Characterization and prevention of the "small-for-size" syndrome in a porcine model of hepatic transplant

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CHARACTERIZATION AND PREVENTION OF THE "SMALL-FOR-SIZE" SYNDROME IN A PORCINE MODEL OF HEPATIC TRANSPLANT

Presented by Amelia J. Hessheimer, MD, in order to apply for the title of Doctor in Medicine and Surgery

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CERTIFICAN:

Que la tesis doctoral "CHARACTERIZATION AND PREVENTION OF THE 'SMALL-FOR-SIZE' SYNDROME IN A PORCINE MODEL OF HEPATIC TRANSPLANT", presentada por Dª Amelia J. Hessheimer, ha sido realizada bajo su dirección,

Y tras valorar el trabajo realizado por la aspirante al Título de Doctor,

AUTORIZAN:

Su presentación y defensa ante el tribunal correspondiente.

En Barcelona, el 21 de febrero de 2011.

Fdo. Constantino Fondevila Campo Fdo. Juan Carlos García-Valdecasas Salgado

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1.1. Introducción

El trasplante hepático es el único tratamiento para pacientes que presentan enfermedad hepática en estado terminal pero la demanda de injertos hepáticos para trasplante excede enormemente el número de órganos disponibles (1-3). Debido a que un hígado sano posee una importante reserva funcional y capacidad regenerativa, el uso de injertos parciales representa una alternativa al trasplante con injertos de hígado completo (4). Sin embargo, el trasplante de injertos parciales requiere un estudio preoperatorio extenso para asegurar la obtención de un injerto adecuado funcionalmente para el receptor, así como una adecuada masa hepática remanente para el donante, y de este modo evitar el desarrollo del síndrome de “small-for-size” (SFS). Dicho síndrome se diagnostica clínicamente cuando aparece colestasis prolongada, coagulopatía, encefalopatía, ascitis intratable, hemorragia gastrointestinal y/o fallo renal en un paciente con una masa hepática <0.8% del peso del receptor o <30-40% del volumen hepático estándar, una vez se han excluido otras causas (5;6). Cuando el síndrome SFS aparece, provoca el fracaso del injerto hasta en el 50% de los receptores (7). La patogénesis del síndrome SFS es multifactorial y se ha asociado no sólo al hecho de trasplantar una masa hepática reducida, también a la presencia de un excesivo flujo portal, a una dificultad en el drenaje venoso a través de las venas hepáticas, a las condiciones físicas y metabólicas del receptor, a la esteatosis hepática y a una funcionalidad insuficiente del propio injerto en general (5;6;8;9). El flujo portal en particular es considerado como un factor esencial involucrado en la aparición de la disfunción y el fallo de un injerto hepático parcial (10-15).

Aparte de una cuidadosa evaluación preoperatoria para evitar el trasplante de un injerto pequeño, se han utilizado varias estrategias adicionales en el ámbito clínico

para evitar la aparición del síndrome SFS. La mayoría de dichas estrategias son quirúrgicas y consisten en procedimientos para favorecer el flujo de salida del injerto mediante la reconstrucción de las venas tributarias de la vena hepática media y/o la realización de venoplastias en las venas hepáticas (16-26), o en la reducción del flujo de entrada portal al injerto a través de la ligadura de la arteria esplénica (27-30), la realización de una esplenectomía (31-36) y/o la creación de un shunt portocaval parcial (13;37-40). Las estrategias farmacológicas para la prevención de síndrome SFS han sido extensamente aplicadas en el contexto experimental, pero la experiencia clínica es muy limitada (41;42).

El estudio del síndrome SFS en humanos es complicado ya que su aparición no es frecuente y cuando se sospecha se utilizan todos los mecanismos para intentar evitarlo. Por tanto, para obtener un mejor conocimiento de la patogénesis del síndrome de SFS en el contexto del trasplante hepático parcial, es importante desarrollar un modelo animal apropiado. Para conseguir este objetivo, varios grupos de investigadores han establecido diferentes modelos animales; algunos con animales pequeños como rata (11;15;43-46) y ratón (47-49). Sin embargo, los modelos con animales grandes presentan características anatómicas y de tamaño que son directamente aplicables al ámbito clínico (50-60).

1.2. Hipótesis

El flujo portal excesivo aparece inmediatamente después de la reperfusión portal en el receptor y es el factor desencadenante de una serie de eventos que provocan la aparición del síndrome de SFS. Un shunt portocaval calibrado podría ser capaz de prevenir la hipertensión portal tras la reperfusión y el desarrollo del síndrome de SFS al trasplantar injertos hepáticos parciales de pequeño tamaño.

1.3. Objetivos

1. Desarrollar un modelo porcino de lesión aguda en un injerto SFS para estudiar la presentación del síndrome SFS y caracterizar la lesión tisular y celular relacionada con la alteración de la hemodinámica hepática.

2. Estudiar los efectos de la modificación del flujo portal a través de la creación de shunts portocavales de diferentes tamaños para conseguir modificar en mayor o menor medida el flujo portal final que alcanzarán los injertos SFS.

3. Determinar cuál es el flujo portal óptimo para prevenir las deletéreas consecuencias de la excesiva perfusión portal y, de este modo, mejorar la función postoperatoria del injerto y la supervivencia de los injertos SFS.

1.4. Material y métodos

En el primer estudio se ha caracterizado la aparición aguda del síndrome SFS en un modelo porcino. Brevemente, realizamos una hepatectomía extensa en un cerdo donante de 15-20 kg de peso para obtener un injerto parcial de pequeño tamaño que se mantiene en la solución de preservación a 4 °C durante 5 horas. Posteriormente se trasplanta a un receptor de 30-35 kg para conseguir que dicho injerto represente aproximadamente un 20% del volumen hepático estándar del receptor (N=17). Como grupo de comparación, realizamos trasplantes de hígado entero utilizando animales, donantes y receptores, de 30-35 kg (N=6).

En el segundo estudio, el lecho vascular portal es descomprimido mediante el establecimiento de un shunt portocaval calibrado antes de la reperfusión en el receptor. Realizamos los trasplantes con injertos SFS igual que en el primer estudio, con pequeñas modificaciones. Después de la extensa hepatectomía, perfusión fría y

extracción del injerto, creamos un shunt portocaval calibrado en el injerto parcial. Para ello, utilizamos un injerto vascular de Gore-Tex® semirrígido con anillos externos de soporte (W.L. Gore & Associates, Inc., Flagstaff, Arizona, USA) de una longitud aproximada de 1,5 cm y de un diámetro de 6 mm (grupo S6, N=6) o de 12 mm (grupo S12, N=6) que anastomosamos entre dos aperturas del correspondiente diámetro entre la vena porta y la vena cava inferior. Después, almacenamos el injerto en la nevera a 4 °C durante 5 horas y entonces realizamos el implante en el receptor.

1.5. Resultados

En el primer estudio, la supervivencia a los cinco días fue del 29% en el grupo de SFS y del 100% en el grupo de injerto completo. El flujo venoso portal, el gradiente de presión y la resistencia fueron significativamente superiores en los receptores de los injertos SFS en comparación con los receptores de injertos completos después de la reperfusión portal y arterial. El flujo arterial, cuantificado como el porcentaje del flujo hepático total, fue significativamente inferior después de la reperfusión en los injertos SFS y esta diferencia se mantuvo en los animales en los que se pudo cuantificar cinco días después. Los marcadores de lesión de la célula endotelial aumentaron inmediatamente después de la reperfusión portal y posteriormente los del daño hepatocelular, pero ambos fueron predictivos de la aparición del fallo del injerto o de su recuperación histológica. La actividad proliferativa celular fue más intensa y temprana en aquellos animales que no sobrevivieron del grupo de SFS. Sin embargo, los injertos SFS que no fracasaron mostraron una actividad regenerativa más lenta y mantenida, aunque su capacidad metabólica no mejoró durante los cinco días del seguimiento.

En el segundo estudio, la supervivencia a los cinco días fue del 29% en el grupo de SFS, del 100% en el grupo S6 y de 0 en el grupo S12. El flujo venoso portal después de la reperfusión se cuadruplicó, se duplicó y se mantuvo similar a las condiciones basales del hígado en el grupo SFS, S6 y S12 respectivamente. En ambos grupos S6 y S12 los shunts fueron capaces de descomprimir el lecho vascular portal y no hubo diferencias en el gradiente de presión venosa portal. La aspartato aminotransferasa y la bilirrubina aumentaron y el tiempo de protrombina disminuyó en todos los animales después de la reperfusión, pero mejoró significativamente al quinto día del postoperatorio en el grupo S6. Los niveles séricos de endotelina-1, un marcador de disfunción endotelial, permanecieron significativamente elevados en el grupo SFS y S12 pero recuperaron sus niveles basales a las doce horas en el grupo S6.

1.6. Discusión

En el primer estudio hemos conseguido reproducir un modelo de trasplante con injerto SFS clínicamente relevante para comprender la patofisiología del síndrome SFS. Es el primer modelo con animal grande descrito que evita el uso del *bypass* veno-venoso, que incluye tiempos de isquemia fría clínicamente relevantes y que estudia las alteraciones en la hemodinámica hepática y su asociación con los resultados postoperatorios, incluyendo la supervivencia y la regeneración hepática, durante un periodo de varios días. Todos los receptores en el primer estudio presentaron un incremento del flujo venoso portal tras la reperfusión. Dicho incremento fue significativamente superior en los receptores de injertos SFS frente a los que recibieron injertos completos. Aunque el incremento en la perfusión portal parece ser un estímulo para la regeneración hepática, simultáneamente causa una lesión irreparable en las células endoteliales que desencadena posteriormente el daño hepatocelular y la muerte del receptor. Los tratamientos del síndrome SFS deben

encaminarse por tanto a la protección de las células endoteliales del estrés físico que produce un excesivo flujo portal y dicho tratamiento debe instaurarse de forma inmediata cuando se revasculariza el injerto.

En el segundo estudio, se demuestra por primera vez el volumen del flujo portal necesario para mantener adecuadamente un injerto SFS sin lesionarlo, que permita su regeneración. De este modo, hemos podido demostrar cómo, aunque un excesivo flujo portal puede causar un daño irreparable a un injerto parcial, estos injertos requieren un flujo portal superior a sus niveles basales. Un incremento de cuatro veces de dicho flujo fue observado en el grupo en el que no se practicaron shunts y el 71% de los injertos fracasaron, pero un incremento de dos veces en el flujo portal basal (grupo S6) no se asoció con ningún caso de fracaso del injerto durante los cinco días de seguimiento. Asimismo, fue muy interesante determinar que unos niveles de flujo portal similares a los basales tras la reperfusión (grupo S12) se asocian con un 100% de fracaso del injerto a los tres días del trasplante.

La endotelina-1 (ET-1) es un péptido que se produce en situaciones de inflamación endotelial y estrés, así como de hipoxia (61;62). El mecanismo por el cual demostramos un incremento de la expresión de ET-1 tras la reperfusión en el grupo SFS sin shunts puede deberse al daño sinusoidal por la excesiva perfusión portal. Sin embargo, su incremento en el grupo S12 no es debido al estrés mecánico de la excesiva perfusión portal ya que la vena porta está descomprimida y el endotelio sinusoidal está bien preservado en estos injertos. En teoría, podría deberse a otras alteraciones fisiológicas asociadas con una reducida perfusión del injerto como la hipoxia.

En los grupos SFS y S6, la administración de glucosa fue sólo necesaria en el periodo postoperatorio inicial cuando los animales no ingerían suficiente alimento por vía oral. Sin embargo, en el grupo S12 todos los animales presentaron episodios repetidos de

hipoglucemia en controles seriados y la frecuencia de dichos controles tuvo que ser aumentada para poder prevenirla. Esta situación de hipoglucemia incorregible recuerda a la que aparece en situaciones de fallo hepático fulminante, en el que hasta un 45% de los pacientes sufren hipoglucemias refractarias a la administración intravenosa de glucosa debido a la depleción de los depósitos hepáticos de glucógeno y a una alterada gluconeogénesis (63).

El modelo descrito en estos estudios corresponde a un modelo agudo de lesión por SFS y su evaluación está limitada por un periodo corto de seguimiento. Sin embargo, sus resultados aportan importante información que puede permitir incrementar el uso de injertos parciales para trasplante hepático.

1.7. Conclusiones

En el trasplante hepático con un injerto SFS, el flujo portal se incrementa alrededor de cuatro veces con respecto a su valor basal. Este aumento en la perfusión portal parece ser un estímulo para la regeneración hepática. Al mismo tiempo, sin embargo, causa un daño endotelial significativo, cuya extensión y severidad determina la aparición de un fracaso del injerto o su recuperación. Aunque un flujo y presión portal excesivos son factores clave para el desarrollo del síndrome de SFS, la presencia de un flujo portal limitado puede ser también un factor perjudicial para los injertos parciales. La realización de un shunt portocaval calibrado que mantiene el flujo venoso portal alrededor de dos veces su valor basal preserva la microarquitectura tisular y mejora los parámetros bioquímicos hepáticos, constituyendo una forma efectiva de prevenir el daño endotelial y el fracaso de los injertos SFS.

2. INTRODUCTION

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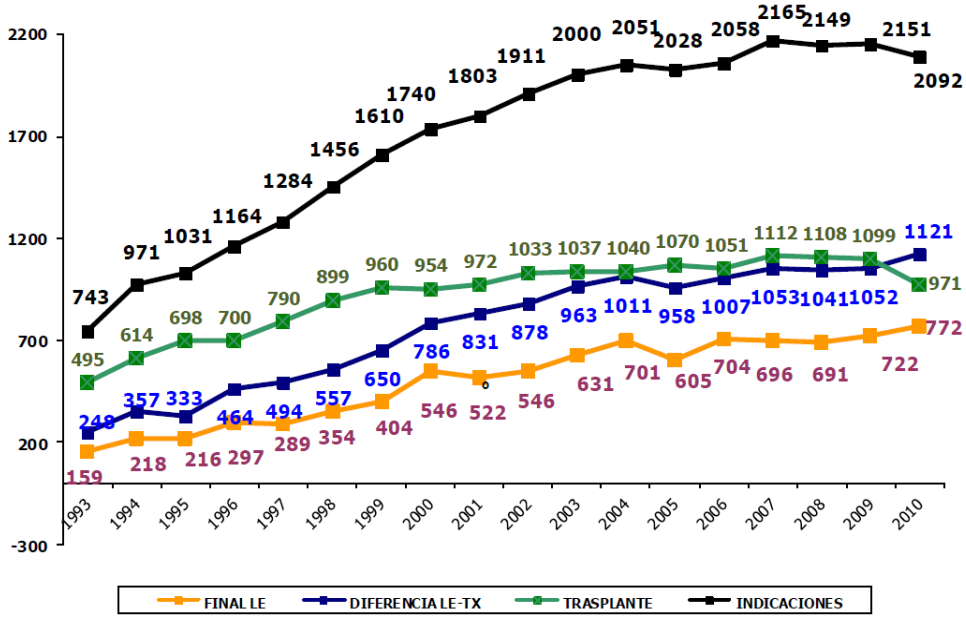
2.1. "Small-for-size" syndrome

Liver transplantation is the only treatment for end-stage liver disease. The demand for livers, however, far surpasses the supply. In the United States in 2008, there were 6,745 liver donors for almost 16,000 patients on the liver transplant waiting list, and approximately 1,800 patients died while awaiting a liver transplant that year (1). The situation in Europe is similar (2). In Spain, the number of liver transplant indications was nearly double the number of liver transplants performed in 2010: 2,092 indications and 971 transplants (FIGURE 1) (3). Based on these facts, means of increasing the number of liver donors are currently of great interest.

FIGURE 1. Evolution of the liver transplant waiting list in Spain, 1993-2010



The black line is the number of liver transplant indications and green the number of actual transplants performed per year. In recent years, there have been approximately twice as many liver transplant indications as liver donors. Figure courtesy of the Spanish National Transplant Organization (Organización Nacional de Trasplantes) (3).

On the basis of the fact that a healthy liver has considerable functional reserve and regenerative capacity (4), reduced-size liver grafting has become an alternative to orthotopic whole liver transplant. The use of partial liver grafts arising from live donation or the splitting of a deceased donation liver currently constitute an important strategy to increase the number of organs. However, reduced-size liver transplantation is a procedure that requires extensive preoperative planning to ensure adequate functional graft and remnant liver mass for the recipient and the donor, respectively, in order to avoid potentially detrimental postoperative sequelae.

In adult-to-adult live donation liver transplantation (ALDLT), the right hemiliver is most commonly used as the transplant graft. Donor right hepatectomy, however, has been associated with a morbidity rate as high as 38-44% and a mortality rate between 0.2 and 0.9% in recent series (64-69). Donor left hepatectomy, in contrast, leads to significantly fewer postoperative complications (70). Left hemiliver grafts offer anatomical advantages over right grafts, as well, including a single bile duct, a single portal vein, and sufficient venous outflow tributaries in most cases (39;40). However, the major and oftentimes prohibitive disadvantage of using left hemilivers is the fact that they frequently result in insufficient hepatic mass for the recipient, leading to the development of “small-for-size” syndrome (SFSS).

“Small-for-size” syndrome arises when a partial liver graft cannot cope with the unique stresses placed on it because of its small size. Though there is no consensus definition, SFSS may be diagnosed clinically when prolonged cholestasis, coagulopathy, encephalopathy, intractable ascites, gastrointestinal bleeding, and/or renal failure arise in a liver representing <0.8% of the recipient’s body weight or <30-40% of the recipient’s standard liver volume, at the exclusion of any other cause (5;6). When it occurs, SFSS leads to graft failure in up to 50% of recipients (7).

The pathogenesis of SFSS is multifactorial and has been associated with not only small functional liver mass but also excessive portal venous inflow, obstructed hepatic venous outflow, the metabolic and physical condition of the recipient, graft steatosis, and insufficient intragraft responses in general (5;6;8;9). Portal inflow, in particular, is thought to be a primary factor involved in the dysfunction and failure of a reduced-size liver graft. The reduction in the intrahepatic vascular bed results in higher portal flow per gram of tissue, a rise in portal pressure, and stress in the hepatic sinusoid (10;12-14). This shear stress is thought to provoke sinusoidal endothelial cell injury, which leads to subsequent processes of hepatocellular damage and death (11;15).

2.2. Clinical approaches to prevent "small-for-size" syndrome

Aside from careful preoperative planning to avoid the potential transplantation of a SFS graft, several additional techniques for the prevention of SFSS have been applied in the clinical setting. These can be divided between two major categories: surgical and pharmacological.

2.2.1. Surgical approaches

The majority of the approaches used to prevent SFSS in the clinical setting are surgical. In large part, these consist in means of maximizing the amount of flow out of or reducing the amount of flow into the partial liver graft. Additionally, there have been reports on the use auxiliary partial orthotopic liver transplantation (71;72) and the transplantation of partial grafts from two different donors into a single recipient (73-75). There are, however, numerous ethical concerns surrounding the latter technique, and experience with both of these techniques is very limited. Hence, they will not be discussed further.

As was mentioned previously, right hemiliver grafts comprise the majority of partial livers transplanted in adults, particularly in Western countries (19). In order to ensure adequate remnant liver in the donors in the context of ALDLT, these grafts are commonly recovered without including the middle hepatic vein (MHV) (18;19;21;22). In doing so, however, adequate drainage from the anterior segments of the graft (i.e., segments V and VIII), may be compromised, thereby leading to congestion and decreasing the amount of functional liver mass in the recipient (16;23;25). To help alleviate this problem, reconstruction of MHV tributaries has been performed using venous interposition grafts to attach large MHV tributaries to the inferior vena cava (IVC) in the recipient (23). In addition, venoplasty of the hepatic vein or veins, with or without cavoplasty, helps to create a wider outflow orifice and reduce intragraft congestion in small transplanted grafts (16;17;20;24;26).

Splenectomy and/or splenic artery ligation (SAL) are the most common approaches used to reduce portal venous inflow in the clinical setting. SAL alone is capable of decreasing portal venous inflow and pressure. The extent to which it does so, however, is unpredictable and varies significantly among patients (27-30). Furthermore, when postreperfusion portal vein flow (PVF) is extremely high, SAL alone may be incapable of reducing PVF to an acceptable level (29). Performing a concomitant splenectomy may offer some added benefit in terms of reducing PVF. However, whether splenectomy should be avoided in the transplant population due to increased septic and thrombotic complications and overall surgical complexity remains an issue of controversy (31-36).

Clinical work has recently been published on ALDLT performed using left hemilivers and a hemiportocaval shunt (HPCS) to decompress the splanchnic bed and prevent irreparable sinusoidal injury (13;37-40). In 2005, Troisi *et al* (13) reported HPCS inflow modification for both small left and right hemilivers (graft weight-to-body weight ratio <0.8%) by prospectively assigning thirteen such grafts to shunted and unshunted

groups. In their study, the authors did not experiment with different standardized shunt sizes or varying levels of inflow but, instead, universally calibrated the shunts to achieve approximately two-times the baseline portal inflow recorded in the donors prior to hepatectomy. Their hypothesis was that twice-baseline would be the most "physiological" flow rate for the reperfusion of a hemiliver. Although SFSS was observed in three recipients of unshunted grafts, it did not arise in any of the recipients of shunted grafts. Furthermore, in the shunted group, at a median follow-up of 17 months, no cases of portal vein or shunt thrombosis were observed, and none of the shunts had to be occluded secondary to the development of hepatic encephalopathy or other signs of excessive portosystemic shunting.

Most recently, Botha *et al* (40) described the first American series on the use of left hemilivers and HPCS for ALDLT. Sixteen patients received left hemilivers, with a median graft weight-to-body weight ratio of 0.67% (range 0.51-1.05). The median portal venous pressure gradient (PVPG) after reperfusion with a HPCS in place was 5 mmHg (range 1-15). None of the shunts were calibrated, and PVF was measured in only four of the sixteen patients, with, again, relatively disparate results among recipients. One of the patients with a HPCS did end up developing SFSS, whereas two others required occlusion of their HPCS secondary to protracted encephalopathy (76). Even though they were already performing HPCS for SFS transplant in the clinical setting, the authors themselves commented that they still did not know what acceptable levels of PVF and/or PVPG would be in the setting of HPCS.

2.2.2. Pharmacological approaches

In experimental studies, numerous pharmacological agents have been applied to SFS grafts. Unlike surgical approaches to prevent SFSS, pharmacological agents offer a reversible means for reducing portal venous flow and/or pressure in the immediate post-reperfusion period, while the partial graft is still adapting to the altered physiology

of the recipient's body. Experience with pharmacological treatment of SFSS in the clinical setting, however, is limited.

Suehiro *et al* described a controlled study of 112 patients on the use of intraportal infusion of nafamostat mesylate, prostaglandin E_1, and thromboxane A_2 synthetase inhibitor in adult recipients of SFS grafts (41). The treated subjects apparently developed less ascites and, thereby, less SFSS than the untreated controls. However, the study was prone to a significant selection bias. All the controls were performed in the first half of the study; the treated subjects, on the other hand, were performed in the second half, whereby the operators' experience had undoubtedly increased. Furthermore, the indication for transplantation was hepatocellular carcinoma in almost 50% of the treated subjects but only 20% of the untreated controls, a fact upon which the authors did not comment but which might be an indication of less severe baseline liver disease in the former group versus the latter.

Somatostatin is a naturally occurring tetradecapeptide that has been shown to decrease portal pressure and splanchnic blood flow in a dose-dependent manner (77-80). Furthermore, at standard doses, somatostatin has minimal-to-no adverse effects on systemic hemodynamics. It appears to function by directly inducing local vasoconstriction in the mesenteric arteries and porto-collateral veins via a nitric oxide-independent mechanism (81-84), as well as by inhibiting the secretion of gut-derived vasodilatory peptides, such as glucagon, vasoactive intestinal peptide, and substance P (85-87). A randomized-controlled study by Feng *et al* investigated the use of perioperative treatment with somatostatin in forty patients undergoing ALDLT (42). The authors demonstrated some early differences between the treated and untreated groups in terms of hepatic transaminases and serum and tissue levels of endothelin-1, a marker of endothelial cell dysfunction. Neither flows nor pressures were mentioned among the results of the study, however, and none of the transplanted grafts met SFS criteria (i.e., all were >0.8% of the respective recipient's body weight).

2.3. Animal models of acute “small-for-size” injury

Studying SFSS in the clinical setting is complicated, as its appearance is rare and considerable attempts are made to avoid it. Hence, in order to gain better insight into the pathogenesis of SFSS in the context of partial liver transplantation, it is important to develop an appropriate animal model. To achieve this end, several different models have been established by various groups.

2.3.1. Small animal models

Acute “small-for-size” (SFS) liver injury has been studied in several small animal models. Chief among these are models in rats and in mice.

The group in Hong Kong has performed extensive work using a rat model of SFS liver transplant. In some of the recipients, they induced cirrhosis by injecting 50% carbon tetrachloride subcutaneously twice a week for six weeks preoperatively (45). Graft size reduction was performed on the backtable using a lobe-ligation technique in order to achieve a final graft weight-to-body weight ratio in the recipient of <0.8%. Grafts were transplanted using a cuff technique for the portal vein and without reanastomosing the hepatic artery (43); this last fact limits the clinical applicability of the model when hemodynamic factors are considered. Using this rat model, both normal (11;15;44;46) and fatty (45) SFS liver transplants have been performed.

The group of Clavien in Zurich developed a mouse model of SFS liver transplant in which partial liver grafts represented approximately 30% of the recipients’ standard liver volume. In contrast to the rat, the mouse requires reanastomosis of the hepatic artery in order to avoid major cholangiocellular and hepatocellular necrosis and recipient death (47;48). Hence, in their model, a patch of the donor aorta containing the celiac trunk was anastomosed end-to-side to the infrarenal aorta of the recipient, while the portal vein was reconnected using a cuff technique. Given that hepatic tissue taken

from SFS liver grafts two days after transplant demonstrated little-to-no proliferative activity based on proliferation cell nuclear antigen (PCNA) immunostaining, the authors concluded that defective regeneration is central to the pathogenesis of SFSS, though they made no comments regarding actual hepatic mass measured at the time of death. The authors have subsequently gone on to discount the portal hyperperfusion theory of SFSS (88), based in part on their observation that postreperfusion PVF decreased to 60% of its baseline value in their SFS mouse model (49). However, using their technique, PVF upon reperfusion was lower in all the recipient mice, regardless of the size of the graft. This finding is not representative of the clinical situation, unless there is a technical complication at the portal vein anastomosis or another cause for prehepatic portal inflow obstruction (e.g., portomesenteric vascular thrombosis). The fact that the authors observed lower-than-baseline postreperfusion flows in their mice suggests that this is not an adequate model in which to study the hemodynamic alterations surrounding acute SFS liver injury in hepatic transplant and, moreover, raises concern as to whether such a small cuffed anastomosis truly allows unimpeded laminar flow through the portal vein.

2.3.2. Large animal models

Experimental studies involving animals are central to advancement in virtually all medical fields. Large animal models are particular useful in the surgical sciences. More than small animal models, they provide appropriate size and anatomy for the establishment of new surgical techniques. Although small animal models require much less of an investment in terms of material, personnel, and space, they are not directly applicable to humans.

Limited work has been performed on partial liver transplant in dogs. Cherqui *et al* used the canine model to develop a technique for recovering and transplanting the left hemiliver from a live donation donor (50;51), which they subsequently applied to adult-

to-pediatric live donation liver transplantation in the clinical setting. The grafts they transplanted, however, were not technically “small-for-size”. Sheep have also been used to practice the technique for recovering partial liver grafts laparoscopically (54). The advantage offered by canine and ovine models is that the retrohepatic IVC is extrahepatic in both species and may, therefore, be separated from the hepatic parenchyma and preserved at the time of donor and recipient hepatectomy.

Overall, however, pigs are the preferred large animal model for liver transplant studies, based on their greater physiological and anatomical similarity to humans, including the absence of hepatic venous sphincters (89;90). All of the major large animal models of SFS liver transplant that have been published to date have been performed using pigs (see TABLE 1, which follows). Though all of these studies involved the transplantation of grafts that represented approximately 20-30% of the recipients’ standard liver volumes, they varied in terms of transection techniques, lengths of cold ischemia, and postoperative survival outcomes. Furthermore, and probably most importantly, all of these studies employed the use of venovenous bypass during the anhepatic phase in the recipient.

In the pig, the retrohepatic IVC is entirely intrahepatic and unable to be separated from the surrounding hepatic parenchyma. Hence retrohepatic caval preservation, also know as the “piggyback” technique, is ironically not able to be performed in the porcine model. The retrohepatic IVC must therefore be clamped infra- and suprahepatically and removed in its entirety with the graft. While the IVC is clamped, the circulating blood volume is essentially reduced to the volume in the head and neck, rostral extremities, and thoracic cavity, and blood flow to the abdominal organs and the caudal extremities becomes increasingly more stagnant. Vascular stasis results in the loss of the substrates needed to fuel the sodium-potassium exchange pump, leading to progressive hyperkalemia and acidosis, which may provoke severe systemic cardiovascular alterations when these ischemic beds are reperfused. Hence, most

groups perform porcine orthotopic liver transplantation (OLT) using venovenous bypass in order to minimize the adverse effects of the anhepatic phase. Venovenous bypass, however, significantly increases the risk of intraoperative complications, such as excess bleeding, hemorrhagic shock, disorders of coagulation, air embolism, and venous thromboembolism (91). By keeping the anhepatic time short, however, both severe, irreversible hemodynamic alterations and the use of venovenous bypass may be avoided.

Based on their larger size, all of the vascular anastomoses in pigs may be performed end-to-end, as they are done in the clinical setting. The cuff technique, which is popular in murine models of liver transplantation, has been described in the context of large animal OLT, as well (92). Although it may be an attractive option to some, based on the decreased level of surgical expertise it theoretically requires, the cuff technique, nonetheless, has not been associated with a shorter anhepatic time. It may, furthermore, lead to progressive anastomotic stenosis and raises concerns about unobstructed, laminar blood flow through the anastomosis (91).

TABLE 1. Porcine "small-for-size" survival models

Institution	Transection	Implant	SLV	CIT (min)	Mortality	Regeneration
Kyushu University (1995) (52)	*Ex situ*	Autograft with VVB	29% (N=5)	125±19	100% (12 hours)	--
University of Athens (2003) (56)	*Ex situ*	Allograft with VVB	18% (N=8)	360	63% (48 hours)	--
University of Pittsburgh (2004) (57)	*In situ*	Allograft with VVB	34% (N=8)	151±22	0 (5 days)	121% (5 days)
			22% (N=15)	139±20	20% (3 days)	147% (3 days)
					60% (5 days)	190% (5 days)
Tohoku University (2002, 2003, 2005 2006) (53;55;58;59)	*In situ*	Allograft with VVB	25% (N=11)	151±51*	100% (24 hours)	--

* Total ischemia.

CIT, cold ischemic time; SLV, standard liver volume; VVB, venovenous bypass.

3. HYPOTHESIS

3. HYPOTHESIS

The hypothesis of this doctoral thesis is two-fold:

1. Excessive portal vein flow is present immediately after portal reperfusion in the recipient and is the instigating factors in a series of events that ultimately lead to the development of SFSS.

2. A calibrated portocaval shunt prevents postreperfusion portal hyperperfusion and, thereby, the development of SFSS in partial liver grafts.

4. OBJECTIVES

4. OBJECTIVES

The objectives of this doctoral thesis are as follows:

1. To produce a porcine model of acute SFS injury that does not use venovenous bypass, in order to study the presentation of SFSS and characterize the cellular lesion in relation to altered hepatic hemodynamics.

2. To study the effects of modifying portal vein flow through the creation of portocaval shunts of different sizes, each calibrated to achieve a distinct decrease in the amount of flow ultimately reaching the SFS liver graft.

3. To determine the optimal decrease in the amount of portal vein flow that is capable of preventing the deleterious consequences of portal hyperperfusion and improving postoperative graft function and survival in SFS liver grafts.

5. MATERIALS, METHODS, AND RESULTS

5. MATERIALS, METHODS, AND RESULTS

5.1. Study 1: Characterization of the acute phase of "small-for-size" syndrome in the porcine model

In the first part of this doctoral thesis, the acute phase of SFSS was characterized in the porcine model. In brief, extended hepatectomy was performed in outbred male weanling pigs weighing 15-20 kg. Reduced-size liver grafts were stored at 4 °C for approximately 5 hours and then transplanted into recipient pigs weighing 30-35 kg, ultimately representing approximately 20% of the recipients' standard liver volumes (N=17). As a basis for comparison, whole liver transplants were also performed, in which both the donors and recipients weighed 30-35 kg (N=6).

A detailed description of the experimental protocol and results can be found in the first article, which follows:

Fondevila C, **Hessheimer AJ**, Taurá P, Sánchez O, Calatayud D, de Riva N, Muñoz J, Fuster J, Rimola A, García-Valdecasas JC. Portal Hyperperfusion: Mechanism of Injury and Stimulus for Regeneration in Porcine Small-for-Size Transplantation. Liver Transpl 2010;16:364-374.

LIVER TRANSPLANTATION 16:364-374, 2010

ORIGINAL ARTICLE

Portal Hyperperfusion: Mechanism of Injury and Stimulus for Regeneration in Porcine Small-for-Size Transplantation

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Understanding the pathogenesis of small-for-size (SFS) syndrome is critical to expanding the applicability of partial liver transplantation. We aimed to characterize its acute presentation and association with alterations in hepatic hemodynamics, microstructure, and regeneration in a porcine model. Eighteen SFS liver transplants were performed. Donors underwent 70% hepatectomy. Partial grafts were implanted into larger recipients. Whole liver transplants were also performed (n = 6). Recipients were followed until death or for 5 days. Hemodynamics were measured, and tissue was sampled intraoperatively and at the study end. Serum was sampled regularly during follow-up. Seventeen SFS transplants and 6 whole liver transplants were included. SFS grafts represented 23.2% (19.3%-25.3%) of the recipients' standard liver volume. The survival rate was 29% and 100% in the SFS and whole liver groups, respectively. The portal venous flow, pressure gradient, and resistance were significantly higher in recipients of SFS grafts versus whole livers after portal and arterial reperfusion. Arterial flow as a percentage of the total liver blood flow was significantly lower after reperfusion in SFS grafts and remained so when measured again after 5 days. Markers of endothelial cell injury increased soon after reperfusion, and those of hepatocellular injury increased later; both predicted the appearance of either graft failure or histological recovery. Proliferative activity peaked earlier and higher among nonsurvivors in the SFS group. Surviving grafts demonstrated a slower but maintained rise in regenerative activity, although metabolic activity failed to improve. In SFS transplantation in the acute setting, portal hyperperfusion is a stimulus for regeneration but may simultaneously cause irreparable endothelial injury. This porcine model not only helps to elucidate the inciting factors in SFS pathogenesis but also offers a clinically relevant means to study its prevention. ***Liver Transpl 16:364-374, 2010.*** © **2010 AASLD.**

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Liver transplantation is the only treatment for end-stage liver disease, although its applicability has progressively decreased over the last decade because of the growing disproportion between donors and potential recipients.[1-3] Recent surgical advances have made possible the utilization of partial liver grafts arising from a living donor or a split cadaveric liver, and their use constitutes an important strategy for increasing the number of organs. The use of partial grafts, however, may be associated with small-for-size syndrome (SFSS). SFSS is a constellation of clinical signs associated with postoperative graft dysfunction: prolonged

Abbreviations: AST, aspartate aminotransferase; CIT, cold ischemic time; H&E, hematoxylin and eosin; HA, hyaluronic acid; HAF, hepatic artery flow; ICG, indocyanine green; IVC, inferior vena cava; P/A, portal/arterial ratio; PDR, plasma disappearance rate; PVF, portal vein flow; PVPG, portal venous pressure gradient; PVR, portal vein resistance; QPT, quick prothrombin time; SFS, small-for-size; SFSS, small-for-size syndrome; SLV, standard liver volume; THBF, total hepatic blood flow; TK, thymidine kinase; TLBF, total liver blood flow; VVB, venovenous bypass; WIT, warm ischemic time.

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cholestasis, coagulopathy, refractory ascites, hepatic aminotransferase elevation, and encephalopathy.[4-7]

As the name implies, SFSS is thought to be attributable to a graft that is too small to cope with the stresses placed on it. Not only factors associated purely with size, such as a graft to recipient body weight ratio < 0.8% and a graft to standard liver weight ratio < 30% to 40%, but also excessive portal venous inflow, obstructed hepatic venous outflow, the metabolic and physical condition of the recipient, and graft steatosis can all contribute to its development.[5,6,8] Portal inflow in particular is thought to be a primary factor involved in the dysfunction and failure of a partial liver graft. The reduction in the intrahepatic vascular bed results in higher portal flow per gram of liver tissue, a rise in portal pressure, and stress in the hepatic sinusoid.[9-12] This shear stress is thought to provoke sinusoidal endothelial cell injury, which leads to subsequent processes of hepatocellular damage and death.[13,14]

Acute small-for-size (SFS) liver injury has been studied in several small-animal models.[13-16] Large-animal models, however, provide a much more clinically relevant means of investigating the pathophysiology of a disease process and treatment options that can be more readily applied in the human setting. The objective of this study was to reproduce the acute phase of SFSS in a porcine model of reduced-size liver transplantation in order to study its presentation and characterize the cellular lesion and its relation to altered hepatic hemodynamics. In doing so, we hoped to be able to identify key targets for the prevention of SFSS and an adequate model in which to study them.

MATERIALS AND METHODS

Study Overview

In order to thoroughly characterize the evolution and survival of the animals after SFS transplantation, 18 SFS liver transplants were performed. In brief, outbred male weanling pigs (15-20 kg) underwent extended hepatectomy. Partial liver grafts were implanted into recipients (30-35 kg) and represented approximately 20% of the recipients' standard liver volume (SLV). As a basis for comparison, whole liver transplants were also performed (n = 6). Whole liver grafts were transplanted from the donors into the recipients, both of which weighed 30 to 35 kg.

Donor Procedure

In the SFS group, the abdominal wall was opened with a midline incision, and vascular branches to the left lateral and paramedian lobes were ligated. After cholecystectomy, crush-clamp resection of 70% of the hepatic tissue was carried out according to the method described by Kelly et al.[17] and the porcine hepatic segmental anatomy.[18] The donor was heparinized (3 mg/kg intravenously), and the liver was perfused with 500 mL of cold University of Wisconsin solution both portally and arterially. The partial liver graft was removed, prepared, weighed, and placed in cold storage at 4°C. In the whole liver group, no resection was performed.

Recipient Procedure

After 5 hours of cold storage, the graft was rinsed with 500 mL of warm Ringer's solution. The portal vein, infrahepatic inferior vena cava (IVC), and suprahepatic IVC were clamped in the recipient. Total hepatectomy was performed, and the recipient liver was weighed. Upon completion of the suprahepatic IVC and portal vein anastomoses, the graft was reperfused. Neither venovenous bypass nor vasoactive agents were used during the anhepatic phase, which was kept below 20 minutes. Immunosuppression (0.04 mg/kg tacrolimus intravenously and 500 mg of methylprednisolone intravenously) was administered during the anhepatic phase. The infrahepatic IVC, hepatic artery, and bile duct anastomoses were performed end to end, with a stent placed in the bile duct to ensure patency.

Intraoperative Monitoring

After an open cutdown of the right carotid sheath, a triple lumen catheter was placed in the internal jugular vein for invasive venous pressure monitoring. In the recipients, the catheter was tunneled subcutaneously to exit at the back of the neck for postoperative access. A single lumen catheter was introduced into the portal vein to monitor portal vein pressure. Ultrasonic flow probes were connected to a flowmeter (HT107, Transonic Systems, Ithaca, NY) to measure hepatic artery flow (HAF) and portal vein flow (PVF). The portal vein pressure, HAF, and PVF were recorded in the donor at the baseline and in the recipient after both portal and arterial reperfusion. At these same times, the internal jugular venous catheter was momentarily advanced into the suprahepatic IVC to record the suprahepatic venous pressure. Liver function was quantified at the baseline and the end of the recipient operation with indocyanine green (ICG) clearance. Results were measured with a LiMON monitor (Pulsion Medical Systems AG, Munich, Germany) and recorded in terms of the plasma disappearance rate (PDR) of ICG and the ICG retention rate after 15 minutes.

Postoperative Management

Recipients were monitored for 5 postoperative days at 3, 6, 12, and 24 hours on the first day and every 8 hours thereafter. They were given free access to water and dry food; animals that did not ingest the food were administered a 10% glucose solution (500 mL intravenously). Immunosuppression was given daily for 4 days (0.04 mg/kg tacrolimus intravenously and 125, 100, 75, and 50 mg of methylprednisolone intravenously). Animals that survived until the fifth day

were anesthetized and reopened. ICG clearance, hemodynamic, and blood-based parameters were reevaluated. Each liver graft was removed, weighed, and sampled, and the animals were euthanized. Nonsurvivors were submitted to an autopsy with graft extirpation.

Blood and Serum Analysis

Blood was sampled in the donor at the baseline and in the recipient at the baseline, at 1, 3, 6, 12, and 24 hours after portal reperfusion, and daily thereafter for the remainder of follow-up. In these samples, the serum levels of aspartate aminotransferase (AST) and total bilirubin and the quick prothrombin time were determined.

Hyaluronic acid (HA) is a polysaccharide synthesized by mesenchymal cells and eliminated chiefly by receptor-mediated endocytosis in the hepatic sinusoidal endothelium[19]; increased serum HA levels reflect sinusoidal endothelial damage.[20-23] HA was measured by a radiometric assay with the Pharmacia HA test (Pharmacia Diagnostics, Uppsala, Sweden) in prereperfusion and postreperfusion serum samples.

Thymidine kinase (TK) activity is an index of hepatic regeneration in both tissue and serum.[24,25] TK activity was measured in serial serum samples with the Liaison TK assay (DiaSorin, Inc., Stillwater, MN) and detected with a Liaison chemiluminescence analyzer (DiaSorin S.p.A., Saluggia, Italy).

Tissue Analysis

Hepatic tissue was sampled in the donor at the baseline and in the recipient 1 hour after portal reperfusion. Each biopsy sample was divided into 2 sections, one preserved in 10% formaldehyde for subsequent inclusion in paraffin and the other embedded in Tissue-Tek optical coherence tomography compound (Sakura Finetek Europe B.V., Zoeterwoude, the Netherlands) and snap-frozen in liquid nitrogen.

CD31 (platelet/endothelial cell adhesion molecule 1) is an immunoglobulin that exists largely as a self-associated cell-surface protein interdigitated with other CD31 molecules on the extracellular borders of endothelial cells to maintain endothelial integrity.[26] Cryosections of hepatic tissue were immunostained with porcine anti-CD31 antibody (MCA1746G, Serotec, Oxford, United Kingdom) to evaluate the microstructural integrity of the hepatic sinusoid. For immunohistochemistry, 4-μm-thick sections were fixed with cold acetone. Endogenous peroxidase activity was inhibited with a peroxidase-blocking solution (S2023, DakoCytomation, Glostrup, Denmark) for 15 minutes. The sections were washed with phosphate-buffered saline and incubated with the primary antibody for 60 minutes. The slides were then washed with phosphate-buffered saline and incubated with biotinylated anti-mouse antibody (K4011, DakoCytomation) for 30 minutes. The peroxidase reaction was developed with a 3,3-diaminobenzidine tetrahydrochloride substrate kit (K4007, DakoCytomation). Finally, the slides were washed with distilled water and counterstained with hematoxylin.

For immunofluorescence, 4-μm-thick sections were fixed with 4% paraformaldehyde in phosphate-buffered saline and blocked with a bovine serum albumin–Triton solution (Sigma-Aldrich, Madrid, Spain). The sections were incubated with the primary antibody for 60 minutes at the ambient temperature. The slides were then washed, incubated with Alexa Fluor 488 green goat anti-mouse antibody (A11029; Commercial Products, Inc., United States) for 60 minutes, and mounted with the Prolong Gold antifade reagent (P36930, Molecular Products, Inc., United States). The slides were stored under dark conditions at 4°C until they were analyzed with a Leica SP5 spectral confocal microscope (Leica Microsystems, Wetzlar, Germany).

Data and Statistical Analysis

All values were expressed as the median and 25% to 75% interquartile range. Differences between groups were compared with the nonparametric Mann-Whitney test for continuous variables, and a 2-tailed P value < 0.05 was considered significant. Calculations were performed with Statistical Package for the Social Sciences version 16.0 (SPSS, Chicago, IL).

Ethical and Humane Considerations

The experimental protocol was approved by the Hospital Clinic Institutional Review Board and the University of Barcelona Committee on Ethics in Animal Experimentation.

RESULTS

Eighteen SFS transplants and 6 whole liver transplants were performed. However, 1 of the recipients in the SFS group died during the operative procedure because of complications of anesthesia and was excluded. In all, 17 SFS transplants and 6 whole liver transplants were included in the study.

Table 1 depicts the average donor, recipient, graft, and transplant characteristics. In the SFS group, 5 pigs (29%) survived the entire 5-day postoperative period (animals 1, 5, 6, 14, and 17 in the series). Death occurred in the SFS groups as follows: 12 to 24 hours (n = 6), 24 to 48 hours (n = 5), and 50 hours (n = 1). In the whole liver group, all 6 animals survived 5 days. At autopsy or euthanasia, no occurrences of vascular thrombosis or bile duct dehiscence were found. Five animals in the SFS group had moderate amounts of ascites (1 at euthanasia and 4 at autopsy). No other procedure-related cause of death (venous outflow obstruction, intra-abdominal abscess, cholangitis, sepsis, gastrointestinal perforation, or hemorrhage) was found in any animal that died prior to the end of follow-up.

TABLE 1. Donor, Recipient, Graft, and Transplant Characteristics

	SFS Group (n = 17)	Whole Liver Group (n = 6)	*P*
Donor weight (kg)	15.9 (14.6-16.7)	30.9 (29.2-33.0)	0.002*
$AST_{D,0}$ (IU/L)	32.4 (27.5-43.1)	28.5 (23-41.5)	0.355
$Bilirubin_{D,0}$ (mg/dL)	0.4 (0.2-0.5)	0.2 (0.1-0.4)	0.444
$QPT_{D,0}$ (%)	100	100	1
Recipient weight (kg)	30.3 (30.0-34.0)	33.8 (31.3-36.0)	0.178
$AST_{R,0}$ (IU/L)	32 (27-34)	29 (21-32.5)	0.395
$Bilirubin_{R,0}$ (mg/dL)	0.3 (0.2-0.4)	0.1 (0.1-0.2)	0.122
$QPT_{R,0}$ (%)	100	100	1
Partial graft (g)	178 (165-205)	653 (640-686)	0.002*
SLV (%)	23.2 (19.3-25.3)	93.4 (89.4-96.8)	0.002*
CIT (minutes)	334 (313-340)	344 (316-384)	0.958
WIT (minutes)	21 (20-25)	20 (19-22)	0.278

NOTE: The subscript "D,0" refers to the donor at baseline, and the subscript "R,0" refers to the recipient at baseline.
*Statistically significant.

Hepatic Hemodynamic Changes

PVF and HAF are reported as milliliters per minute per kilogram of hepatic tissue. The portal venous pressure gradient (PVPG) was calculated as the difference between the portal and suprahepatic venous pressures. The portal vein resistance (PVR), a measure of microstructural alterations that reduce the hepatic sinusoidal radius and inhibit portal inflow,[27] was calculated according to the following formula[28]:

$$\mathrm{PVR(dyn\ s\ m^2/cm^5)} = 80 \times \mathrm{PVPG}/(\mathrm{PVF} \times 0.001)$$

PVF, PVPG, and PVR were all significantly higher in the SFS group versus the whole liver group after both portal and arterial reperfusion; however, there were no significant differences between the surviving members of the SFS group (n = 5) and the whole liver group in any of these 3 parameters at euthanasia. Alternatively, HAF did not vary significantly between the 2 groups after arterial reperfusion but was significantly higher in the whole liver group at euthanasia.

The portal/arterial ratio (P/A) at the baseline was similar in both the SFS and whole liver groups: 2.9 (1.6-4.0) and 2.6 (2.0-4.2), respectively. After reperfusion, P/A increased to 17.6 (8.6-19.0) in the SFS group and 8.3 (7.1-9.2) in the whole liver group. By the end of follow-up, P/A had decreased in both groups to 13.0 (12.1-14.3) in the SFS group and 3.9 (3.0-6.4) in the whole liver group ($P = 0.015$; Table 2). Secondary to extreme portal hypertension, the P/A ratio was markedly altered in SFS livers after portal reperfusion and improved slightly in those animals that survived to the end of the follow-up period.

In order to look for hemodynamic differences predictive of outcome, the SFS group was subdivided into survivors (n = 5) and nonsurvivors (n = 12). The results were compared between the 2 groups, but no statistically significant differences in any of the 5 parameters at any intraoperative time were found [PVF: 4349 (2837-5481) mL/minute/kg in survivors versus 3389 (2748-4467) mL/minute/kg in nonsurvivors after portal reperfusion, $P = 0.671$, and 3435 (2998-3577) versus 3056 (2296-3688) mL/minute/kg after arterial reperfusion, $P = 0.695$; PVPG: 8.0 (8.0-10.5) versus 12.0 (8.0-14.0) mm Hg after portal reperfusion, $P = 0.556$, and 8.0 (7.5-9.0) versus 10.0 (8.0-12.0) mm Hg after arterial reperfusion, $P = 0.296$; PVR: 1022 (701-1454) versus 1394 (1064-1864) dyn s m^2/cm^5 after portal reperfusion, $P = 0.192$, and 1180 (910-1218) versus 1370 (999-1775) dyn s m^2/cm^5 after arterial reperfusion, $P = 0.302$; HAF: 205 (197-255) versus 180 (119-237) mL/minute/kg after arterial reperfusion, $P = 0.176$; P/A: 12.6 (12.0-15.3) versus 18.6 (8.6-32.5) after arterial reperfusion, $P = 0.389$].

Hepatic Endothelial Cell Injury

From baseline to 1 hour after portal reperfusion, the HA concentration in serum increased in all the recipients: 490 (448-792) and 311 (260-403) μg/L in the SFS and whole liver groups, respectively ($P = 0.093$). When SFS group survivors were separated from nonsurvivors, the increase was significantly higher in the latter group: 337 (327-362) versus 633 (465-868) μg/L, respectively ($P = 0.011$).

An evaluation of Masson's trichrome–stained and hematoxylin and eosin–stained tissue sampled 1 hour after portal reperfusion revealed periportal edema and hemorrhage, with a loss of the endothelial lining on some of the portal vein branches in animals of the SFS group (Fig. 1A,D). SFS livers also had congestion and extension of the periportal hemorrhage into the hepatic parenchyma. In SFS group nonsurvivors, hemorrhage was severe enough that it extended into the sinusoids all the way to the centrilobular veins (Fig. 1B). In SFS group survivors, although the intraparenchymal hemorrhage was less extensive, there was significant

TABLE 2. Evolution of Hepatic Hemodynamic Parameters

		SFS Group (n = 17)	Whole Liver Group (n = 6)	*P*
PVF (mL/minute/kg)	Baseline	982 (686-1159)	1146 (944-1431)	0.325
	Portal reperfusion	3389 (2748-5015)	2097 (1719-2435)	0.034*
	Arterial reperfusion	3314 (2486-3612)	1736 (1263-1910)	0.003*
	Euthanasia†	1555 (1277-1815)	1026 (919-1142)	0.212
PVPG (mm Hg)	Baseline	2 (2-3)	2 (1-3)	0.211
	Portal reperfusion	11.5 (8-13.5)	4.5 (3-6)	0.002*
	Arterial reperfusion	9 (7.5-11)	3.8 (2.8-4.5)	0.002*
	Euthanasia†	6 (5-7)	4 (2-5)	0.112
PVR (dyn s m^2/cm^5)	Baseline	345 (283-410)	267 (212-348)	0.073
	Portal reperfusion	1345 (877-1647)	226 (167-292)	0.003*
	Arterial reperfusion	1180 (980-1726)	237 (171-340)	0.002*
	Euthanasia†	582 (380-812)	318 (247-493)	0.157
HAF (mL/minute/kg)	Baseline	331 (275-488)	401 (280-608)	0.591
	Arterial reperfusion	156 (101-211)	198 (153-208)	0.737
	Euthanasia†	121 (107-144)	285 (201-309)	0.031*
HAF (% THBF)	Baseline	25 (20-39)	28 (21-34)	0.771
	Arterial reperfusion	5 (4-9)	9 (10-11)	0.049*
	Euthanasia†	8 (7-9)	21 (15-25)	0.041*
P/A	Baseline	2.9 (1.6-4.0)	2.6 (2.0-4.2)	0.981
	Arterial reperfusion	17.6 (8.6-19.0)	8.3 (7.1-9.2)	0.052
	Euthanasia†	13.0 (12.1-14.3)	3.9 (3.0-6.4)	0.015*

*Statistically significant.
†SFS group (n = 5).

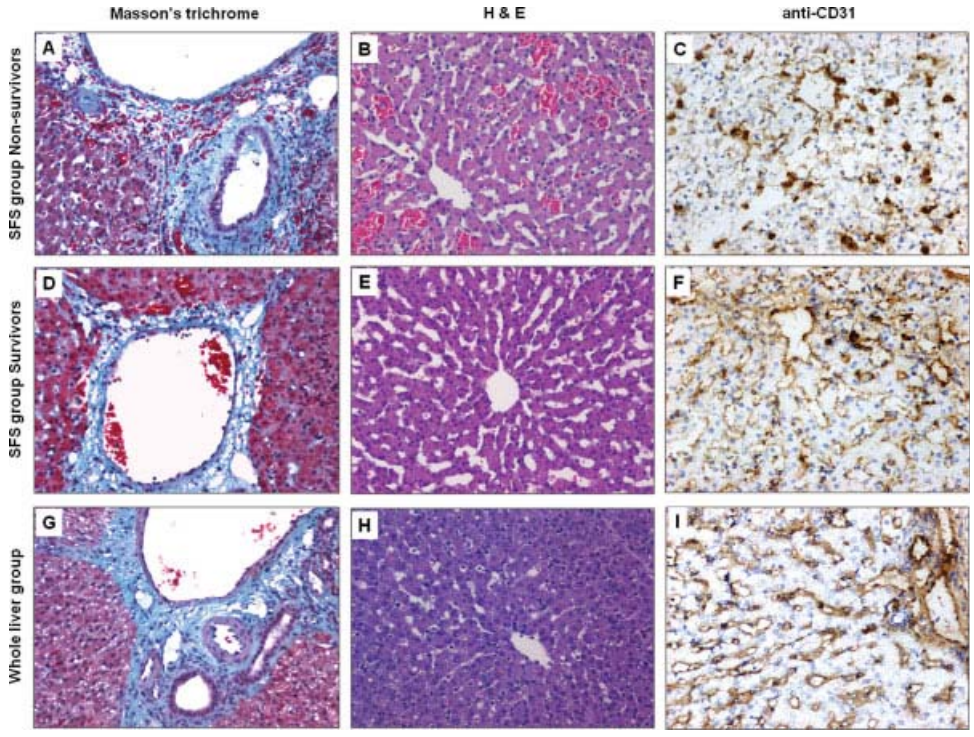

Figure 1. Masson's trichrome, H&E, and CD31 immunohistochemical staining of paraffin-embedded tissue samples taken 1 hour after portal reperfusion. (A,B) Among the SFS group nonsurvivors, severe periportal edema and hemorrhage extending into the hepatic parenchyma occurred. (D,E) These changes were less severe in the SFS group survivors, and (G,H) they were absent in animals in the whole liver group. CD31 immuno- histochemistry showed (C,F) various degrees of destruction of the endothelial cell lining in the SFS group and (I) normal preservation of the sinusoidal architecture in the whole liver group.

sinusoidal dilatation, which was not present in postreperfusion samples from the whole liver group. Among animals in the whole liver group, all the aforementioned changes were minimal (Fig. 1G,H).

On CD31 immunohistochemical staining of endothelial cells 1 hour after reperfusion, there was extensive destruction of the normal hepatic microarchitecture in SFS group nonsurvivors, with numerous foci

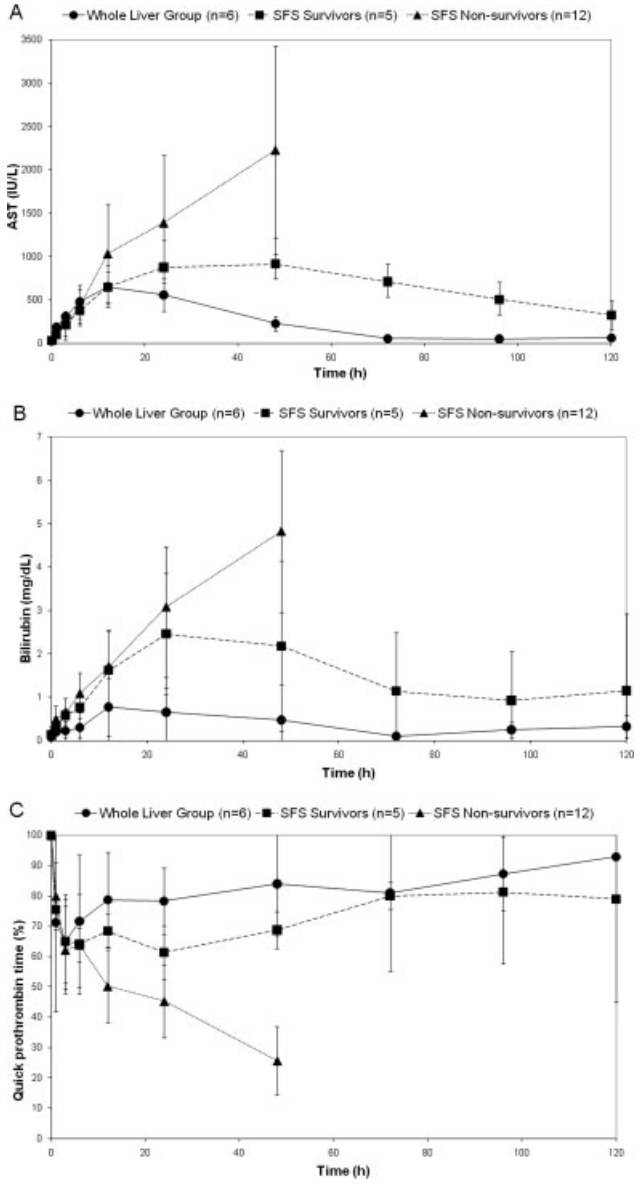

Figure 2. Postoperative evolution of (A) AST, (B) bilirubin, and (C) the quick prothrombin time. SFS group survivors (n = 5) were separated from nonsurvivors (n = 12) in order to demonstrate the very different trends that were present in each. All the nonsurvivors in the SFS group died with significant alterations in their liver markers by the end of the third postoperative day.

of detached endothelial cells clustered throughout the samples (Fig. 1C). Among the survivors in the SFS group, the endothelial cell reperfusion injury was present but less severe (Fig. 1F). In the whole liver group, well-delineated sinusoidal spaces lined by preserved endothelial cells could clearly be seen, and clustering of detached endothelial cells was minimal (Fig. 1I).

Hepatocellular Injury

Hepatic aminotransferase and bilirubin levels rose and the quick prothrombin time fell during the first postoperative day in all animals. In the whole liver group, AST peaked at 12 hours, rapidly declined subsequently, and normalized by 72 hours. In the SFS group, AST continued to rise to significantly higher values than in the whole liver group and ultimately peaked at approximately 48 hours. When SFS survivors were separated from nonsurvivors, there were significant differences between the 2 groups in terms of aminotransferase evolution. In the survivors, AST followed an evolution similar to that of the whole liver group. In the nonsurvivors, however, AST rose dramatically after 6 hours and continued to rise until death. Analogous and inverse trends were present in the evolutions of bilirubin and the quick prothrombin time, respectively (Fig. 2).

Hepatic Regeneration

All of the grafts increased in size during follow-up, even in the whole liver group. Because animals survived for disparate lengths of time, the change in the hepatic mass during follow-up was divided by the survival time in days. Grafts in the SFS group grew 100 (72-154) g/day, whereas those in the whole liver group grew 70 (48-75) g/day ($P = 0.153$). An analysis of the SFS subgroups showed that the growth rate was significantly higher among nonsurviving members of the SFS group than it was among either the survivors or the recipients of whole liver grafts [154 (124-195) g/day for nonsurvivors versus 72 (63-75) g/day for survivors, $P < 0.01$; nonsurvivors versus whole livers, $P < 0.05$].

To better quantify hepatic regeneration, TK activity was measured in serial serum samples. In the whole liver group, the level did not increase upon reperfusion and remained stable throughout the postoperative period. In the SFS group, however, there was a dramatic rise between 3 and 12 hours, and this was followed by a decline until 48 hours and relative stabilization thereafter. Analyzing the survivors and nonsurvivors separately, we found that the manner in which TK evolved in each was quite distinct. Among survivors, TK rose gradually throughout the follow-up period. In contrast, among nonsurvivors, TK rose dramatically from 6 to 12 hours to values over 2 times those measured in either the survivors or the whole liver group, after which point it declined until death (Fig. 3).

Normal hepatic histology was preserved during the process of regeneration in the surviving livers. Tissue taken from the donor prior to the start of hepatic resection revealed diffuse CD31 immunofluorescence staining of the endothelial cells and an orderly sinusoidal pattern. Five days after graft reperfusion, the hepatic microarchitecture was still well preserved, the differences with respect to the baseline being that the cords of hepatocytes were broader and the sinusoids were somewhat narrower and more intensely stained (Fig. 4A).

Quantitative Hepatic Function

Quantification of hepatic function using the PDR of ICG did not demonstrate any differences between the

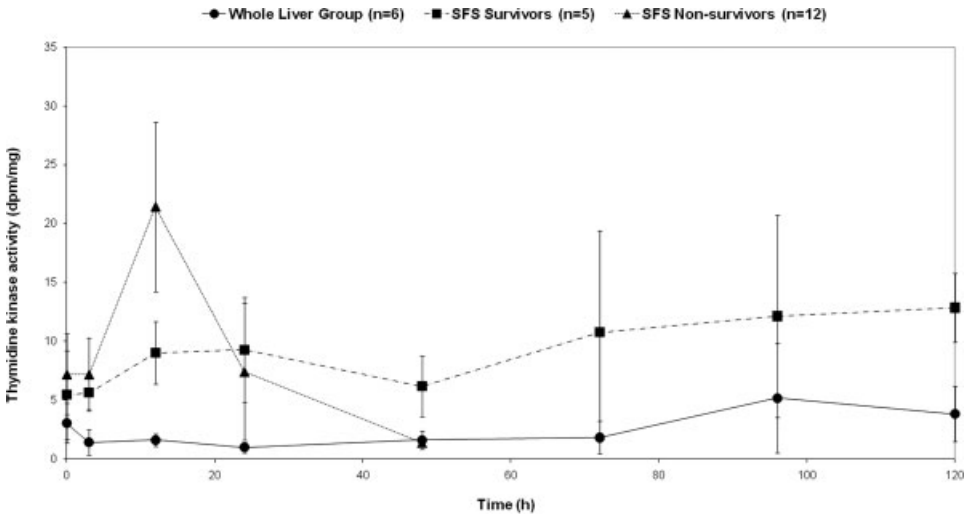

Figure 3. Postoperative evolution of the serum thymidine kinase activity, a marker of cellular proliferation. There was a dramatic increase in thymidine kinase activity after reperfusion in SFS group nonsurvivors, whereas in SFS group survivors and animals in the whole liver group, activity remained relatively stable throughout the follow-up period.

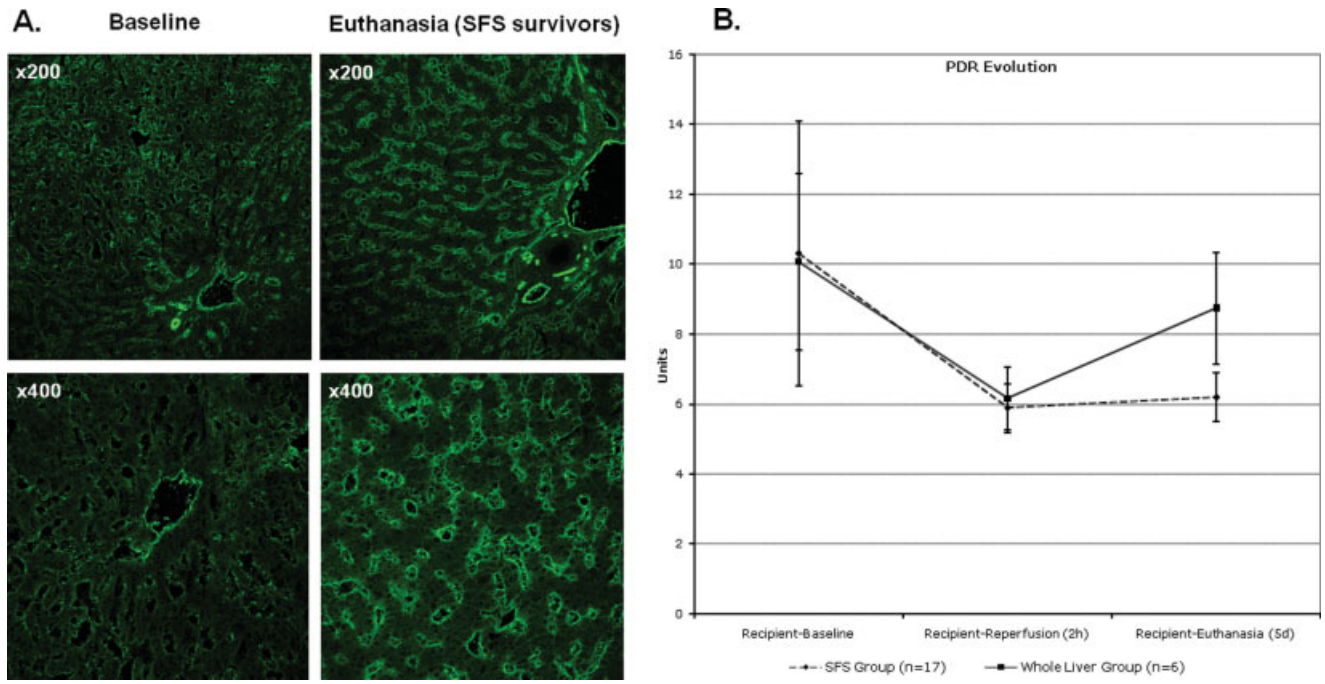

Figure 4. (A) CD31 immunofluorescence of hepatic tissue at the baseline and the end of follow-up among survivors in the SFS group. During regeneration, the same hepatic microarchitecture and microvascular density were preserved, although the hepatocyte cords grew thicker because of regeneration-associated cellular hyperplasia. Even though the hepatic microstructure was preserved during the process of regeneration in SFS livers, metabolic function was not. (B) PDR of indocyanine green was measured as a marker of quantitative hepatic function. Two hours after portal reperfusion, PDR was reduced from the baseline in all the recipients. At the end of follow-up, PDR remained depressed in the surviving animals in the SFS group, but it had recovered almost to the baseline in the animals in the whole liver group.

SFS and whole liver groups at the baseline: 10.5 (8.3-11.1) %/min and 10.4 (8.3-11.8) %/min, respectively. Upon completion of the recipient procedure, PDR was significantly and similarly reduced in both groups: 6.0 (5.7-6.2) %/min and 6.1 (5.2-6.3) %/min, respectively. Survivors and nonsurvivors in the SFS group did not show differences in terms of PDR at the end of the operative procedure: 6.0 (5.9-6.4) %/min and 5.4 (4.8-6.0) %/min, respectively.

On the fifth postoperative day, PDR remained low among survivors in the SFS group, whereas it had essentially returned to baseline in the whole liver

TABLE 3. Porcine Small-for-Size Survival Models

Authors	Transection	Implant	SLV	CIT (minutes)	Mortality	Regeneration
Yanaga et al.[55] (1995)	Ex situ	Autograft with VVB	29.4% (n = 5)	125 ± 19	100% (12 hours)	—
Asakura et al.[34,35] (2003, 2002)	In situ	Allograft with VVB	25.0% (n = 7)	114 ± 33*	86% (24 hours)	—
Smyrniotis et al.[36] (2003)	Ex situ	Allograft with VVB	18.3% (n = 8)	360*	63% (48 hours)	—
Kelly et al.[17] (2004)	In situ	Allograft with VVB	33.6% (n = 8)	151 ± 22	0% (5 days)	121% (5 days)
			22.1% (n = 15)	139 ± 20	20% (3 days)	147% (3 days)
					60% (5 days)	190% (5 days)
Wang et al.[56,57] (2005, 2006)	In situ	Allograft with VVB	25.1% (n = 11)	151 ± 51†	100% (24 hours)	—
Fondevila (2010)	In situ	Allograft (no VVB)	24.1% (n = 17)	322 ± 37	71% (5 days)	149% (5 days)

*Total ischemia.
†Time to perform a back-table split.

group: 4.2 (3.8-4.6) %/min and 9.3 (7.7-10.0) %/min, respectively (Fig. 4B). Measurements of the retention rate after 15 minutes revealed a similar pattern.

DISCUSSION

This large-animal model is straightforward, reproducible, and clinically relevant. It provides the appropriate size and an anatomy similar to that of humans for the establishment and practice of new surgical techniques. Although small-animal models (mice and rats in particular) require much less of an investment in terms of material, personnel, space, and time, they are not directly applicable to humans. Table 3 details other major porcine SFS survival models published to date. Although all the studies involved the implantation of grafts with approximately 20% to 30% of the recipients' SLV, they varied in terms of transection techniques, lengths of cold ischemia, and postoperative survival outcomes. The current study is the first of its kind to avoid the use of venovenous bypass, include clinically relevant lengths of cold ischemia, and study hepatic hemodynamic alterations and their association with early postoperative outcomes, including survival and regeneration, over a period of several days. We decided to euthanize the animals in order to obtain tissue samples and perform hemodynamic measurements at a point when the trajectory of the recipient (ie, graft failure or recovery) appeared to have been established. Nonetheless, a longer period of follow-up could have allowed us to observe other complications associated with the use of grafts of such small size.

SFSS has been described as occurring in grafts of all sizes, even those representing >30% of the SLV or >0.8% of the graft weight to body weight ratio, presumably because of other factors such as steatosis or the underlying disease in the recipient. However, our aim was to specifically study the acute failure that occurs as a direct result of an extremely small graft size. The clinical relevance of this model has to do with the fact that extremely reduced-size transplantation with grafts representing 20% to 30% of SLV is not performed on any meaningful scale in Western centers. It would be highly advantageous, however, for living donors to be able to donate the left hepatic lobe instead of the right because the risks associated with a left lobectomy are significantly reduced. Similarly, we could double the yield of cadaveric liver transplants if we were able to consistently split livers for 2 adults. These types of transplants are not routinely performed, however, because of the not unfounded fear that SFS graft failure will arise in the recipient of a left liver lobe. Because we obviously cannot study these grafts in the clinical setting, we established this experimental model to study the early series of events that precede their failure in order to identify points at which we can intervene to prevent it.

In human liver transplantation with a whole graft, PVF typically increases upon reperfusion; this involves hyperdynamic splanchnic circulation and the replacement of a cirrhotic organ with a noncirrhotic one with lower resistance to portal flow.[29,30] In the case of partial liver transplantation, the vascular bed through which this high flow has to pass is significantly diminished. Although portal inflow is considered to be a stimulus for hepatic regeneration,[31,32] excessive PVF can be detrimental to the function and survival of the reduced-size organ.[6,10,33]

All the recipients in this study experienced increased PVF upon reperfusion. Postreperfusion PVF was considerably higher among the recipients of SFS livers versus those of whole livers, and 71% of the SFS grafts ultimately failed. Severe graft damage and dysfunction and the exclusion of other causes of death indicated that graft failure was the cause of death in these animals. The survival rate among our SFS recipients was consistent with the rates reported in similar studies,[17,34-36] and survival in the SFS

group occurred randomly; this indicated that the experience of the operators did not determine survival.

The primary target in the entire process of SFS liver injury appears to be the sinusoidal endothelial cell. One hour after portal reperfusion, serum levels of HA were already significantly higher in the group of animals that went on to develop SFS graft failure, whereas significant increases in markers of hepatocellular injury (AST, bilirubin, and prothrombin time) occurred later. The histological and immunohistochemical examination of tissue sampled 1 hour after reperfusion was noteworthy for extensive rupture of the endothelial cells of the portal vein branches and dissection of blood into the periportal spaces and beyond.

Although PVF did not vary significantly between the nonsurvivors and survivors within the SFS group, injury to the sinusoidal endothelium was significantly greater in the former group of animals in comparison with the latter. One possible explanation for this is that PVF was, in fact, higher immediately after portal reperfusion in the nonsurvivors. In our study, we did not measure PVF until we had completed the infrahepatic IVC anastomosis and were certain that we had achieved adequate postreperfusion hemostasis with respect to both the vascular anastomoses and the transection surface. Postreperfusion PVF was actually measured approximately 15 minutes after the portal reperfusion event. It is plausible that by the time we measured PVF, significant stress-induced endothelial cell injury had already occurred, injury that altered the hepatic microstructure to the extent that portal resistance increased and flow actually decreased. In fact, postreperfusion portal resistance tended to be higher among nonsurvivors versus survivors, although the differences did not reach statistical significance. Furthermore, extensive damage to the endothelial lining was evident on Masson's trichrome and anti-CD31 staining of postreperfusion liver biopsy samples from the SFS grafts that ultimately failed, much more so than in those that did not.

Suboptimal HAF is poorly tolerated by transplanted livers. Unlike native organs, transplanted grafts lack alternative sources of arterial inflow, such as the peribiliary arterial plexus. In the posttransplant setting, arterial hypoperfusion is associated with ischemia and cholestasis, clinical signs that have also been described in SFS grafts.[6] It has been hypothesized that insufficient HAF may be an important contributor to SFS liver injury.[12,37] In the study by Marcos et al.[38] on hepatic hemodynamics in a series of 16 adult living donor liver transplants, HAF composed 2% to 16% of the total liver blood flow (TLBF) after portal reperfusion. In particular, among grafts that met SFS criteria (a graft weight to body weight ratio of 0.8 or less, n = 5), HAF represented 3% to 10% of the postreperfusion TLBF. All of the grafts functioned well, regardless of either the proportion of TLBF that was from the hepatic artery or the portal-to-arterial flow ratio. In our study, 100% of the grafts in the whole liver group survived the follow-up period, whereas 71% of the grafts in the SFS group failed prior to the end of follow-up. HAF as a proportion of TLBF did vary between SFS grafts and whole livers after arterial reperfusion and at euthanasia. PVF seems to play a primary role in affecting the outcome of reduced-size liver transplantation, but our data also suggest that a reduction in HAF, particularly with respect to TLBF, may play a role in the pathogenesis of SFS graft dysfunction as well. Secondary to the significant graft growth in the SFS group, the arterial flow per tissue mass decreased by the fifth postoperative day, whereas in the whole liver group, it remained stable or, in some cases, even increased slightly. The delayed effects of this decline in arterial flow and whether it would be maintained over time were not evaluated in our model, and a longer period of follow-up would be needed in order to do so.

Whether hepatic regeneration is augmented or impaired after SFS liver transplantation remains controversial.[10,17,39-41] We observed that, per day, the increase in hepatic mass was greater in grafts that failed than in those that survived. Serum TK activity was therefore evaluated to better quantify proliferative activity at each postoperative time. Among nonsurvivors, TK activity rose considerably during the first 12 hours. In survivors and the recipients of whole liver grafts, TK activity rose much more slowly and reached maximum levels between the third and fifth days in accordance with data previously published on hepatic regeneration following extended hepatectomy in the porcine model.[24,42,43] In the failed grafts, it appears that the stimulus for regeneration was present, but the reperfusion injury was apparently too severe for them to recover. Many of the same cytokines and cellular signaling pathways have been implicated in both inflammation and regeneration,[44,45] and it is plausible that the considerable stress to which SFS grafts were subjected simultaneously conditioned hepatocellular proliferation.

Surviving grafts had preserved hepatic architecture on postoperative day 5. Significant tissue regeneration also occurred. Regeneration-associated hyperplasia was evident in the relative thickening of the hepatocyte cords seen in the final samples in comparison with those taken at the baseline. In addition, the greater intensity of anti-CD31 immunofluorescence observed on day 5, in the absence of any apparent increase in the microvascular density, seems to indicate that mechanical stress in the sinusoid up-regulated the expression of molecules responsible for maintaining endothelial stability.

Even though all of the partial grafts grew in size and those that survived recovered from the acute ischemia-reperfusion lesion, they did not regain normal metabolic function during the follow-up period. Other authors have reported similar findings in the experimental setting demonstrating, in particular, a reduction in the metabolic activity of cytochrome P-450 isozymes[46-49] and ICG clearance.[50] Moreover, in clinical living donor liver transplantation, recipients have been reported to achieve higher relative drug levels versus the recipients of whole cadaveric livers,[51] even when hepatic regeneration is complete.[52,53] The restoration of metabolic activity does not coincide with that of liver mass when a reduced-size liver regenerates.

Future treatments for SFSS should include strategies primarily aimed at protecting the hepatic endothelial cell from the physical stress of excessive PVF. These may include surgical therapies to mechanically reduce sinusoidal hypertension, such as portosystemic flow derivations, or pharmacological therapies to augment the capacity of the endothelial cell to resist injury. Furthermore, portal hyperflow in the immediate postreperfusion period implies not only great tangential force against the walls of the hepatic sinusoids but also elevated delivery of gut-derived endotoxins and substrates for the production of reactive oxygen species.[6,54] Hence, antioxidant and anti-inflammatory agents can also play an important role in ameliorating reperfusion injury in partial livers.

Studying SFSS in the clinical setting is complicated, as its appearance is rare and considerable attempts are made to avoid it. However, SFSS is one of the primary obstacles preventing the wider use of partial livers arising from not only living donors but also cadaveric donors in the context of split liver transplantation. The data that we present herein provide not only further information regarding the pathophysiology of the acute phase of this poorly understood process but also a well-characterized, reproducible, and clinically relevant model for the future study of SFS liver transplantation and new means of improving its success.

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5.2. Study 2: Decompression of the portal bed and the prevention of “small-for-size” syndrome with a calibrated portocaval shunt

In the second part of this doctoral thesis, the portal vascular bed was prophylactically decompressed by establishing a calibrated portocaval shunt prior to reperfusion in the recipient. SFS liver transplants were performed as described in 5.1, with a few modifications. After extended hepatectomy, cold perfusion, and extraction, a calibrated portocaval shunt was created in the partial liver graft on the backtable. A Gore-Tex® Vascular Graft with external supporting rings (W.L. Gore & Associates, Inc., Flagstaff, Arizona, USA), measuring approximately 1.5 cm in length and either 6 mm (N=6) or 12 mm (N=6) in diameter, was anastomosed between openings cut to corresponding sizes in the portal vein and infrahepatic IVC, respectively. Once the portocaval shunt was complete, the graft was placed in cold storage for approximately 5 hours and then transplanted into the recipient pig.

A detailed description of the experimental protocol and results can be found in the second article, which follows:

Hessheimer AJ, Fondevila C, Taurá P, Muñoz J, Sánchez O, Fuster J, Rimola A, García-Valdecasas JC. Decompression of the Portal Bed and Twice-Baseline Portal Inflow Are Necessary for the Functional Recovery of a “Small-for-Size” Graft. Ann Surg 2011;253.

Decompression of the Portal Bed and Twice-Baseline Portal Inflow Are Necessary for the Functional Recovery of a "Small-for-Size" Graft

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Background. In partial liver transplant, a reduction in the intrahepatic vascular bed produces a rise in the portal vein flow and the portal venous pressure gradient, leading to endothelial and, thereby, hepatocellular injury and death in a process known as "small-for-size" (SFS) syndrome.

Objective. To demonstrate that a calibrated portocaval shunt prevents superfluous inflow in a porcine model of SFS transplant.

Methods. Donor pigs (15–20 kg) underwent 70% hepatectomy. In 2 groups, a 6 mm (S6) (n = 6) or 12 mm (S12) (n = 6) Gore-Tex shunt was placed between the portal vein and infrahepatic inferior vena cava. In a third group, no portocaval shunt was placed (SFS) (n = 17). Grafts were stored for 5 hours at 4°C and then transplanted into recipients (30–35 kg).

Results. Five-day survival was 29% in SFS, 100% in S6, and 0 in S12. Postreperfusion portal vein flow was 4-, 2-, and 1-times flow at baseline in SFS, S6, and S12, respectively. With respect to portal venous pressure gradient, both the 6- and 12-mm shunts effectively decompressed the portal bed. Aspartate aminotransferase and bilirubin rose and the Quick prothrombin time fell in all animals after reperfusion but improved significantly by day 5 in S6. Serum levels of endothelin-1 remained elevated in SFS and S12 but returned to baseline by 12 hours in S6: 2.76 (2.05–4.08) and 2.04 (1.97–2.12) versus 0.43 (0.26–0.50) pg/mL, respectively ($P < 0.05$ for both comparisons).

Conclusions. A calibrated portocaval shunt that maintains portal vein flow about twice its baseline value produces a favorable outcome after SFS liver transplantation, avoiding endothelial injury due to portal hyperperfusion or to hypoperfusion because of excess shunting.

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Adult-to-adult living donor liver transplantation (ALDLT) and split liver transplantation both involve the implantation of a partial graft. On the basis of the fact that a healthy liver has considerable functional reserve and regenerative capacity, partial or reduced-size liver grafting is a feasible treatment option for end-stage liver disease. However, reduced-size liver transplantation is a procedure that requires extensive preoperative planning to assure adequate functional graft and remnant liver mass for the recipient and donor, respectively, to avoid potentially detrimental postoperative sequelae.

Adult-to-adult living donor liver transplantation using the left hemiliver or left lateral segment is a procedure that is rarely performed in Western medical centers. Right hepatectomy for ALDLT is associated with a 20% morbidity rate and a 0.5% mortality rate.[1] Left hepatectomy for ALDLT, on the contrary, has a better safety profile for the donor, leaving behind twice as much remnant liver as right hepatectomy, but has been associated with a more difficult postoperative course and twice the incidence of postoperative mortality in the recipient.[2] With respect to this last fact, many left liver grafts implanted into anyone but children or the smallest adult recipients fail secondary to the development of "small-for-size" syndrome (SFSS).

"Small-for-size" syndrome arises when a partial graft cannot cope with the unique stresses placed on it because of its small size. Though there is no consensus definition, SFSS may be diagnosed clinically when prolonged hyperbilirubinemia, coagulopathy, and/or encephalopathy arise in a liver that represents less than 0.8% of the recipient's graft weight-to-body weight ratio (GWBWR).[3] It is a diagnosis of exclusion that can only be made after other technical, infectious, and immunological causes have been ruled-out. Though the pathogenesis of SFSS is multifactorial, it has been demonstrated that grafts representing less than 0.8% of the recipient's body weight (GWBWR) or less than 30% to 40% of the recipient's standard liver volume (SLV) fare more poorly than larger grafts. We previously studied the pathophysiological processes associated with the appearance of SFSS in a clinically relevant porcine model and determined that excessive portal flow and pressure in the immediate postreperfusion period trigger sinusoidal endothelial cell injury, the extent and severity of which predict the ultimate appearance of either "small-for-size" (SFS) graft failure or histological recovery.[4] In this study, using the same experimental model, we aim to prophylactically decompress the portal vascular bed by establishing a calibrated portocaval shunt (PCS) prior to reperfusion in the recipient. In doing so, we hope to prevent the deleterious consequences of superfluous portal inflow and improve postoperative graft function and survival, without, simultaneously, compromising regenerative, and metabolic parameters due to excessive portosystemic shunting.

MATERIAL AND METHODS

Study Overview

"Small-for-size" liver transplants were performed as described previously,[4] with a few modifications. Male outbred weanling pigs (15–20 kg) underwent extended hepatectomy. Partial liver grafts were included in 1 of 3 experimental groups. In 2 of the groups, a Gore-Tex Vascular Graft with external supporting rings (W. L. Gore & Associates, Inc., Flagstaff, AZ), measuring 1 to 2 cm in length and either 6 mm (group "S6," n = 6) or 12 mm (group "S12," n = 6) in diameter, was anastomosed between openings cut to a corresponding size

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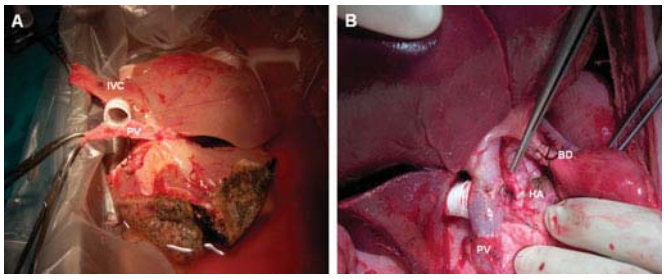


FIGURE 1. A, Partial liver graft, consisting in the right lateral lobe and half of the right paramedian lobe, after cold perfusion and extraction. During the back-table preparation, a Gore-tex graft is placed between the PV and the infrahepatic IVC. Tachosil, a fibrin-collagen-thrombin sponge placed in the donor upon completion of the liver resection, remains adherent to the zone of transection. B, Partial liver graft with 12-mm shunt, after completion of the PV, HA, and BD anastomoses in the recipient. PV indicates portal vein; IVC, inferior vena cava; HA, hepatic artery; and BD, bile duct.

in the portal vein and the infrahepatic inferior vena cava, respectively (Fig. 1). In the third group, no portocaval shunt was placed (group "SFS," n = 17). Partial liver grafts were implanted into recipients (30–35 kg), representing approximately 20% of the recipients' standard liver volume.

Intraoperative Monitoring

After an open cut-down of the right carotid sheath, a triple lumen catheter was placed in the internal jugular vein for invasive venous pressure monitoring. In the recipients, the catheter was tunneled subcutaneously to exit at the back of the neck for postoperative access. A single lumen catheter was introduced into the portal vein to monitor portal venous pressure. Ultrasonic flow probes were connected to a flowmeter (HT107, Transonic Systems, Ithaca, NY) to measure hepatic artery flow (HAF) and portal vein flow (PVF), the latter both proximal and distal to the portocaval shunt in S6 and S12. Portal venous pressure, HAF, and PVF were recorded in the donor at baseline and in the recipient after both portal and arterial reperfusion. The venous line was also momentarily positioned in the suprahepatic inferior vena cava to record the suprahepatic venous pressure. Liver function was quantified at baseline and the end of the recipient operation using indocyanine green (ICG) clearance. Results were measured using a LiMON monitor (Pulsion Medical Systems AG, Munich, Germany) and recorded in terms of the plasma disappearance rate (PDR) of ICG and ICG retention rate after 15 minutes (R15).

Postoperative Management

Recipients were monitored during 5 postoperative days: at 3, 6, 12, and 24 hours the first day and every 8 hours thereafter. They were given free access to water and dry food. Food and water intake and serum glucose levels were evaluated at each postoperative assessment, and animals that had limited or no intake *per os* and/or low serum glucose levels (<70–80 mg/dL) were administered 50 g of intravenous (IV) glucose (500 mL of a 10% glucose solution). Immunosuppression was given daily for 4 days (tacrolimus 0.04 mg/kg IV, methylprednisolone 125–100-75–50 mg IV.). Animals that survived until the fifth day were anesthetized and reopened. Indocyanine green clearance, hemodynamic, and blood-based parameters were re-evaluated. The liver graft was removed, weighed, and sampled, and the animals were euthanized. Nonsurvivors were submitted to an autopsy.

Blood and Serum Analysis

In serum samples collected serially during the follow-up period, levels of aspartate aminotransferase and total bilirubin and the Quick prothrombin time were determined.

Thymidine kinase (TK) activity is an index of hepatic regeneration in both tissue and serum.[5] Thymidine kinase activity was measured in serial serum samples using the LIAISON Thymidine Kinase assay (DiaSorin, Inc., Stillwater, OK) and detected with a LIAISON chemiluminescence analyzer (DiaSorin SpA, Saluggia, Italy).

Endothelin-1 (ET-1) is a potent vasoconstrictive peptide derived from endothelial cells.[6] In the liver, ET-1 is synthesized by and released from sinusoidal endothelial cells to help regulate hepatic blood flow.[7] Several studies have implicated ET-1 in the hepatic microcirculatory disturbances seen after hepatic ischemia-reperfusion.[8–11] Endothelin-1 was measured in serum samples using the endothelin ELISA kit BI-20052 (Biomedica Midizinprodukte GmbH, Vienna, Austria).

Interleukin-6 (IL-6) is a proinflammatory cytokine. Serum levels of IL-6 were measured using the Porcine IL-6 Quantikine ELISA Kit P6000 (R& D Systems, Minneapolis, MN).

The serum glucose concentration was serially measured throughout the course of follow-up. Serial serum glucose measurements from 3 to 72 hours, by which point the animals had either died or were able to consume dry food autonomously, were graphed on an *xy* plot. Time was the *x* coordinate, measured in hours, and serum glucose was the *y* coordinate, measured in mg/dL. The area under the curve was calculated and divided by the sum, in grams, of the glucose administered during the same time period, thereby, generating the glucose efficiency index (GEI).

Tissue Analysis

Hepatic tissue was sampled in the donor at baseline and in the recipient 1 hour after portal reperfusion. Biopsies were each divided into 2 sections, one preserved in 10% formaldehyde for subsequent inclusion in paraffin and the other embedded in Tissue-Tek OCT compound (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands) and snap frozen in liquid nitrogen.

Cluster of differentiation molecule 31 (CD31) immunoglobulin helps maintain endothelial stability by interdigitating with other CD31 molecules at the extracellular border of adjacent cells.[12] Cryosections of hepatic tissue were immunostained with porcine anti-CD31 antibody (MCA1746G; Serotec, Oxford, UK) to evaluate the integrity of the endothelial cells in the hepatic sinusoid, as described previously.[4]

For ET-1 staining, paraffin-embedded tissue was cut, dewaxed in xylene, and rehydrated. Sections were washed with phosphate-buffered saline and boiled in ethylenediamine tetra-acetic acid buffer for 2 minutes for antigen retrieval. Slides were then incubated with the primary antibody against ET-1 (MA3–005, ABR-Affinity BioReagents, Golden, CO) for an hour at ambient temperature. Endogenous peroxidase activity was inhibited with peroxidase-blocking solution (S20223, DakoCytomation, Glostrup, Denmark) for 10 minutes. Slides were washed and incubated with biotinylated antimouse antibodies (K4011, DakoCytomation, Glostrup, Denmark) for 30 minutes. The peroxidase reaction was developed with 3,3-diaminobenzidine tetrahydrochloride DAB substrate kit (K4007, DakoCytomation, Glostrup, Denmark) for 2 minutes, and the slides were finally washed with water and counterstained with hematoxylin.

Data and Statistical Analysis

All values were expressed as the median and the 25% to 75% interquartile range, unless otherwise specified. Differences between

groups were compared using the Mann-Whitney test for continuous variables, and a 2-tailed *P* value of 0.05 or less was considered significant. Calculations were performed using Statistical Package for the Social Sciences version 16.0 (SPSS, Chicago, IL).

Ethical and Humane Considerations

The experimental protocol was approved by the Hospital Clinic institutional review board and the University of Barcelona Committee on Ethics in Animal Experimentation.

RESULTS

Twenty-nine SFS transplants were performed: SFS group, n = 17; S6 group, n = 6; and S12 group, n = 6. Table 1 depicts the donor, recipient, and transplant characteristics for each of the 3 groups. Five-day survival was 100% in S6, 29% in SFS, and 0 in S12 (Fig. 2). At autopsy or euthanasia, no incidences of vascular thrombosis or bile duct dehiscence were found. Five animals in the SFS group had moderate amounts of ascites (1 at euthanasia and 4 at autopsy). No other procedure-related cause of death (venous outflow obstruction, intraabdominal abscess, cholangitis, sepsis, gastrointestinal perforation, or hemorrhage) was found in any of the animals that died prior to the end of follow-up.

Hepatic Hemodynamic Changes

Portal vein and HAFs are reported as milliliters per minute per kilogram (mL/min/kg) of hepatic tissue. The portal vein pressure gradient (PVPG) was calculated as the difference between the portal and suprahepatic venous pressures.

Upon portal reperfusion, PVF increased significantly in SFS and S6 but not in S12 (Table 2). Postreperfusion PVF was approximately 4 times the baseline value in SFS, versus approximately 2 times baseline in S6 and more or less its baseline value in S12. It was significantly lower in S12 versus SFS ($P = 0.003$) and S6 ($P = 0.021$) and in S6 versus SFS ($P = 0.042$). On arterial reperfusion, PVF remained significantly higher in SFS versus S12 ($P = 0.008$). By the end of 5 days, PVF had decreased from postportal reperfusion values in the 2 surviving groups (Fig. 3A).

There were no significant differences between the groups in PVPG at baseline. After portal reperfusion, the gradient increased roughly the same extent in S6 and S12 but was significantly higher-–almost twice as high-–in the SFS group versus the other 2 ($P = 0.001$ vs. S6 and S12). By the end of the 5-day follow-up period, PVPG had decreased almost to baseline in S6 but remained significantly elevated in SFS ($P < 0.001$) (Fig. 3B).

Hepatic artery flow was similar in all 4 groups at baseline. It decreased significantly on arterial reperfusion in SFS but was unchanged in S6 and S12. After reperfusion, HAF was significantly lower in SFS versus S6 ($P = 0.005$) and S12 ($P = 0.017$). By the end of 5 days, HAF had decreased significantly in both SFS and S6, because of the fact that the great increase in hepatic mass was not accompanied by a parallel increase in arterial flow. There was no significant difference between the 2 groups at euthanasia (Table 2).

The portal/arterial (P/A) ratio did not vary among the groups at baseline. After reperfusion, P/A increased significantly in SFS and to a much lesser extent in S6 and S12. At the end of follow-up, P/A had decreased in SFS but actually increased in S6. Likewise, HAF as a percent of the total hepatic blood flow was similar in all the groups at baseline; decreased considerably in SFS and to a lesser extent in S6 and S12 upon reperfusion; and increased in SFS but decreased in S6 after 5 days of follow-up (Table 2).

Hepatic Endothelial Cell Injury

Light microscopy findings 1 hour after portal reperfusion are depicted in Figure 4. In the SFS group, there was significant endothelial cell denudation and detachment in the portal vein branches and hepatic sinusoids, resulting in hepatic congestion and parenchymal necrosis and generalized disruption of the normal hepatic architecture. However, in the S6 and S12 groups, the sinusoidal endothelium was effectively preserved, as seen on immunostaining with CD31 (Fig. 4).

Endothelin-1 is a vasoconstrictive peptide upregulated in situations of endothelial-cell stress and hypoxia, and levels of ET-1 were measured in serum samples both before and after hepatic reperfusion. Endothelin-1 levels did not vary significantly between the SFS, S6, and S12 groups at baseline. Though levels rose following reperfusion and peaked at 3 hours in all 3 groups, they rose to a significantly lesser extent in S6 versus SFS and S12 ($P = 0.02$ and 0.05, respectively). Furthermore, levels of ET-1 returned to baseline by 12 hours in S6 but remained significantly elevated in SFS and S12: 0.43 (0.26–0.50) versus 2.76 (2.05–4.08) and 2.04 (1.97–2.12), respectively ($P < 0.05$ for both comparisons) (Fig. 5A).

These findings in serum were confirmed with ET-1 immunostaining of post-reperfusion tissue samples. Endothelin-1 expression was upregulated in tissue samples taken 1 hour after portal reperfusion from the SFS grafts, and there was significant uptake of ET-1 into the distorted hepatocyte cords. In the S12 group, as well, ET-1 expression was upregulated 1 hour after portal reperfusion. Though the hepatocyte cords remained well-preserved, there was significant uptake of ET-1 in the cells. In the S6 group, however, ET-1 immunostaining of the hepatic tissue was similar to that of the donor, with minimal staining of the sinusoidal endothelial-cell lining (Fig. 5B).

Hepatocellular Injury

Upon reperfusion, AST and bilirubin increased and the Quick prothrombin time decreased in all of the animals. Aspartate aminotransferase peaked before the end of the second postoperative day in all of the animals. Aspartate aminotransferase was significantly higher in SFS versus S6 at 48 and 72 hours and in S12 versus S6 at 24 hours ($P < 0.05$ for all comparisons) (Fig. 6A).

Total bilirubin peaked by the second postoperative day in all 3 groups, though differences between the groups did not reach statistical significance at any postoperative time point (Fig. 6B).

In all animals, the Quick prothrombin time declined significantly during the initial 6 to 12 hours after portal vein reperfusion and then began to recover between 24 and 48 hours. Recovery, however, was slower in the S6 group animals versus those that survived in the SFS group. Quick prothrombin time was significantly higher in SFS versus S6 on postoperative days 3, 4, and 5 (Fig. 6C).

Inflammatory Response

Plasma levels of IL-6 rose after portal reperfusion in all the animals. The rise was the greatest in the S12 group, in which it peaked at 6 hours. At 6 hours, levels of IL-6 were 378 (299–402), 235 (169–327), and 780 (685–1037) in the SFS, S6, and S12 groups, respectively ($P < 0.05$ for S12 versus SFS and S6).

Increase in Hepatic Mass and Stimulus for Regeneration

All of the grafts increased in size during the follow-up period. Because total survival time varied by group, the increase in mass was divided by the survival time in days. Growth per day was significantly higher in the SFS group, 100 grams per day (g/d) (72–154), versus S6, 60 g/d (50–66), and S12 groups, 72 g/d (59–79) ($P = 0.002$ and 0.046, respectively) (Fig. 7A).

Thymidine kinase is a phosphotransferase upregulated by actively dividing cells. Thymidine kinase activity was measured in serial serum samples to determine the stimulus for regeneration during the follow-up period. Among animals in the SFS group, TK

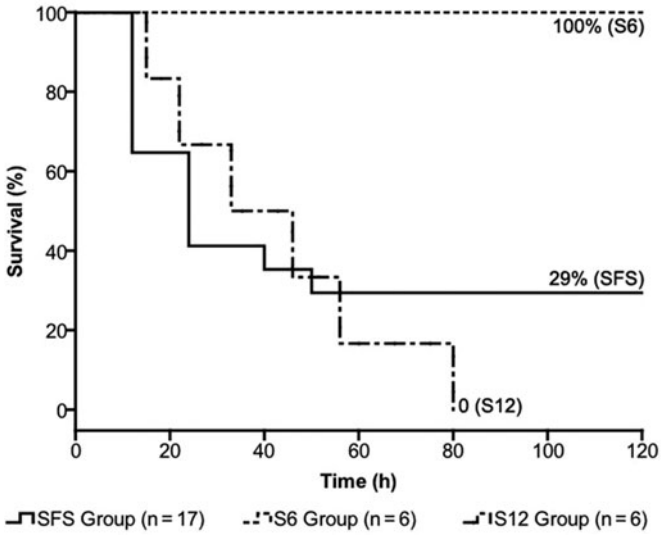


FIGURE 2. Survival curves for the 3 groups. Five-day survival was 100%, 29%, and 0 in the S6, SFS, and S12 groups, respectively.

TABLE 1. Donor, Recipient, Graft, and Transplant Characteristics

	SFS (n = 17)	S6 (n = 6)	S12 (n = 6)	ANOVA
Donor weight, kg	15.9 (14.6−16.7)	16.3 (15.0−19.4)	15.4 (14.6−15.9)	NS
$AST_{D,0}$, IU/L	32 (28−43)	38 (29−42)	39 (37−41)	NS
$Bilirubin_{D,0}$, mg/dL	0.4 (0.2−0.5)	0.1 (0.1−0.3)	0.4 (0.3−0.5)	NS
$QPT_{D,0}$, %	100	100	100 (99−100)	NS
Recipient weight, kg	30.3 (30.0−34.0)	33.0 (32.0−33.8)	31.8 (30.5−33.8)	NS
$AST_{R,0}$, IU/L	32 (27−34)	34 (25−38)	24 (18−34)	NS
$Bilirubin_{R,0}$, mg/dL	0.3 (0.2−0.4)	0.2 (0.1−0.3)	0.2 (0.1−0.2)	NS
$QPT_{R,0}$, %	100 (98−100)	96 (94−100)	96 (96−98)	NS
SLV, %	23.2 (19.3−25.3)	21.2 (20.3−24.4)	20.8 (18.7−26)	NS
GWBWR, %	0.57 (0.51−0.65)	0.59 (0.56−0.64)	0.60 (0.52−0.64)	NS
CIT, min	334 (313−340)	301 (288−321)	307 (282−318)	NS
WIT, min	21 (20−25)	23 (22−24)	24 (23−25)	NS

Subscript "D,0" refers to the donor at baseline and "R,0" to the recipient at baseline. NS indicates not significant; AST, aspartate aminotransferase; CIT, cold ischemic time; GWBWR, graft weight-to-body weight ratio; QPT, Quick prothrombin time; SLV, standard liver volume; WIT, warm ischemic time.

activity initially peaked at 12 hours and was significantly higher in the SFS group versus S6 and S12 at this time point ($P < 0.050$ for both comparisons). Among the survivors in the SFS group, TK activity underwent a second more gradual rise from 48 hours to the end of follow-up. The same gradual rise after 48 hours was observed in the S6 group (Fig. 7B).

Glucose Efficiency Index

An observation we made during the course of the experiment was that animals in the S12 group all suffered from recurrent hypoglycemic episodes in spite of frequent glucose monitoring and infusions. Thus, we devised the GEI to quantify this observation. The GEI was significantly higher in SFS, 40 (27–55), and S6, 25 (18–32), versus S12, 11 (9–14) ($P = 0.016$ and 0.047, respectively), indicating less effective glucose utilization per gram of glucose administered in the 12-mm shunt group.

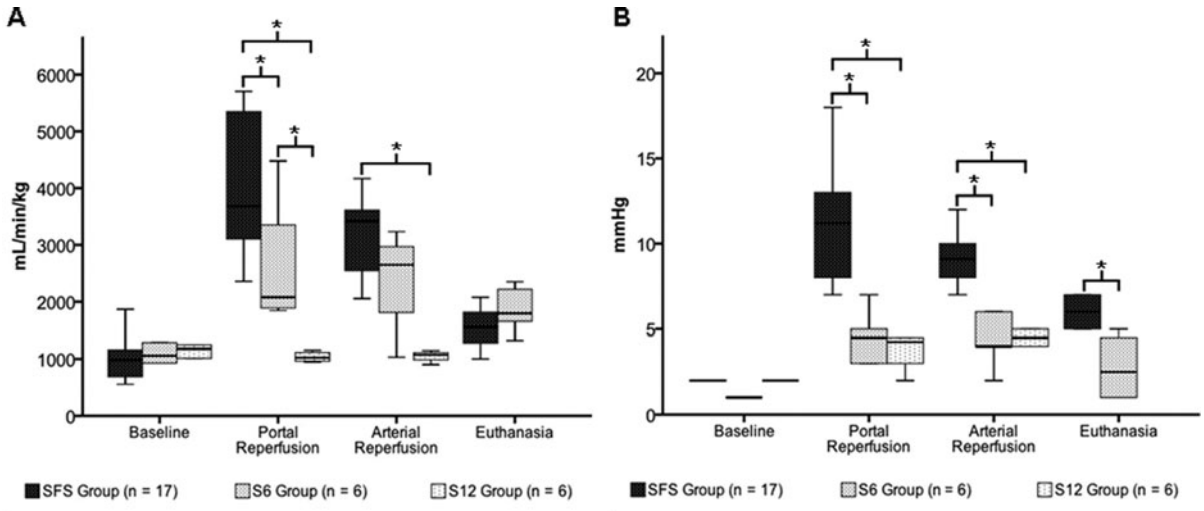
Quantitative Hepatic Function

Hepatic function was quantified according to the PDR of ICG. There were no significant differences between any of the groups at baseline: SFS 10.5 (8.3–11.1), S6 11.6 (11.2–12.0) and S12 9.7 (9.4–10.1). After reperfusion, PDR declined to a similar extent in all 3 groups: SFS 6.0 (5.5–6.4), S6 6.2 (6.1–7.2), and S12 5.1 (3.7–6.5). At the end of 5 days of follow-up, PDR remained stable in S6 and declined even further among SFS survivors: 6.2 (6.1–7.2) and 4.2 (3.7–4.7), respectively ($P = 0.031$). Measurements of the retention rate after 15 minutes revealed a similar pattern.

DISCUSSION

In the present study, we demonstrate for the first time the amount of portal vein flow necessary to sustain a "small-for-size" liver graft. We used the same clinically relevant, large animal model, in which transplants were performed with adequate periods of cold ischemia and without venovenous bypass, that we validated previously.[4] Experimental models have the distinct advantage of offering homogeneous conditions for the study group, a situation that is very difficult to reproduce in the human setting.

Though it is a known fact that too much PVF can cause severe and irreparable damage to a partial graft, we found, interestingly, that the graft still requires PVF superior to its normal baseline value. A 4-fold increase in PVF, which was observed in the unshunted SFS



	SFS (n=17)	S6 (n=6)	S12 (n=6)
Baseline	982 (686-1159)	1057 (927-1287)	1180 (1011-1247)
Portal Reperfusion	3389 (2748-5015)	2077 (1888-3350)	1024 (964-1112)
FOLD-CHANGE	3.8 (2.9-4.9)	2.1 (2.0-2.8)	0.9 (0.9-1.0)

FIGURE 3. The evolutions of PVF A, and the PVPG B, among the 3 groups. Postreperfusion PVF was approximately 4-times baseline in SFS, versus approximately 2-times baseline in S6 and unchanged from baseline in S12. Flow values are reported in mL/min/kg hepatic tissue. To convert to mL/min/100 g hepatic tissue, these values may be divided by a factor of 10. Data are expressed as the median, with the 25% to 75% percentiles in boxes and the 5% to 95% percentiles as whiskers. $^*P < 0.05$.

TABLE 2. Evolution of Hepatic Hemodynamic Parameters*

		SFS (n = 17)	S6 (n = 6)	S12 (n = 6)	ANOVA
PVF, mL/min/kg	BAS	982 (686−1159)	1057 (927−1287)	1180 (1011−1247)	NS
	PR	3389 (2748−5015)	2077 (1888−3350)	1024 (964−1112)	0.003
	AR	3314 (2486−3612)	2649 (1812−2970)	1071 (986−1109)	0.018
	EUT	1555 (1277−1815)†	1796 (1654−2218)	–	NS
PVPG, mmHg	BAS	2 (2−3)	1	2	NS
	PR	12 (8−14)	5 (3−5)	4 (3−5)	0.000
	AR	9 (8−11)	4 (4−6)	4 (4−5)	0.000
	EUT	6 (5−7)†	3 (1−5)	–	0.017
HAF, mL/min/kg	BAS	331 (275−488)	401 (347−425)	360 (329−590)	NS
	AR	156 (101−211)	372 (249−593)	452 (323−464)	0.005
	EUT	121 (107−144)†	158 (133−231)	–	NS
HAF, %THBF	BAS	25 (20−39)	29 (21−35)	28 (24−31)	NS
	AR	5 (4−9)	15 (9−23)	18 (18−19)	0.05
	EUT	8 (7−9)†	9 (9−10)	–	NS
P/A	BAS	2.9 (1.6−4.0)	3.0 (2.1−3.7)	2.6 (2.3−3.3)	NS
	AR	17.6 (8.6−19.0)	6.2 (3.5−10.5)	4.5 (4.5−5)	0.05
	EUT	13.0 (12.1−14.3)†	11.8 (11.7−13.6)	–	NS

*This table depicts the evolution of hemodynamic parameters measured at baseline in the donor, after portal and arterial reperfusion in the recipient, and 5 days after reperfusion in the surviving animals. All flow values are reported in mL/min/kg hepatic tissue. To convert to mL/min/100 g hepatic tissue, these values may be divided by a factor of 10.

NS indicates not significant; AR, arterial reperfusion; BAS, baseline; EUT, euthanasia; HAF, hepatic artery flow; P/A, portal-to-arterial flow ratio; PR, portal reperfusion; PVF, portal vein flow; PVPG, portal vein pressure gradient; THBF, total hepatic blood flow.

†n = 5 for SFS at euthanasia.

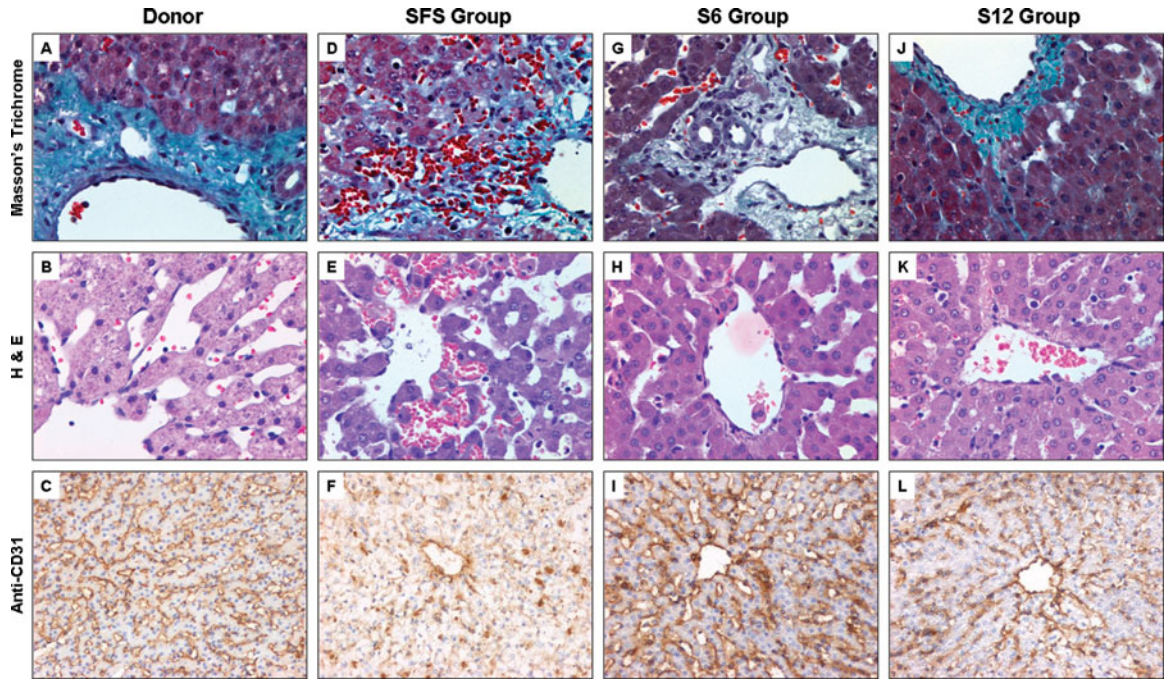


FIGURE 4. Masson's trichrome, H&E, and CD31 immunohistochemical staining of paraffin-embedded tissue samples taken 1 hour after portal reperfusion. In the SFS group, there was significant endothelial denudation in the medium-sized portal vein branches and hemorrhage into perivenular connective tissue (D), which extended into the hepatic parenchyma when severe (E). The partial portocaval shunts in the S6 and S12 groups facilitated preservation of the sinusoidal endothelial and the periportal connective tissue (G & J), and there was no intraparenchymal hemorrhage present in either group (H & K). CD31 immunostaining was notable for destruction of the endothelial lining among animals in the SFS group (F), in contrast to preservation of the normal sinusoidal microarchitecture in the S6 and S12 groups (I & L).

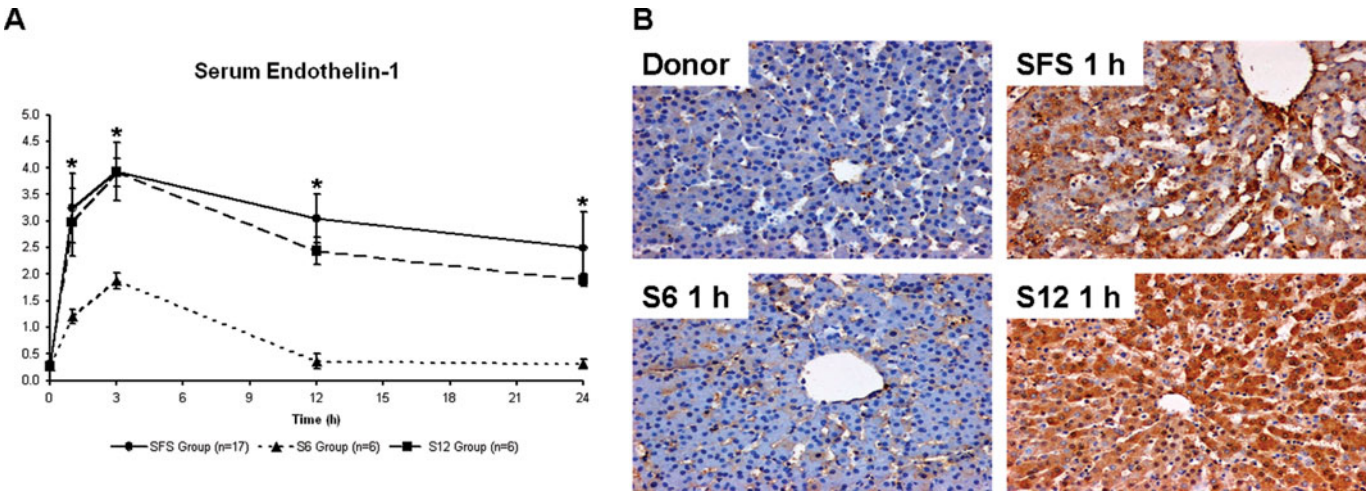


FIGURE 5. A, Postoperative evolution of serum ET-1, a marker of endothelial-cell stress and hypoxia. Here, data are expressed as the mean and the standard error of the mean as whiskers, whereas they are expressed as the median and the 25% to 75% interquartile range in the text.*$P < 0.05$ for both SFS and S12 versus S6. B, ET-1 immunostaining of postreperfusion tissue samples. ET-1 expression was significantly upregulated in both the SFS and S12 groups, though the hepatocyte cords remained intact in the latter. S6 grafts, on the other hand, were notable for very minimal ET-1 staining of the endothelial lining.

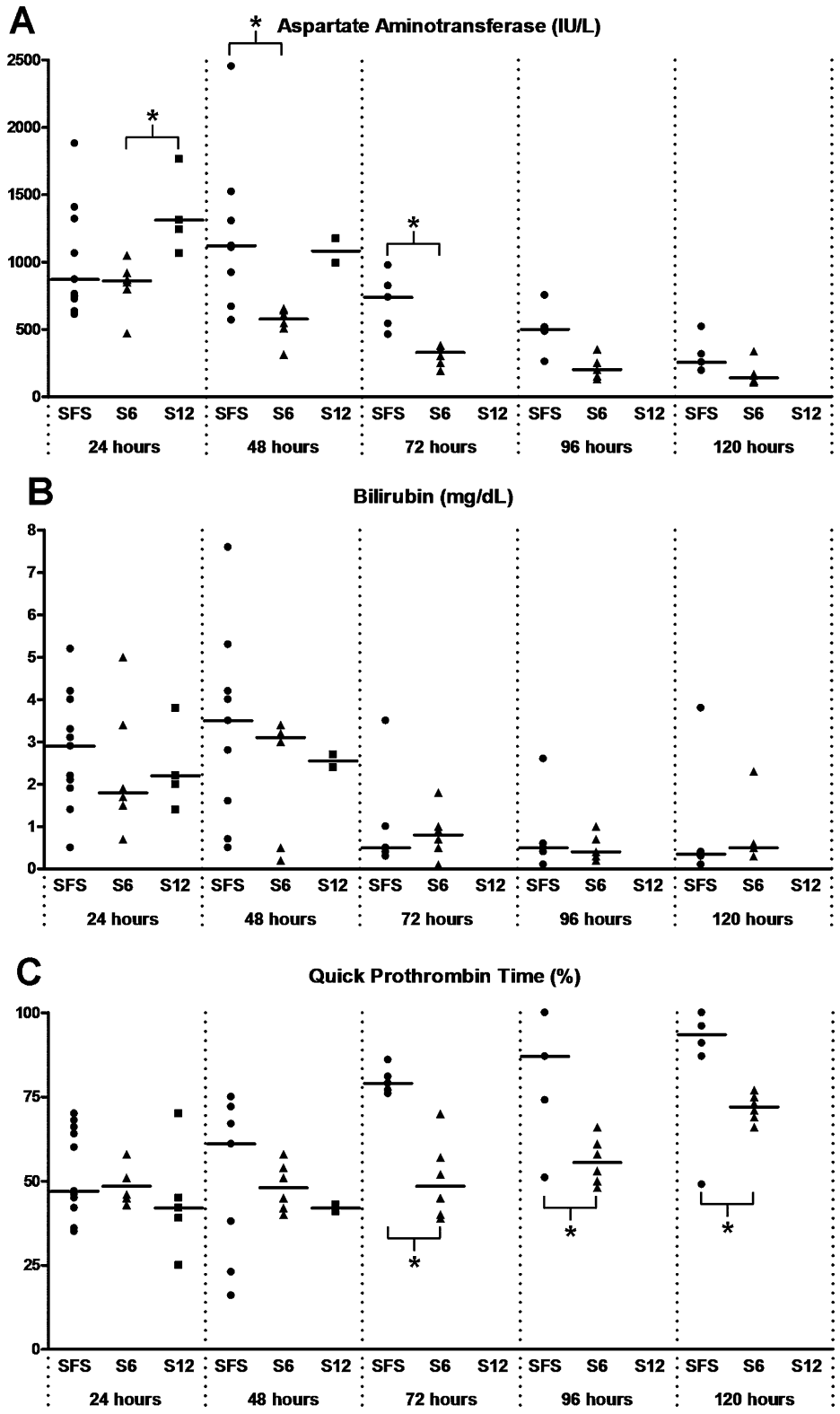


FIGURE 6. A, Postoperative evolutions of AST, B, bilirubin, and C, Quick prothrombin time. Bars represent the median values. $^{*}P < 0.05$.

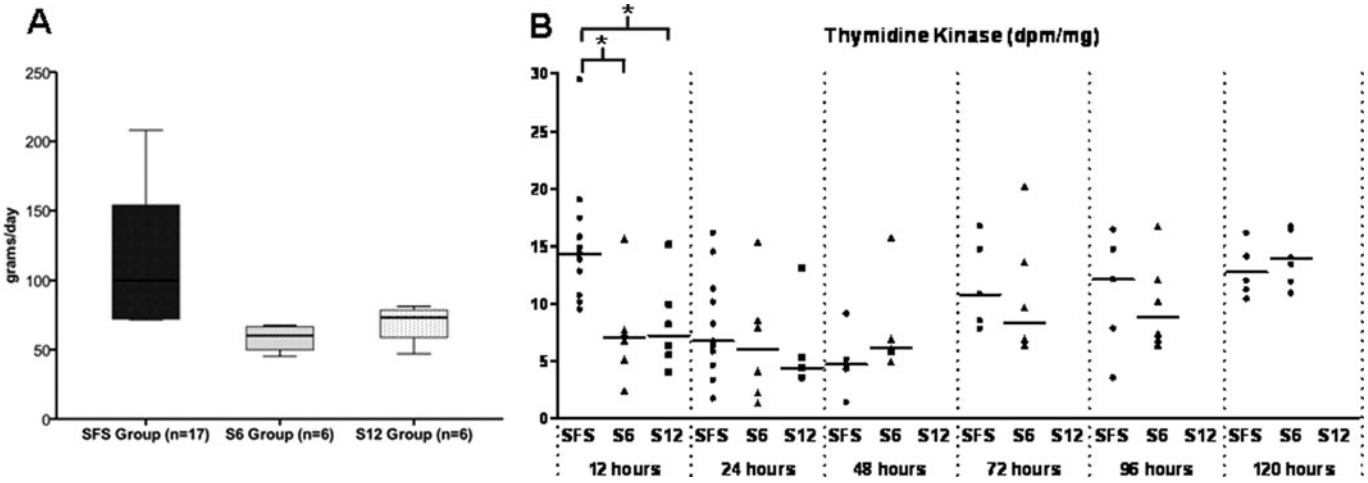


FIGURE 7. A, Increase in the mass of the graft, measured in grams per day, was significantly higher in the SFS group versus the S6 and S12 groups. Data are expressed as the median, with the 25% to 75% percentiles in boxes and the 5% to 95% percentiles as whiskers. $^{*}P < 0.05$. B, Postoperative evolution of serum thymidine kinase activity, a marker of cellular proliferation. There was a dramatic increase in TK activity after reperfusion among the SFS grafts. After the first day, however, SFS group survivors and animals in the S6 group had a similar evolution in TK activity for the remainder of the follow-up period. Bars represent the median values. $^{*}P < 0.05$.

group, resulted in SFS graft failure in 71% of the animals, whereas a 2-fold increase in PVF, which was seen in the S6 group, was associated with 100% survival. However, a postreperfusion PVF unchanged from baseline, as was seen in the S12 group, was associated with 100% mortality at 3 days. In terms of PVPG, both the 6- and 12-mm shunts successfully decompressed the portal venous bed and avoided the portal venous hypertension that caused significant hepatic endothelial cell injury in the unshunted recipients. Thus, based on this data, it appears that a shunt that maintains PVF at or around twice baseline is an appropriate means of preventing a rise in portal pressure and, thereby, the development of SFSS in partial liver grafts.

Clinical work has already been published on successful ALDLT with left liver lobes through the use of a hemiportocaval shunt (HPCS) to decompress the splanchnic bed and prevent irreparable sinusoidal injury.[13–17] In 2005, Troisi et al[15] reported HPCS inflow modification for both small left and right lobe grafts (graft weight-to-body weight ratio <0.8%) by prospectively assigning 13 such grafts to shunted and nonshunted groups. Interestingly, they calibrated the shunts to achieve approximately 2-times the baseline portal inflow recorded in the donors prior to hepatectomy. In their study, they did not experiment with different standardized shunt sizes or varying levels of inflow but, instead, universally opted for a goal of twice baseline based on the hypothesis that it would be the most "physiological" flow rate for the reperfusion of a hemiliver. Although SFSS was observed in 3 recipients of unshunted grafts, it did not arise in any of the recipients of shunted grafts. Furthermore, in the shunted group, at a median follow-up of 17 months, no cases of portal vein or shunt thrombosis were observed, and none of the shunts had to be occluded secondary to the development of hepatic encephalopathy or other signs of excessive portosystemic shunting.

Most recently, Botha et al[17] described the first American series on the successful use of left liver lobes and HPCS for ALDLT. Sixteen patients received left liver lobes, with a median GWBWR of 0.67% (range 0.51%–1.05%). The median PVPG after reperfusion with a HPCS in place was 5 mmHg (range 1–15 mmHg). None of the shunts were calibrated, and PVF was measured in only 4 of 16 patients, with, again, relatively disparate results among recipients. One of the patients with a HPCS did end up developing SFSS, whereas 2 others required occlusion of their HPCS secondary to protracted encephalopathy.[18] Even though they are already performing HPCS for SFS transplant in the clinical setting, the authors themselves comment on the fact that it still remains to be determined what acceptable PVF and/or PVPG are in the setting of HPCS.

In terms of other large-animal experimental models, the group from the University of Tsukuba has published several updates on their series of porcine SFS transplants with PCS.[19–22] In their most recent manuscript, the authors described 11 SFS transplants performed with a 6-mm side-to-side PCS versus 11 without. Upon reperfusion in the group without PCS, PVF was roughly twice its baseline value, although it was about one-third of baseline in the group with PCS. The authors reported 9% survival at 24 hours in the group without PCS versus 73% survival at the end of 4 days in the group with PCS. Included among the causes of death within the PCS group was portal vein thrombosis.

This study is the first to purposefully vary the amount of blood shunted through a PCS to obtain distinct levels of portal vein inflow and study their effects on the partial liver graft. It confirms the positive results of the only clinical study in which the HPCS was calibrated to achieve twice the baseline portal venous inflow.[15] Furthermore, it confirms the fact that too much portosystemic shunting can have adverse effects, as well, as has been observed by other authors.[17,22]

In human whole liver transplant, blood flow to the graft increases upon reperfusion in the recipient.[23] Flows can more than double due to the loss of normal vascular tone and the persistence of abnormal splanchnic hemodynamics, in particular. Furthermore, these altered hemodynamics can persist up to a year after transplant.[24] Clearly, the human liver is capable of supporting approximately twice the amount of its normal portal venous inflow. Beyond that, in terms of partial grafts in particular, it appears that the chances of developing SFS graft failure increase significantly.

In addition to improving portal venous hemodynamics, both the 6- and 12-mm shunts also improved flow through the hepatic artery. Hepatic artery flow was significantly higher in the S6 and S12 groups after reperfusion than it was in the SFS group. Likewise, P/A did not vary significantly from baseline to graft reperfusion, as it did in the SFS group. Other authors have shown that insufficient

HAF may be an important factor in the development of SFSS.[24–26] It appears that PCS, perhaps by reducing flow through the portal vein, can help maintain adequate HAF, as well.

Both the 6- and 12-mm shunts successfully corrected the unremitting hypertransaminasemia and hyperbilirubinemia that developed in the majority of the unshunted controls, though it did take the shunted grafts slightly longer to regenerate functional clotting factors. Furthermore, with both the 6- and 12-mm shunts, the hepatic sinusoidal endothelium was protected from the hyperperfusion injury, as could clearly be seen on light microscopy and immunostaining with CD31. However, only the 6-mm shunt preserved sufficient PVF to the partial graft to avoid the deleterious effects of portal hypoperfusion.

Endothelin-1 is upregulated in situations of endothelial-cell inflammation and stress as well as hypoxia. The mechanism for the increased expression of ET-1 observed in postreperfusion samples in the unshunted SFS group likely had to do with shear stress in the hepatic sinusoids, which could clearly be seen on CD31-immunostaining of tissue samples taken 1 hour after portal reperfusion. However, the increased expression of ET-1 observed among postreperfusion samples in the S12 group could not have been due to mechanical injury, being that the portal vein was decompressed and the sinusoidal endothelium in these grafts remained well preserved. Rather, the increased expression of ET-1 observed in this group could have been due to other physiological alterations associated with graft hypoperfusion, such as hypoxia. This is theory, however, and remains to be elucidated.

Levels of the proinflammatory cytokine IL-6 were measured in postreperfusion samples. Interestingly, the levels were the highest among animals in the S12 group, even higher than those in the SFS group. A possible explanation for this is that hypoperfusion led to ongoing hypoxia and ischemia in these partial grafts, leading to greater local injury and propagation of the inflammatory cascade.

In the SFS and S6 groups, glucose administration was only necessary in the initial postoperative period when the animals were taking limited intake *per os*. In the S12 group, however, not only did each of the 6 animals have repeated episodes of hypoglycemia on serial checks, but we also had to increase the frequency with which we checked serum glucose levels to stay ahead of their hypoglycemia. The GEI was calculated as an objective means of quantifying these differences and was found to be significantly lower in the S12 group versus the other 2. This situation of unremitting hypoglycemia is reminiscent of that found in fulminant liver failure, in which up to 45% of patients suffer from hypoglycemia refractory to IV glucose administration, secondary to depletion of hepatic glycogen stores and impaired gluconeogenesis.[27] It appears that the extreme amount of portal venous shunting to which they were subjected resulted in either impaired hepatic glycogenolysis or gluconeogenesis in the livers in the S12 group. This is a very interesting finding and one that deserves further investigation.

In terms of hepatic regeneration, both the per daily increase in graft mass and serum TK activity were higher in the SFS group than in either of the 2 groups of shunted animals, though it is possible that at least part of the increase in mass in the former group was due to graft congestion and edema. Perhaps because the injury was greater, the stimulus for regeneration was also greater in the SFS group. Because many of the cytokines and cellular signaling pathways implicated in inflammation and regeneration are the same,[28–30] it is conceivable that the injury in the SFS grafts conditioned cellular proliferation. On the other hand, there were no significant differences between S6 and S12 in terms of either measure of hepatic regeneration. In both the S6 group and SFS animals that survived, TK activity reached maximum levels between the third and fifth postoperative days, in accordance with data published previously in the porcine model on hepatic regeneration after extended hepatectomy.[31–33]

In spite of the fact that all of the partial grafts increased in size during the follow-up period and were able to recover from the acute ischemia-reperfusion injury, grafts in the SFS group demonstrated a decline in quantitative hepatic function upon portal reperfusion that continued to the end of follow-up. In contrast, metabolic function of the grafts in the S6 group had not improved by 4 days, but it also had not deteriorated further.

This is an acute model of small-for-size injury, and the study is limited by the follow-up period. Further work and, ultimately, clinical trials need to be performed to study the long-term effects of a portocaval shunt in the setting of reduced-size liver transplant. Nonetheless, the results of this study offer promise for the broader application of transplant with partial liver grafts, including left hemilivers.

The fear of SFSS is the primary obstacle preventing the widespread use of partial liver transplantation. The data we present herein, however, provide the hope that this syndrome may be avoided. The use of a portocaval shunt that decompresses the portal bed while still maintaining roughly 2 times the baseline portal venous flow appears to be an effective means of preventing liver dysfunction and failure in SFS liver transplant.

ACKNOWLEGMENTS

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6. DISCUSSION

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6.1. Study 1

This large animal model is straightforward, reproducible, and clinically relevant. Other porcine SFS survival models published to date vary in terms of transection techniques, lengths of cold ischemia, and postoperative outcomes (52;53;55-58;60). This is the first study of its kind to avoid the use of venovenous bypass, include clinically relevant lengths of cold ischemia, and study hepatic hemodynamic alterations and their association with early postoperative outcomes, including survival and regeneration, over a period of several days. The decision was made to euthanize the animals in order to obtain tissue samples and perform hemodynamic measurements at a point when the trajectory of the recipient (i.e., graft failure or recovery) appeared to have been established. Nonetheless, a longer period of follow-up could have allowed for the observation of other complications associated with the use of grafts of such small size.

“Small-for-size” syndrome has been described as occurring in grafts of various sizes, even those representing >30-40% of the recipients’ standard liver volume or >0.8% of the recipients’ body weight. This is presumably due to other factors, such as steatosis or the underlying disease in the recipient. However, the aim of this study was to specifically study acute failure that occurs as a direct result of an extremely small graft size. The clinical relevance of this model has to do with the fact that reduced-size transplantation with grafts representing 20-30% of the recipients’ standard liver volume is not performed on any meaningful scale in Western centers. It would be highly advantageous, however, for live donation donors to be able to donate the left hemiliver instead of the right because the risks associated with a left hepatectomy are significantly reduced, as described in the Introduction. Similarly, the yield of deceased donation liver transplantation could be doubled if these livers could be consistently split

for two adults. These types of transplants are not routinely performed because of the not unfounded fear that SFS graft failure will arise in the recipient of a left hemiliver. Because it is very difficult to study the use of these grafts in the clinical setting, this experimental model was established to study the early series of events that precede their failure, in order to identify points at which to intervene and prevent it.

In human liver transplantation with a whole graft, portal vein flow typically increases upon reperfusion. This is the result of hyperdynamic splanchnic circulation in the recipient and the replacement of a cirrhotic organ with a noncirrhotic one, with lower resistance to portal flow (93;94). In the case of partial liver transplantation, the vascular bed through which this high flow has to pass is significantly diminished. Although portal inflow is considered to be a stimulus for hepatic regeneration (95;96), excessive PVF can be detrimental to the function and survival of the reduced-size organ (8;13;96).

All the recipients in this study experienced an increase in PVF upon reperfusion. Postreperfusion PVF was considerably higher among the recipients of SFS livers versus those of whole livers, and 71% of SFS grafts ultimately failed. Severe graft damage and dysfunction and the exclusion of any other cause of death indicate that graft failure was the cause of death in these animals. The survival rate among SFS recipients was consistent with the rates reported in similar studies (53;55-57). Furthermore, survival in the SFS group occurred randomly, indicating that the experience of the operators was not a major determinant.

The primary target in the entire process of SFS liver injury appears to be the sinusoidal endothelial cell. One hour after portal reperfusion, serum levels of hyaluronic acid were already significantly higher in the subgroup of animals that went on to develop SFS graft failure, whereas significant increases in markers of hepatocellular injury (aspartate aminotransferase, bilirubin, and the Quick prothrombin time) occurred later. The histological and immunohistochemical examination of tissue sampled 1 hour after

reperfusion was noteworthy for extensive rupture of the endothelial cells of the portal vein branches and dissection of blood into the periportal spaces and beyond.

Although PVF did not vary significantly between the nonsurvivors and survivors within the SFS group, injury to the sinusoidal endothelium was significantly greater in the former group of animals versus the latter. One possible explanation for this is that PVF was, in fact, higher immediately after portal reperfusion among nonsurvivors. In the study, PVF was not measured until the infrahepatic IVC anastomosis was complete and adequate hemostasis with respect to both the vascular anastomoses and the transection surface of the liver had been achieved. Postreperfusion PVF was actually measured approximately fifteen minutes after the portal reperfusion event. It is plausible that by the time PVF was measured, significant stress-induced endothelial cell injury had already occurred, injury that altered the hepatic microstructure to the extent that portal resistance increased and flow actually decreased. In support of this theory, extensive damage to the endothelial lining was evident on Masson's trichrome and anti-CD31 staining of postreperfusion liver biopsies from the SFS grafts that ultimately failed, much more so than in those that did not. Furthermore, postreperfusion portal resistance tended to be higher among nonsurvivors versus survivors, though the difference did not reach statistical significance.

Suboptimal hepatic artery flow (HAF) is poorly tolerated by transplanted livers. Unlike native organs, transplanted grafts lack alternative sources of arterial inflow, such as the peribiliary arterial plexus. In the posttransplant setting, arterial hypoperfusion is associated with ischemia and cholestasis, clinical signs that have also been described in SFS grafts (8). It has been hypothesized that insufficient HAF may be an important contributor to SFS liver injury (14;97). However, in the study by Marcos *et al* (98) on hepatic hemodynamics in a series of sixteen ALDLT, HAF comprised 2-16% of total hepatic blood flow (THBF) after portal reperfusion. In particular, among grafts that met SFS criteria (graft weight-to-body weight ratio <0.8, N=5), HAF represented 3-10% of

postreperfusion THBF. All of the grafts functioned well, regardless of either the proportion of THBF that was from the hepatic artery or the portal-to-arterial flow ratio.

In the present study, all of the grafts in the whole liver group but only 29% of those in the SFS group survived the follow-up period. HAF as a proportion of THBF did vary significantly between SFS grafts and whole livers after arterial reperfusion and at euthanasia. PVF seems to play a primary role in affecting the outcome of reduced-size liver transplantation, but this data also suggests that a reduction in HAF, particularly with respect to THBF, may play a role in the pathogenesis of SFS graft dysfunction, as well. Secondary to significant graft growth in the SFS group, the arterial flow per tissue mass decreased by the fifth postoperative day, whereas in the whole liver group it remained stable or, in some cases, even increased slightly. The delayed effects of this decline in arterial flow and whether it would be maintained over time were not evaluated; a longer period of follow-up would be needed in order to do so.

Whether hepatic regeneration is augmented or impaired after SFS liver transplantation remains an issue of controversy (13;48;57;99;100). In the present study, the increase in hepatic mass per day was greater in grafts that failed than in those that survived, though it is likely that at least some of this increase was due to graft congestion and edema. Serum thymidine kinase (TK) activity was therefore evaluated to better quantify proliferative activity at each postoperative time point. Among nonsurvivors, TK activity rose considerably during the first twelve hours and declined thereafter; by two days, there was virtually no measurable serum TK activity in this subgroup. Since many of the same cytokines and cellular signaling pathways have been implicated in both inflammation and regeneration (101;102), the considerable stress to which SFS grafts were immediately subjected could have simultaneously conditioned hepatocellular proliferation. When the reperfusion injury was severe, however, this proliferative stimulus was not enough to allow them to recover. Among survivors and the recipients of whole liver grafts, on the other hand, TK activity rose much more slowly and reached

maximum levels between the third and fifth days, in accordance with data published previously on hepatic regeneration following extended hepatectomy in the porcine model (103-105).

Surviving grafts had preserved hepatic architecture on postoperative day five. Significant tissue regeneration had also occurred. Regeneration-associated hyperplasia was evident in the relative thickening of the hepatocyte cords seen in the final samples in comparison with those taken at baseline. In addition, the greater intensity of anti-CD31 immunofluorescence observed on day five, in the absence of any apparent increase in the microvascular density, seems to indicate that mechanical stress in the sinusoid upregulated the expression of molecules responsible for maintaining endothelial stability.

Even though all of the partial grafts increased in size and those that survived recovered from the acute ischemia-reperfusion lesion, they did not regain normal metabolic function during the follow-up period. Other authors have reported similar findings in the experimental setting, demonstrating, in particular, a reduction in the metabolic activity of cytochrome P-450 isozymes (106-109) and ICG clearance (110) after extended hepatectomy. Moreover, in clinical ALDLT, recipients have been reported to achieve higher relative drug levels versus the recipients of whole deceased donation livers (111), even when hepatic regeneration is complete (112;113). The restoration of metabolic activity does not coincide with that of liver mass when a reduced-size liver regenerates.

Future treatments for SFSS should include strategies primarily aimed at protecting the hepatic endothelial cell from the physical stress of excessive PVF. These may include surgical or pharmacological therapies to reduce sinusoidal hypertension and/or augment the capacity of the sinusoidal endothelial cells to resist injury. Furthermore, portal hyperflow in the immediate postreperfusion period implies not only great

tangential force against the walls of the hepatic sinusoids but also elevated delivery of gut-derived endotoxins and substrates for the production of reactive oxygen species (8;44). Hence, antioxidant and anti-inflammatory agents can also play an important role in ameliorating reperfusion injury in partial livers.

SFSS is one of the primary obstacles preventing the wider use of partial livers arising from not only live donation but also deceased donation in the context of split liver transplant. The data from this first study provides not only further information regarding the pathophysiology of the acute phase of this poorly understood process but also a well-characterized, reproducible, and clinically relevant model for the future study of SFS liver transplantation and new means of improving its success.

6.2. Study 2

In the second study, the amount of portal vein flow necessary to sustain a “small-for-size” liver graft has been demonstrated for the first time. The same clinically relevant large animal model, in which transplants were performed with adequate periods of cold ischemia and without venovenous bypass and which was validated in the first study, was used.

Though it is a known fact that too much PVF can cause severe and irreparable damage to a partial liver graft, these grafts still require PVF superior to their normal baseline values. A four-fold increase in PVF, which was observed in the unshunted SFS group, resulted in SFS graft failure in 71% of the animals, whereas a two-fold increase in PVF, which was seen in the S6 group, was not associated with a single case of graft failure during five days of follow-up. However, a postreperfusion PVF unchanged from baseline, as was seen in the S12 group, was associated with 100% graft failure by three days. In terms of the portal venous pressure gradient, both the 6- and 12-mm shunts successfully decompressed the portal venous bed and avoided the portal venous hypertension that caused significant hepatic endothelial cell injury in the unshunted recipients. Thus, based on this data, it appears that a shunt that maintains PVF at or around twice baseline is an appropriate means of preventing a rise in portal pressure and, thereby, the development of SFSS in partial liver grafts.

The group from Tohoku University in Japan has published several updates on their series of porcine SFS transplants with portocaval shunt (53;55;58;59). In their most recent manuscript, the authors described eleven SFS transplants performed with a 6-mm side-to-side portocaval shunt versus eleven without. Upon reperfusion in the group without portocaval shunt, PVF was roughly twice its baseline value, while it was about one-third of baseline in the group with portocaval shunt. The authors reported 9% survival at twenty four hours in the group without portocaval shunt versus 73% survival

at the end of four days in the group with portocaval shunt. Included among the causes of death within the portocaval shunt group was portal vein thrombosis.

This study is the first to purposefully vary the amount of blood shunted through a portocaval shunt to obtain distinct levels of portal vein inflow and study their effects on the partial liver graft. It confirms the positive results of the only clinical study in which HPCS was calibrated to achieve twice the baseline portal venous inflow (13). Furthermore, it confirms the fact that too much portosystemic shunting can have adverse effects, as well, as has been observed by other authors (40;58).

In human whole liver transplant, blood flow to the graft increases upon reperfusion in the recipient (93). Flows can more than double due to the loss of normal vascular tone and the persistence of abnormal splanchnic hemodynamics. Furthermore, these altered hemodynamics can persist up to a year after transplant (114). Clearly, the human liver is capable of supporting approximately twice the amount of its normal portal venous inflow. Beyond that, in partial grafts in particular, it appears that the chances of developing SFS graft failure increase significantly.

In addition to improving portal venous hemodynamics, both the 6- and 12-mm shunts also improved flow through the hepatic artery. Hepatic artery flow was significantly higher in the S6 and S12 groups after reperfusion than it was in the SFS group. Likewise, the portal-to-arterial flow ratio did not vary significantly from baseline to graft reperfusion, as it did in the SFS group. Other authors have shown that insufficient HAF may be an important factor in the development of SFSS (97;114;115). It appears that a portocaval shunt, perhaps by reducing flow through the portal vein, can help maintain adequate HAF, as well.

Both the 6- and 12-mm shunts successfully corrected the unremitting hypertransaminasemia and hyperbilirubinemia that developed in the majority of the unshunted controls, though it did take the shunted grafts slightly longer to regenerate

functional clotting factors. Furthermore, with both the 6- and 12-mm shunts, the hepatic sinusoidal endothelium was protected from the hyperperfusion injury, as could clearly be seen on light microscopy and immunostaining with anti-CD31. However, only the 6-mm shunt preserved enough PVF to the partial graft to avoid the deleterious effects of portal hypoperfusion.

Endothelin-1 (ET-1) is upregulated in situations of endothelial cell inflammation and stress as well as hypoxia. The mechanism for the increased expression of ET-1 observed in postreperfusion samples in the unshunted SFS group likely had to do with shear stress in the hepatic sinusoids, which could clearly be seen on anti-CD31 immunostaining of liver biopsies taken one hour after portal reperfusion. However, the increased expression of ET-1 observed among postreperfusion samples in the S12 group could not have been due to mechanical injury, being that the portal vein was decompressed and the sinusoidal endothelium in these grafts remained well-preserved. Rather, the increased expression of ET-1 observed in this group could have been due to other physiological alterations associated with graft hypoperfusion, such as hypoxia. This is a theory, however, and remains to be confirmed.

Levels of the proinflammatory cytokine interleukin-6 were measured in postreperfusion samples. Interestingly, levels were the highest among animals in the S12 group, even higher than those in the SFS group. A possible explanation for this is that hypoperfusion led to ongoing hypoxia and ischemia in these partial grafts, thereby creating greater local injury and propagation of the inflammatory cascade.

In the SFS and S6 groups, glucose administration was only necessary in the initial postoperative period when the animals were taking limited intake *per os*. In the S12 group, however, not only did each of the six animals have repeated episodes of hypoglycemia on serial checks, but the frequency with which their serum glucose levels were checked had to be increased, in order to stay ahead of their hypoglycemia. The

glucose index was calculated as an objective means of quantifying these differences and was found to be significantly lower in the S12 group versus the other two. This situation of unremitting hypoglycemia is reminiscent of that found in fulminant liver failure, in which up to 45% of patients suffer from hypoglycemia refractory to intravenous glucose administration, secondary to depletion of hepatic glycogen stores and impaired gluconeogenesis (63). It appears that the extreme amount of portal venous shunting to which they were subjected resulted in impaired hepatic glycogenolysis and/or gluconeogenesis in the livers in the S12 group.

In terms of hepatic regeneration, both the per daily increase in graft mass and serum TK activity were higher in the SFS group than in either of the two groups of shunted animals, though it is possible that at least part of the increase in mass in the former group was due to graft congestion and edema. Perhaps because the injury was greater, the stimulus for regeneration was also greater in the SFS group. Because many of the cytokines and cellular signaling pathways implicated in inflammation and regeneration are the same (101;102;116), it is conceivable that the injury in the SFS grafts conditioned cellular proliferation. On the other hand, there were no significant differences between S6 and S12 in terms of either measure of hepatic regeneration. In both the S6 group and SFS animals that survived, TK activity reached maximum levels between the third and fifth postoperative days, in accordance with data published previously in the porcine model on hepatic regeneration after extended hepatectomy (103-105).

In spite of the fact that all of the partial grafts increased in size during the follow-up period and were able to recover from the acute ischemia-reperfusion injury, grafts in the SFS group demonstrated a decline in quantitative hepatic function upon portal reperfusion that continued until the end of follow-up. In contrast, metabolic function of the grafts in the S6 group had not improved by five days, but it also had not deteriorated further.

This is an acute model of SFS injury, and the study is limited by the follow-up period. Further work and, ultimately, clinical trials need to be performed to study the long-term effects of a portocaval shunt in the setting of reduced-size liver transplant. Nonetheless, the results of this study offer promise for the broader application of transplant with partial liver grafts, including left hemilivers.

7. CONCLUSIONS

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The conclusions of this doctoral thesis are the following:

1. In “small-for-size” liver transplant in the acute setting, portal vein flow rises to three- to four-times its baseline value.

2. Portal hyperperfusion appears to be a stimulus for regeneration. Simultaneously, however, it causes significant sinusoidal endothelial cell injury, the extent and severity of which ultimately predict either SFS graft failure or histological recovery.

3. Though excessive portal vein flow and pressure are key factors leading to the development of SFSS, too little portal vein flow can also be detrimental to a partial liver graft.

4. A calibrated portocaval shunt that maintains portal vein flow around twice its baseline value improves histological and biochemical parameters and is an effective means of preventing sinusoidal endothelial cell injury and SFS graft failure.

8. BIBLIOGRAPHY

8. BIBLIOGRAPHY

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9. APPENDIX

9. APPENDIX

Over the course of completing this doctoral thesis, I also participated in the creation of the following scientific publications:

ORIGINAL ARTICLES

1. Shah SA, Chark D, Williams J, **Hessheimer A**, Huh J, Wu YC, Chang PA, Scholl FG, Drinkwater DC. Retrospective Analysis of Local Sensorimotor Deficits after Radial Artery Harvesting for Coronary Artery Bypass Grafting. J Surg Res 2007;139(2):203-8.
2. Fondevila C, **Hessheimer AJ**, Ruiz A, Calatayud D, Ferrer J, Charco R, Fuster J, Navasa M, Rimola A, Taurá P, Ginés P, Manyalich M, García-Valdecasas JC. Liver transplant using donors after unexpected cardiac death: novel preservation protocol and acceptance criteria. Am J Transplant 2007;7(7):1849-55.
3. Forner A, Ayuso C, Varela M, Rimola J, **Hessheimer AJ**, Rodríguez-Lope C, Reig M, Bianchi L, Llovet JM, Bruix J. Evaluation of tumor response after locoregional therapies in hepatocellular carcinoma (HCC): Are RECIST criteria reliable? Cancer 2009;115(3):616-23.
4. **Hessheimer A**, Parramón D, Guimerá A, Erill I, Rimola A, García-Valdecasas JC, Villa R, Fondevila C. A rapid and reliable means of assessing hepatic steatosis in vivo via electrical bioimpedance. Transplantation 2009; 88(5):716-722.
5. **Hessheimer AJ**, Forner A, Varela M, Bruix J. Metabolic risk factors are a major comorbidity in patients with cirrhosis independent of the presence of hepatocellular carcinoma. Eur J Gastroenterol Hepatol 2010;22(10):1239-44.

6. Fondevila C, **Hessheimer AJ**, Flores E, Vendrell M, Muñoz J, Escobar B, Taurá P, Fuster J, García-Valdecasas JC. Step-by-step guide for a simplified model of porcine orthotopic liver transplant. J Surg Res 2011. (In press)
7. Fondevila C, **Hessheimer AJ**, Maathuis MHJ, Muñoz J, Taurá P, Calatayud D, Leuvenink H, Rimola A, Ploeg RJ, García-Valdecasas JC. Superior preservation of DCD livers with continuous normothermic perfusion. Ann Surg 2011. (In press)

REVIEW ARTICLES:

1. Varela M, Forner A, **Hessheimer A**. Revisión crítica de la clasificación del hepatocarcinoma. Gastroenterología Práctica 2006;15(2):4-9.
2. Bruix J, **Hessheimer AJ**, Forner A, Boix L, Vilana R, Llovet JM. New aspects of diagnosis and therapy of hepatocellular carcinoma. Oncogene 2006;25(27):3848-56.
3. Forner A, **Hessheimer AJ**, Isabel Real M, Bruix J. Treatment of hepatocellular carcinoma. Crit Rev Oncol Hematol 2006;6(2):89-98.

BOOK CHAPTERS:

1. **Hessheimer AJ**, Earl TM, Chapman WC. Extrahepatic surgery in the cirrhotic patient. In Blumgart, Ed. Surgery of the Liver, Biliary Tract, and Pancreas, 5th edition. (In press)

One of these articles, which is of particular relevant to the work of this doctoral thesis, has been attached.

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Step-by-Step Guide for a Simplified Model of Porcine Orthotopic Liver Transplant

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Background. **Based on similar anatomy, physiology, and size to humans, pigs provide an excellent means for studying new therapies related to orthotopic liver transplant (OLT). Techniques that have been described to date, however, are unnecessarily complex and increase the likelihood of morbidity and adverse outcome.**

Materials and Methods. **Male outbred weanling pigs underwent OLT according to our procedure, with a short anhepatic time (<20 min) and without veno–venous bypass or vasoactive substances during the anhepatic phase. Vascular anastomoses were performed identical to the clinical setting, and a simple stented choledochocholedochostomy was created.**

Results. **The authors have performed this procedure 130 times using four transplant models: standard, whole-liver ($n = 10$), small-for-size ($n = 48$), donor after cardiac death ($n = 44$), and donor adenoviral gene transfection ($n = 28$). The average cold ischemic and anhepatic times were 302 ± 43 and 17 ± 3 min, respectively. Hypotension was successfully treated with intravenous fluids. In all cases, the recipient survived the operation and was extubated. Survival to the end follow-up varied according to the model and was 56% (73/130) for all cases. At autopsy or euthanasia, no vascular thrombosis or outflow obstruction was found. Survival was 100% for pigs transplanted with standard, whole-liver grafts ($n = 10$). In this group, AST and bilirubin rose during the first 24 h after graft reperfusion, while the Quick prothrombin time (QPT) fell. By the fifth postoperative day, these parameters had returned to baseline.**

Conclusions. **This model is straightforward and reproducible and offers surgeons and researchers the opportunity to perform OLT studies under clinically relevant conditions.**

Key Words: **pig; liver transplant; anhepatic; veno–venous bypass; preclinical study.**

INTRODUCTION

Experimental studies involving animals are central to advancement in virtually all medical fields. Large animal models are particularly useful in the surgical sciences. More than small animal models, they provide appropriate size and anatomy for the establishment and practice of new surgical techniques.

Pre-clinical studies of liver transplantation were first performed in the dog model by Goodrich and colleagues in 1956 [1]. In this seminal work, the hepatic allograft was implanted heterotopically, and the native liver was left in place. Subsequent investigations by Moore and colleagues in 1959 [2] and Kaupp and Starzl in 1960 [3] employed a more complex surgical technique, in which total hepatectomy was performed with the use of veno–venous bypass in the recipient.

Although the initial liver transplant studies involved dogs, pigs later became the preferred large animal model based on their greater physiological and anatomical similarity to humans, including the absence of hepatic venous sphincters [4, 5]. Since the late 1960s, numerous groups have described their own techniques of liver transplantation in pigs [4, 6–14]. Most of these models, however, are technically complex, leading to unnecessarily long operative times, and needlessly

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employ steps that increase recipient morbidity and mortality. Therefore, in spite of the numerous descriptions of porcine orthotopic liver transplant (OLT) already in existence, we continue to encounter surgical groups of the opinion that the pig model of OLT is extremely difficult to perform and associated with a prohibitively high number of recipient deaths.

Since the mid 1990s, our group has used a simplified technique to perform hundreds of liver transplants in pigs with consistent success [15–20]. The purpose of this work is to communicate, in detail, how to reproduce this technically straightforward model, from the induction of anesthesia to the postoperative follow-up.

MATERIALS AND METHODS

Animals

In general, outbred male weanling pigs, 25–35 kg, are used as both the donors and the recipients. The pigs are housed in temperature- and light-controlled cages on a 12–12 h light-dark cycle and provided with food and water *ad libitum*. Food is withheld 12 h prior to surgery.

Anesthesia

The pig is premedicated with azaperone (Stressnil) (10 mg/kg i.m.). Thirty minutes after premedication, the pig is weighed and taken to the operating table, still spontaneously breathing. The pig is preoxygenated with 100% oxygen and administered inhaled isoflurane (2%–5%, titrated to effect) to deepen the sedation. A peripheral ear vein is cannulated with an intravenous catheter, and anesthesia is induced with sodium thiopental (15 mg/kg i.v.). The pig is placed in the prone position, with the head and front legs hanging slightly off the edge of the operating table. An assistant helps by retracting the tongue down and extending the head back slightly to bring the oropharynx and trachea into alignment. Using an extra long Miller laryngoscope, the oropharynx is carefully swept from left to right until the epiglottis is brought into view. The epiglottis is retracted downward, revealing the vocal cords. With extreme care, a 6.5- or 7-mm endotracheal tube is passed through the cords. Force should not be applied during the intubation, as the porcine trachea is very fragile and easily fractured. Once the endotracheal tube has been placed and its position confirmed *via* capnography, the tube is secured, and the pig is placed in the supine position.

The pig is ventilated with volume-controlled intermittent positive pressure ventilation to maintain the end tidal CO_2 between 35 to 40 mmHg and PaO_2 between 200 to 240 mmHg. Anesthesia is maintained with isoflurane (1%–2%, titrated to effect). Prior to the opening skin incision, fentanyl (100 μg followed by 50 μg/h i.v.) is administered for analgesia, and cefoxitin (1 g i.v.) is given for antibiotic prophylaxis. Cisatracurium (0.2–0.3 mg/kg followed by 1.5 mg/h i.v.) is given for paralysis. Warm crystalloid solution (Plasmalyte, 20–25 mL/kg/h) is administered prior to and following the anhepatic phase, while during the anhepatic phase, a bolus of warm colloid solution (Voluven, 500 mL) is given. At no point are any vasoactive substances administered.

Access and Intraoperative Monitoring

The donor procedure may be performed with nothing more than the intravenous catheter placed at the start of anesthesia. However, the recipient procedure requires more invasive monitoring devices. A paratracheal incision is made on the right side of the neck, approximately one to two fingerbreadths below the angle of the mandible, and the internal jugular vein (IJV) and internal carotid artery (ICA) are carefully dissected. A triple-lumen catheter is placed in the IJV for drug and fluid administration, with one port connected to a pressure transducer for venous pressure monitoring. A single-lumen catheter is placed in the ICA and connected to a pressure transducer for arterial pressure and heart rate monitoring. At the end of the transplant procedure in the recipient, the neck incision is closed, and a Witzel tunnel is created to fix the catheters to the back of the neck for postoperative access.

Donor Operation

The abdomen is opened from the xiphoid process to the end of the nipple line with a midline laparotomy, avoiding the urethra, and a self-retaining Balfour retractor is placed. The small bowel is retracted caudally, and the right lobe of the liver is carefully retracted to the left. The membrane covering the retrohepatic inferior vena cava (IVC) is opened from the level of the right hepatic vein to the infrahepatic IVC. The right hepatic lobe and small bowel are allowed to fall back into place, and attention is then turned to the left side of the liver. The spleen and left lobe of the liver are retracted caudally, and the left triangular ligament is opened from the level of the left hepatic vein to the gastrohepatic ligament. The operator's nondominant hand may be passed under the hepatic hilum to assist in the palpation of vascular structures, such as the right gastric artery. At this point, the right gastric artery may be doubly ligated and cut.

Next, attention is turned to the portal triad structures in the hepatoduodenal ligament, which are dissected as close to the duodenum as possible. The common bile duct is gently dissected but not skeletonized. The portal vein is also dissected, and two heavy silk ligatures are passed around it and secured loosely. An antegrade or retrograde cholecystectomy is then performed, depending on the operator's preference.

The abdominal aorta is prepared for cannulation. The small bowel is retracted cephalad, and the aorta is dissected just proximal to the bifurcation. Two heavy silk ligatures are passed around it and secured loosely in preparation for cannulation. Next, the supraceliac aorta is prepared for cross clamp. The stomach and spleen are retracted caudally, and the abdominal aorta is palpated at a point just distal to the aortic hiatus. The membranous portion of the diaphragm is opened, and the aorta is dissected. A heavy silk ligature is passed around it and secured loosely. The donor is then heparinized (3 mg/kg i.v.).

Two 1-L bags of the cold preservation solution (we use University of Wisconsin solution) are hung and connected to rapid perfusion cannulae, which are then flushed of air and closed. Once the heparin has been circulating for approximately 2–3 min, the abdominal aorta is ligated distally, an arteriotomy is made just distal to the ligature, and one of the perfusion cannulae is introduced into the vessel and secured tightly with the remaining ligature. The portal vein is cannulated in a similar manner. The supraceliac aorta is then ligated. The perfusion cannulae are opened as the diaphragm and the supradiaphragmatic and infrahepatic IVC are incised. The liver effluent is allowed to drain freely into the thoracic cavity, and the liver is surrounded with crushed ice.

Once the liver has been flushed for a few minutes, the common bile duct is transected distally in the hepatic hilum. The hepatic artery is then dissected to its origin at the celiac trunk, the gastroduodenal, splenic, and left gastric arteries being transected distally to their origins in the process. Once a liter of the cold preservation solution has passed through the portal vein, the vein is transected at the level of the venotomy. The remainder of the diaphragm is then transected so that the liver can be extracted. On the backtable, the liver is prepared and placed in cold storage at 4°C for 4 to 5 h.

Recipient Operation

The abdomen is opened with a right-sided "J" incision to ensure adequate exposure to both the supra- and infrahepatic IVC. The liver is

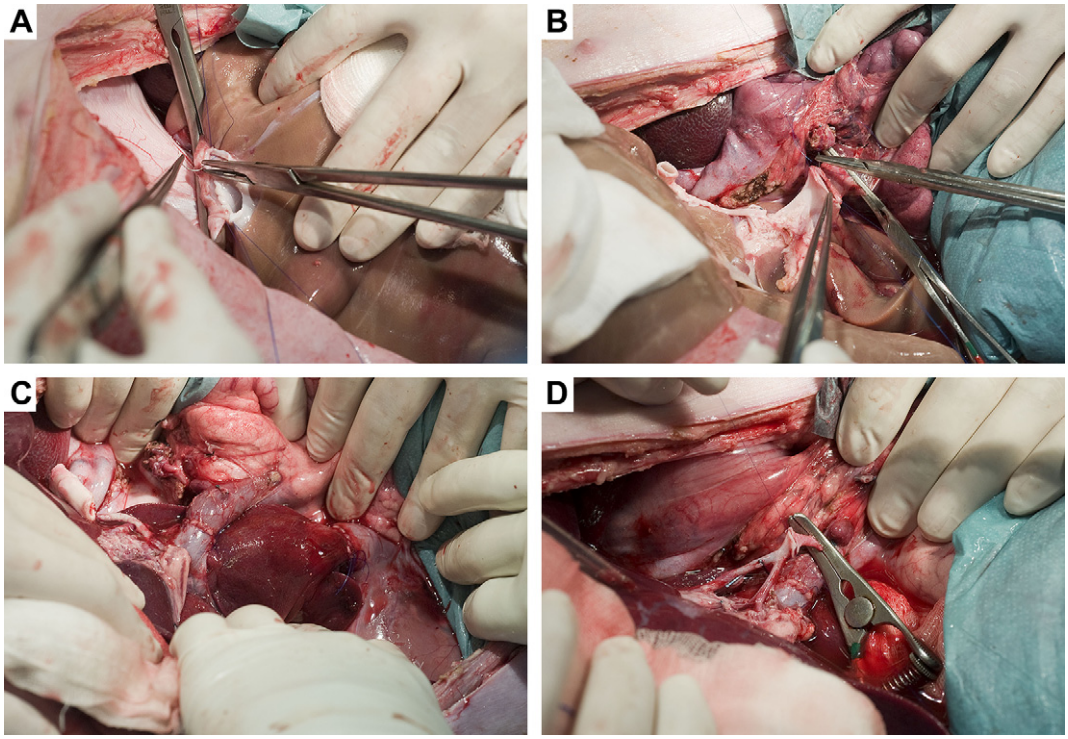

FIG. 1. Suprahepatic IVC (A) and portal vein (B) anastomoses are performed end-to-end with running 4-0 and 5-0 Prolene sutures, respectively. Upon completion of the portal vein anastomosis, the graft is reperfused. Infrahepatic IVC anastomosis is performed in a similar fashion (C). Finally, hepatic artery anastomosis is performed with a running 6-0 Prolene suture using patches of the donor and recipient common hepatic artery (D).

freed from its ligamentous attachments as described in the donor operation. In the hepatic hilum, structures are dissected as close to the liver as possible. The bile duct is doubly ligated and cut. Terminal branches of the hepatic artery (from left to right, cystic artery, right hepatic artery, left hepatic artery, and right gastric artery) are also doubly ligated and cut. (Note: The gastroduodenal artery is left intact, as it will be used later to fashion a patch for the arterial anastomosis.) At this point, the only remaining structure in the hilum is the portal vein, and all other connective tissue, nerves, and lymphatics are cut.

The liver allograft is taken out of cold storage and flushed with 1 L of a warm (37°C) crystalloid solution. The portal vein, infrahepatic IVC, and suprahepatic IVC are then clamped in that order. This marks the start of the anhepatic time. The recipient portal vein and infrahepatic IVC are then cut as far distal as possible, while the suprahepatic IVC is cut as far proximal as possible, initially leaving some of the hepatic parenchyma attached to the vessels.

The suprahepatic IVC is prepared by uniting the hepatic vein orifices, if applicable, and removing any remaining bits of hepatic parenchyma. The suprahepatic IVC anastomosis is performed end-to-end to the suprahepatic IVC of the graft with a running 4-0 polypropylene (Prolene) suture (Fig. 1A). Special care is taken not to "back-wall" any of the stitches placed on the anterior face of the vessel, in order to avoid inadvertent stenosis of the suprahepatic IVC. The donor and recipient portal veins are then cut to appropriate lengths to ensure that there is no redundancy, which could lead to kinking and thrombosis when the liver is allowed to fall back into its natural position. (Note: With the liver retracted cephalad, this anastomosis should barely reach and should feel very tight to the operator.) The portal vein anastomosis is performed end-to-end with a running 5-0 Prolene suture (Fig. 1B). Before the sutures are tied, the portal vein is filled with saline, and the assistant helps the operator tie a 0.5- to 1-cm air knot. This "growth factor," which should be about half of the diameter of the anastomosis, allows the vein to expand when it is fully reperfused and helps avoid stenosis. A clamp is then placed on the donor infrahepatic IVC, the suprahepatic IVC and portal vein clamps are removed, and the graft is reperfused. This marks the end of the anhepatic phase. In all, the anhepatic phase cannot last any longer than 20 min.

Once the graft is reperfused, it is likely that the anesthesiologist with have to administer intravenous boluses due to post-reperfusion hypotension. In the meantime, the infrahepatic IVC is performed end-to-end with a running 4-0 Prolene suture (Fig. 1C).

Next, the hepatic artery is prepared. A patch including the donor gastroduodenal artery is anastomosed to a similar patch including the recipient gastroduodenal artery with a running 6-0 Prolene suture (Fig. 1D).

For the biliary reconstruction, a piece of silastic tube, approximately 2-cm long and 3-mm in diameter, is placed in the open ends the donor and recipient common bile duct and tied in place with a silk ligature (Fig. 2A). The tube is then introduced into the recipient common bile duct and secured in a similar fashion (Fig. 2B). Finally, the two silk ligatures are tied to one another to keep the cut ends of the bile ducts apposed (Fig. 2C and D).

Final hemostasis is performed, and the abdomen is then closed in layers. The pig is given a dose of buprenorphine (0.1 mg i.m.) for analgesia. Anesthesia is then weaned, and the pig is extubated.

Postoperative Management

Recipient pigs are typically followed for a period of up to 5 d: at 3, 6, 12, and 24 h the first day and every 8 h thereafter. They are given free access to water and dry food; those that do not ingest the food or are noted to be hypoglycemic (serum glucose $\leq$ 70 mg/dL) are administered 25–50 g of glucose (250–500 mL of 10% glucose solution i.v. Q8 h, as indicated). Immunosuppression (tacrolimus 0.04 mg/kg i.v. Q24 h, methylprednisolone 125–100–75–50 mg i.v. Q24 h) and antibiotics (cefoxitin 1 g i.v. Q8 h) are given for four days. Buprenorphine

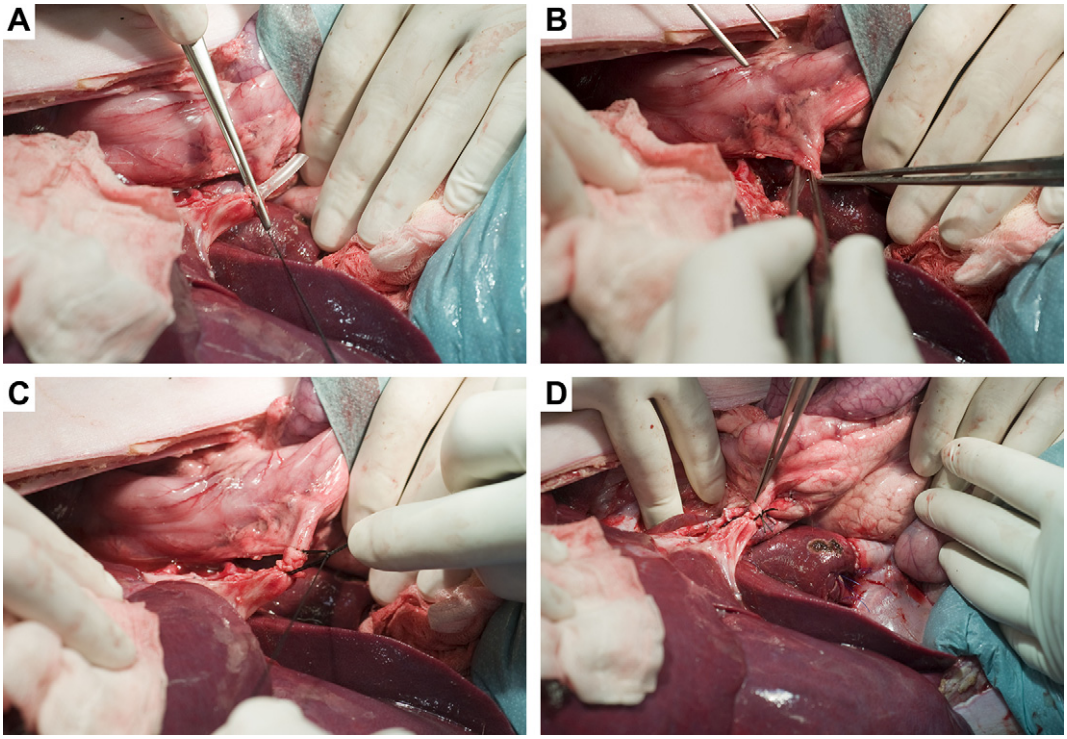

FIG. 2. A simple choledochocholedochostomy is performed. A silastic tube is introduced into the donor common bile duct and secured with a silk ligature (A). The tube is then introduced into the recipient common bile duct and secured in a similar manner (B). Finally, the two ligatures are tied to one another (C), completing the anastomosis (D).

(0.1 mg i.m. Q8 h) is given for analgesia. Animals that survive until the fifth day are re-opened under anesthesia, in order to examine the vascular and biliary anastomoses, and then euthanized with an overdose of anesthesia.

Serum Analysis

In serum samples collected serially during follow-up, levels of aspartate aminotransferase (AST), total bilirubin, and the Quick prothrombin time (QPT) are determined with an automatic analyzer.

Data

Values are expressed as the mean and standard deviation or the median and 25%–75% interquartile range, as appropriate. Continuous variables were analyzed using the Student *t*-test for parametric variables or the Manny-Whitney test for nonparametric variables. A two-tailed P value <0.05 was considered significant.

Ethical and Human Considerations

This experimental transplant protocol has been approved by the Hospital Clinic Institutional Review Board and the University of Barcelona Committee on Ethics in Animal Experimentation.

RESULTS

Since the mid 1990s, several different groups of people in our department have used this porcine model of orthotopic liver transplant. At the time of this writing, the authors of the present manuscript have performed this transplant procedure 130 times, using a total of 260 pigs. All of the 130 transplants were technically successful, i.e., the recipient pig survived the operation and was able to be extubated at the end. Though the basic details of the transplant procedure were the same, the 130 transplants were ultimately performed according to one of four experimental transplant models: standard, whole-liver transplant ($n = 10$), small-for-size liver transplant ($n = 48$) [20], donor after cardiac death (DCD) liver transplant ($n = 44$), and liver transplant following adenoviral gene transfection in the donor ($n = 28$) [19]. For all of the transplant procedures, the average cold ischemic time was 302 ± 43 min and anhepatic time 17 ± 3 min (range 10–22). No incidences of vascular thrombosis, venous outflow obstruction, or bile duct dehiscence were discovered at autopsy or euthanasia in any of the recipients. In all of the 130 transplants, although the donor procedures varied somewhat, the recipient procedure was exactly the same as that described herein. Regardless of the status of the liver graft, based on the fact that the anhepatic period was consistently short, the immediate post-reperfusion systemic hemodynamics were very similar in all cases. Hence, data from the entire 130-animal cohort was used to evaluate immediate pre- and post-reperfusion hemodynamic parameters. During

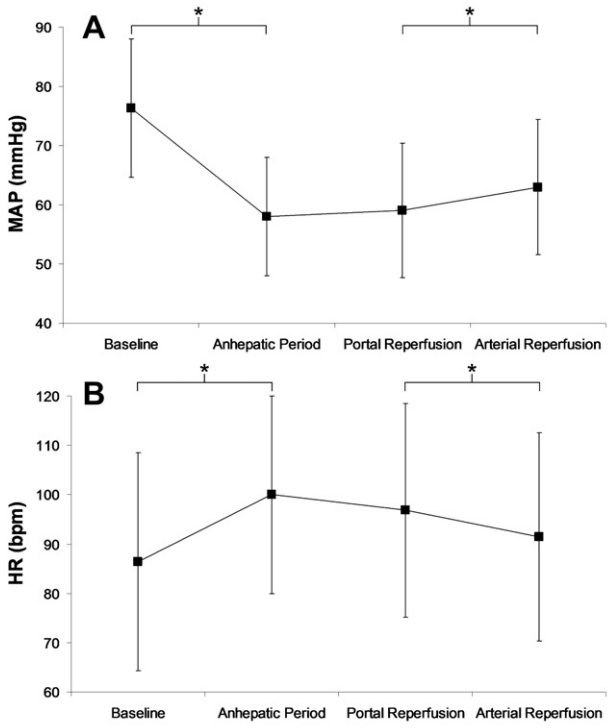

FIG. 3. Mean arterial pressure (MAP) and heart rate (HR) at baseline, after portal reperfusion, and after arterial reperfusion in pigs undergoing orthotopic liver transplant without veno-venous bypass ($n = 130$). The anhepatic time was kept below 20 min. Intravenous fluids were given during and after the anhepatic phase, and no vasoactive substances were used. Though MAP declined and HR increased significantly during the anhepatic phase, both of these parameters were improved by the time the hepatic artery was reperfused. Dots represent the mean and whiskers the standard deviation. *$P < 0.05$.

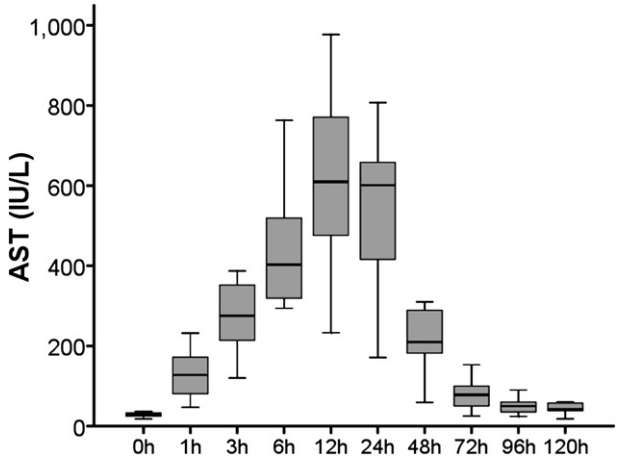

FIG. 4. The postoperative evolution of serum aspartate aminotransferase (AST) in pigs undergoing standard, whole-liver transplant ($n = 10$). Values peaked between 12 and 24 h and returned to normal by the fifth postoperative day. Lines represent the median, bars the 25%–75% interquartile range, and whiskers the 95% confidence interval.

the anhepatic phase, the mean arterial pressure (MAP) was significantly lower than it was at baseline ($P < 0.001$). Immediately after the portal vein was unclamped and the graft was reperfused, MAP remained essentially unchanged from the anhepatic phase. By the time the hepatic artery was reperfused, however, MAP had improved significantly from the post-portal reperfusion value ($P < 0.001$) (Fig. 3A). Heart rate (HR), on the other hand, increased significantly during the anhepatic phase ($P < 0.05$), remained relatively unchanged immediately after portal reperfusion, and decreased significantly by the time the arterial anastomosis was complete ($P < 0.05$) (Fig. 3B).

Recipient mortality was highly dependent on the size and quality of the implanted graft. For the recipients of standard, whole-liver grafts, the 5-d survival rate was 100% (10/10). For the recipients of grafts treated with adenoviral gene transfection, the 4-d survival rate was 71% (20/28). For the recipients of DCD grafts, the 5-d survival rate was 59% (26/44). Finally, for the recipients of SFS grafts, the 5-d survival rate was 35% (17/48). Overall, recipient survival to the end of the follow-up period occurred in 56% of cases.

As reflected by the survival statistics, the recipients of standard, whole-liver grafts had a homogeneous postoperative evolution, whereas the postoperative course varied significantly among animals transplanted according to the three other models (SFS, DCD, and adenoviral gene transfection). Hence, only the recipients of standard, whole-liver grafts were used to evaluate the evolutions of AST, total bilirubin, and QPT during the follow-up period ($n = 10$). Serum AST rose progressively between reperfusion and 12 h, peaked between 12 and 24 h, and subsequently declined, reaching normal preoperative values by the fifth postoperative day (Fig. 4). Similarly, serum bilirubin peaked between 12 and 24 h and then declined subsequently (Fig. 5). QPT declined immediately after reperfusion, reaching a nadir at 1 h, and then progressively rose, reaching normal values by the fourth or fifth day (Fig. 6).

DISCUSSION

In our experience, the porcine model is an excellent model in which to study new techniques and genetic, mechanical, and pharmacological therapies related to liver transplantation. Several models of porcine OLT have already been described in the literature over the course of the past 50 y, including purportedly simplified versions. Nonetheless, even these simplified versions include unnecessarily complex steps, such as complicated vascular and biliary reconstructions [13]. Our model is simple, straightforward, and has been

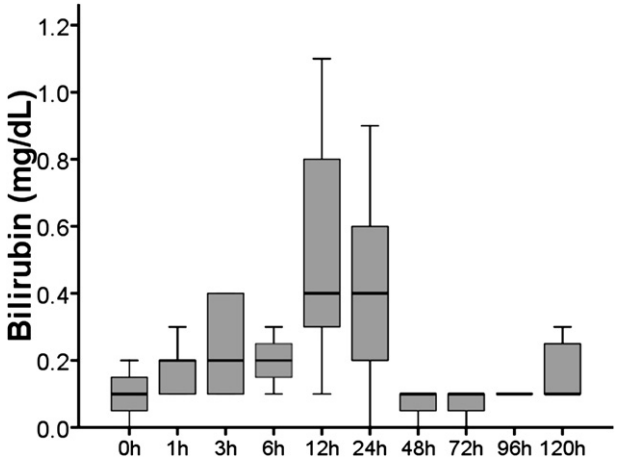

FIG. 5. The postoperative evolution of serum total bilirubin in pigs undergoing standard, whole-liver transplant ($n = 10$). Values peaked between 12 and 24 h and returned to normal thereafter. Lines represent the median, bars the 25%–75% interquartile range, and whiskers the 95% confidence interval.

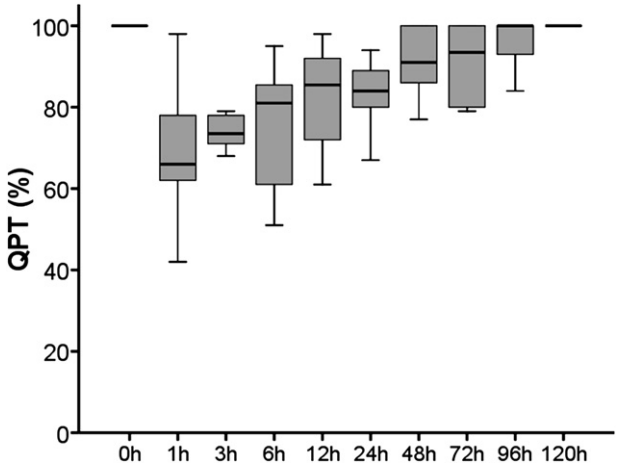

FIG. 6. The postoperative evolution of the Quick prothrombin time (QPT) in pigs undergoing standard, whole-liver transplant ($n = 10$). Values reached a nadir at 1 h and returned to normal between the fourth and fifth postoperative day. Lines represent the median, bars the 25%–75% interquartile range, and whiskers the 95% confidence interval.

replicated by not only different surgeons in our own group but also groups from other centers that have learned our technique [14, 21]. Our objective in this article was to describe the entire procedure, from the induction of anesthesia to the postoperative management, in sufficient detail so that other groups interested in performing preclinical liver transplant studies in a clinically pertinent model might be able to do so.

Some of the factors that we have found key to achieving consistent success in porcine OLT include an appropriate anesthetic management and a short anhepatic period. Pigs are very susceptible to the effects of vasoactive substances, and the administration of even small doses during periods of relative hemodynamic instability may ultimately result in cardiac arrest [22]. In our experience, if the anhepatic time is maintained to be less than 20 min, post-reperfusion hypotension with reflex tachycardia is transient and is adequately treated with volume alone. Furthermore, these hemodynamic alterations are invariably significantly improved by the time the arterial anastomosis is complete approximately 30 min later.

In the pig, the retrohepatic IVC is entirely intrahepatic and unable to be separated from the surrounding hepatic parenchyma. Hence, retrohepatic caval preservation, also known as the "piggyback" technique, is ironically not able to be performed in the porcine model. The retrohepatic IVC must be clamped infra- and suprahepatically and removed in its entirety with the graft. While the IVC is clamped, the circulating blood volume is essentially reduced to the volume in the head and neck, upper extremities, and thoracic cavity, and blood flow to the abdominal organs and the lower extremities becomes increasingly more stagnant. Vascular stasis results in the loss of the substrates needed to fuel the sodium-potassium exchange pump, leading to progressive hyperkalemia and acidosis, which may provoke severe systemic cardiovascular alterations when these ischemic beds are reperfused. Hence, most groups perform porcine OLT using veno–venous bypass in order to minimize the adverse effects of the anhepatic phase. Veno–venous bypass, however, significantly increases the risk of intraoperative complications, such as excess bleeding, hemorrhagic shock, disorders of coagulation, air embolism, and venous thromboembolism [13]. By keeping the anhepatic period short, both severe, irreversible hemodynamic alterations and the use of veno–venous bypass can be avoided.

Based on the fact that anhepatic period needs to be kept as short as possible, the flush of the graft with warm lactated Ringer's solution is performed before rather than after vascular clamping in the recipient, which is different from the clinical setting. In human liver transplant, the warm flush is typically performed immediately prior to the completion of the portal vein anastomosis and graft reperfusion, in order to minimize warm ischemia in the graft. In our model, flushing the graft with the vena cava and portal vein clamped, however, would add several more minutes of splanchnic venous stasis, leading to an even greater release of cytokines and inflammatory mediators upon the restoration of flow.

In the pig, we perform vascular anastomoses as we do in the clinical setting, in an end-to-end fashion using a running polypropylene suture. The cuff technique, which is popular in murine models of liver transplantation, has also been described in large-animal

models of OLT [23]. Although an attractive option based on the decreased level of surgical expertise it theoretically requires, the cuff technique, nonetheless, has not been associated with a shorter anhepatic time, and may furthermore lead to progressive portal stenosis as the recipient pig grows during the period of follow-up [13].

When we initially started performing the biliary anastomosis in the pig, we performed an interrupted choledochocholedochostomy with polydioxanone suture (PDS). However, we subsequently experimented with different techniques and found that the simple anastomosis we describe herein requires a shorter operative time and leads to the same results and no adverse effects for follow-up periods of up to a week. More entailed biliary reconstructions, such as cholecystoduodenostomy [14], cholecystojejunostomy [13], or external biliary drainage [10] only serve to add unnecessary time and complexity to the procedure and are, furthermore, not representative of current clinical practice.

In conclusion, although liver transplantation in pigs may be regarded with trepidation even by experienced human liver surgeons, it does not have to be. By adhering to a few simple principles—keeping the anhepatic phase short and not using veno-venous bypass or vasoactive substances—good outcomes can consistently be achieved, and the liver surgeon or researcher is provided with an invaluable tool to study new therapies and techniques in a clinically relevant transplant model.

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