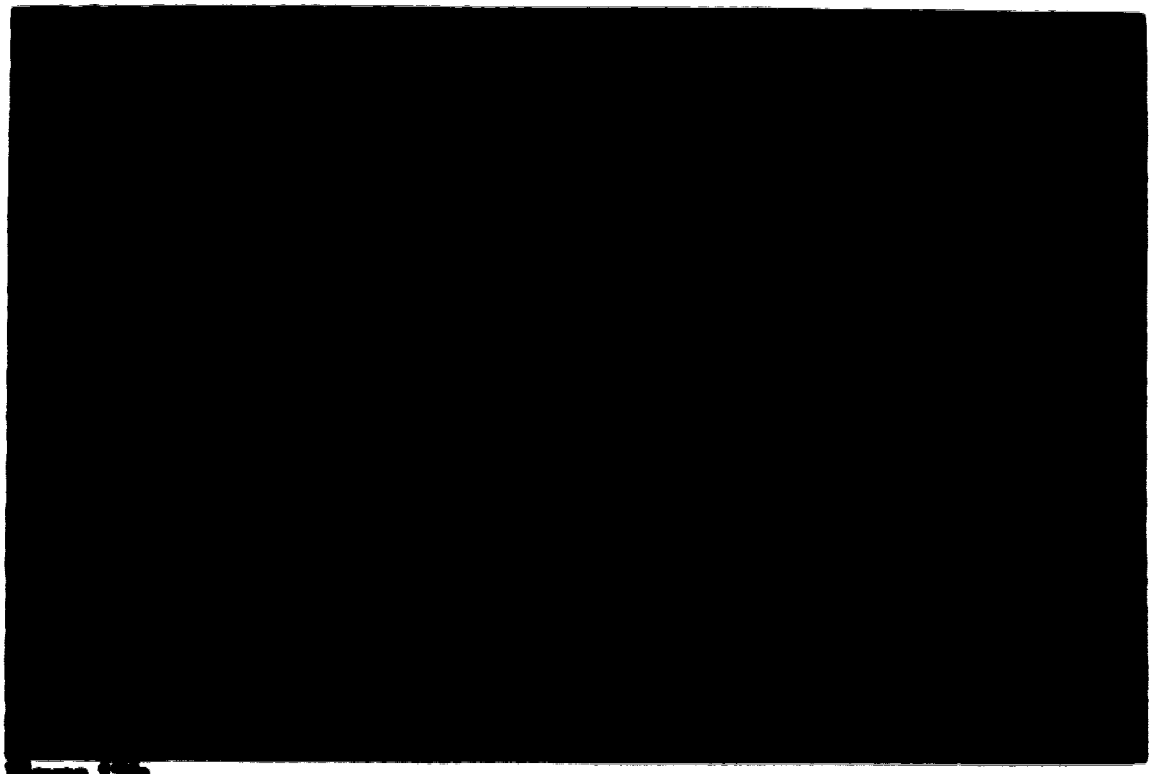

Figure 12.5

Figure 12.6

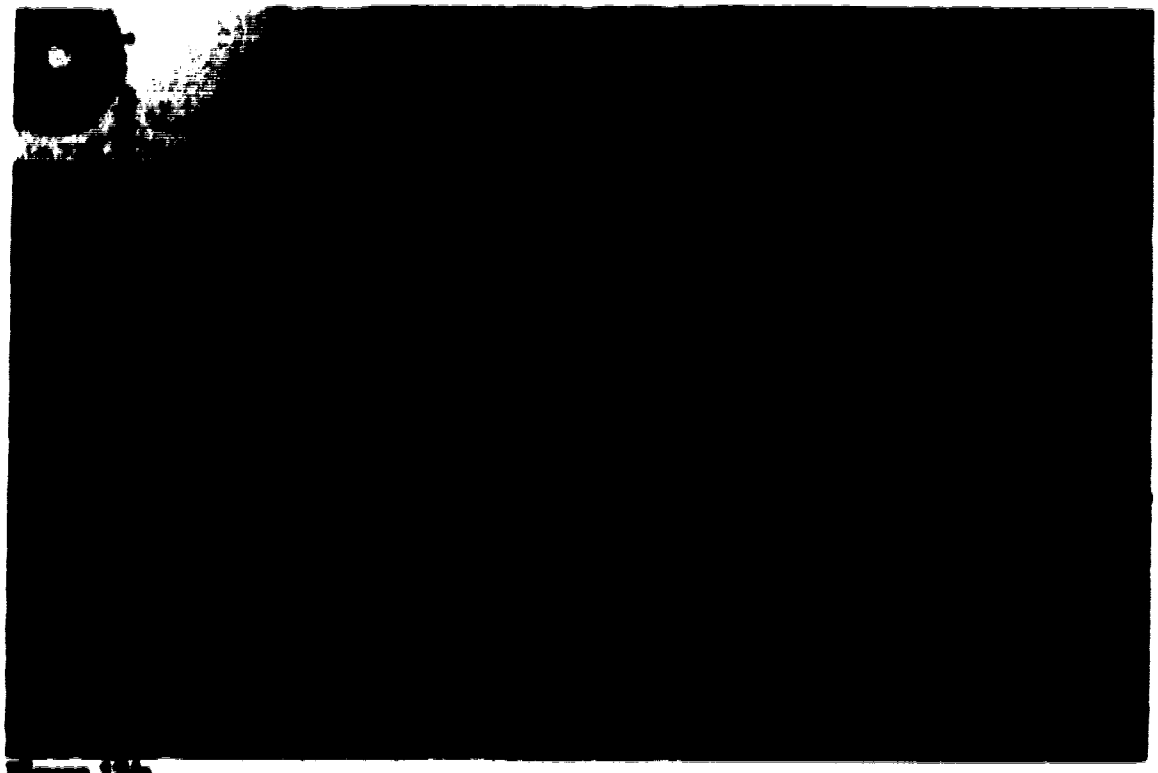

Figure 13.5

Figure 13.6

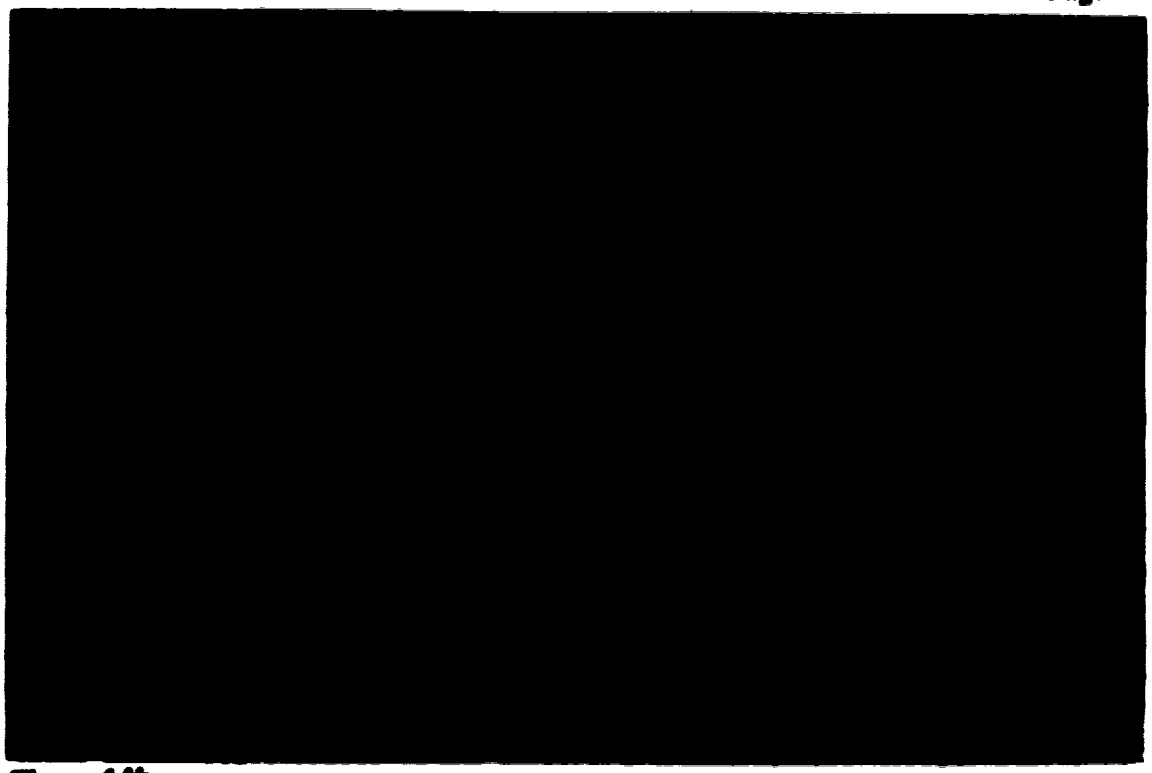

Figure 14b

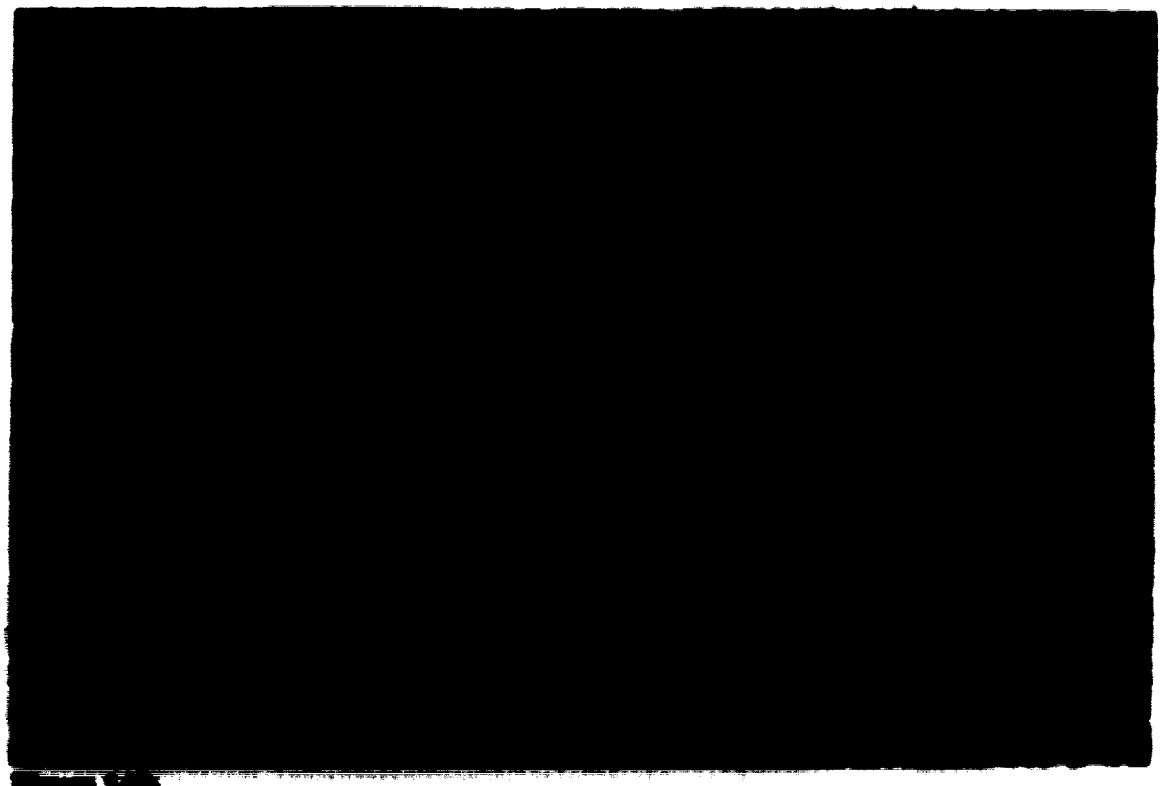

Figure 14c

Figure 15/a

Table 1
Experimental Groups

Group	Donor Strain	Recipient Strain	No. Tx	Technique	Term
1	BN	Lewis	12	Heterotropic	Two way
2	BN	Lewis	12	Orthotropic	Two way
3	Lewis	Lewis	12	Heterotropic	Control

Table 2
Clinical Grading for GVHD

Grade	Heterotopic	Orthotopic
1	6 (50%)	12 (100%)
2	6 (50%)	-
3	-	-

Grade 1: Light redness of ears, snout & paws.
Grade 2: Moderate redness of ears, snout, paws, light hair loss & diarrhea.
Grade 3: Severe redness of ears, snout & paws, alopecia, generalized dermatitis and profuse diarrhea.

Table 3
Relative Spleen Weights

Group		Heterotopic	Orthotopic	Control
7 Days*		0.3706	0.2985	0.1993
		0.3351	0.2343	0.2215
		0.3288	0.2373	0.2396
		0.3515	0.2790	0.2054
	Mean±SD	0.3465±0.018	0.2622±0.031	0.2164±0.018
14 Days				
		0.2557	0.2461	0.2081
		0.2600	0.2077	0.1992
		0.2750	0.2400	0.2131
		0.3045	0.2621	0.1917
	Mean±SD	0.2738±0.022	0.2389±0.023	0.2030±0.09
21 Days				
		0.2212	0.1946	0.2106
		0.2479	0.2355	0.1980
		0.2996	0.2321	0.2079
		0.2573	0.2089	0.2004
	Mean±SD	0.2565±0.016	0.2177±0.019	0.2055±0.06
Normal Lewis Rat	Mean±SD			0.2120±0.020

* Statistically significant, p= 0.0037

Table 4
Popliteal Lymph Node Enlargement Index

Group	Animal	Heterotopic		Orthotopic
7 Days	1	1.681		1.474
	2	1.634		1.248
	3	1.431		1.376
	4	1.689		1.368
	Mean±SD	1.608±0.12	p= 0.019*	1.366±0.09
14 Days	1	1.452		0.529
	2	1.251		1.295
	3	1.656		1.023
	4	1.632		0.552
	Mean±SD	1.497±0.18	p= 0.020*	0.849±0.37
21 Days	1	2.503		1.050
	2	2.327		1.577
	3	1.828		0.951
	4	1.729		0.742
	Mean±SD	2.096±0.37	p=0.007*	1.080±0.35

* Statistically significant

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