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Improvement of Liver Transplantation by reducing Preservation - Reperfusion Injury

by

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Abstract

The liver differs from other solid organs in that it has a dual blood supply, receiving arterial blood via the hepatic artery and venous blood via the portal vein. The reperfusion injury which occurs after ischemia, has been studied to only a limited extent in the liver. In particular, the relative contribution of the portal venous blood and the hepatic arterial blood to the reperfusion injury has not been documented previously.

During liver transplantation, implantation of the new liver is achieved by anastomosing the suprahepatic vena cava, the infrahepatic vena cava and the portal vein. At this stage, the liver is reperfused with portal venous blood only. Thereafter the hepatic arterial anastomosis is undertaken. The delay in providing the liver allograft with arterial blood will depend upon the difficulty in the dissection of the hepatic artery. The impact of the delay in rearterialization of the liver allograft has not been studied previously.

Currently, the University of Wisconsin Solution is the gold standard for liver preservation. Celsior is a new cardioplegic solution, which has also been suggested for use for liver preservation. However, its role as a liver preservation solution has been studied to a limited extent.

The aim of this study was:

1. To document the reperfusion injury after liver transplantation.
2. To document the relative contribution of the portal venous blood and the hepatic arterial blood to the reperfusion injury.
3. To investigate the impact of early rearterialization on the reperfusion injury after liver transplantation.
4. To investigate the effect of the new preservation solution, Celsior, on the reperfusion injury after liver transplantation.

Large White X-Landrace pigs were subjected to orthotopic liver transplantation. The donor liver was stored in Eurocollins solution for 3 hours. The animals were randomly allocated to either rearterialization 60 minutes after portal reperfusion, rearterialization 20 minutes after portal reperfusion, simultaneously portal and arterial reperfusion, and rearterialization 20 minutes before portal venous reperfusion.

In another experiment, the donor livers were stored in either Eurocollins solution, University of Wisconsin Solution, or Celsior.

Blood samples were taken at various intervals and subjected to the following biochemical investigations. Malondialdehyde and vitamin A were used as markers of reperfusion injury. Hyaluronic acid levels were used as markers of endothelial cell function. Serum AST was used as a marker of hepatocellular injury.

In summary, these studies showed that there was a significant reperfusion injury after portal venous reperfusion with no additional injury after rearterialization. Early rearterialization also resulted in a lesser reperfusion injury. There was also less hepatocellular injury with early rearterialization. Histological evidence of injury was also less in the livers which were rearterialized early. In addition, the livers preserved in Celsior had evidence of a lesser reperfusion injury. Thus in conclusion, in liver transplantation early rearterialization might result in better early graft function.

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ABBREVIATIONS

ADP	adenosine diphosphate
ALT	alanine aminotransferase
AMP	adenosine monophosphate
AST	aspartate amino transferase
ATP	adenosine triphosphate
C	Celsius
CEL	celsior solution
CVP	central venous pressure
DNA	deoxyribonucleic acid
EC	endothelial cell
ELAM	endothelial leukocyte adhesion molecule
EM	electron microscopy
ESR	electron spin resonance
ET	endothelin
ET-1	endothelin-1
Ha	hyaluronic acid
HA	hepatic artery revascularization
HABP	hyaluronic acid binding protein
H&E	hematoxylin and eosin
HTK	histidine-tryptophane-ketoglutarate
ICAM	intercellular adhesion molecule
ICU	intensive care unit
IFN	interferon
IL	interleukin
LDH	lactate dehydrogenase
LM	light microscopy
MDA	malondialdehyde
LT	leucotriene
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
OLT	orthotopic liver transplantation
PI	preservation injury
PL	phospholipase
PAF	platelet activating factor
PG	prostaglandin
PMN	polymorphonuclear leukocyte
PNF	primary non-function
PV	portal vein revascularization
PRI	preservation-reperfusion injury
ROI	reactive oxygen intermediates
RI	reperfusion injury
SEC	sinusoidal endothelial cell
SLC	sinusoidal lining cell
SOD	superoxide dismutase
TBA	2-thiobarbituric acid
TNF	tumor necrosis factor
TOR	toxic oxygen radicals
UW	university of Wisconsin
XD	xanthine dehydrogenase
XO	xanthine oxygenase

Definitions

Preservation injury (PI):

The injury sustained to parenchymal and reticulo-endothelial cells which occurs from the time of bloodflow arrest in the donor to the time of reperfusion in the recipient.

Reperfusion injury (RI):

The injury which occurs at the time of revascularization of the graft.

Preservation-reperfusion injury (PRI):

The combination of preservation injury and reperfusion injury.

CHAPTER 1

Liver Transplantation - *General Review*

Introduction

In the twenty years after the first reported human liver transplantation by Thomas Starzl [1] in 1963, liver transplants were performed in only 10 centres worldwide, the total number being only 500. In 1993, thirty years after the first liver transplant over 3500 liver transplants were performed in a total of 88 centers [2 3]. It has now become established as the only definitive cure for acute and chronic end-stage liver disease. All other forms of therapy can be described as either disease prevention, prophylaxis or a palliation [4 5].

The increasing numbers of liver transplants being performed have forced both physicians and surgeons to confront the complex and unique clinical problems of the liver transplanted patients [2]. The results of liver transplantation were initially poor. The reasons for this included surgical and technical problems, graft rejection, poor organ function, overwhelming infection and poor organ availability [6 7]. Currently, more than 5000 liver transplants are performed annually [8 9 10 11], with an overall 5 year success rate of approximately 75-80% [12], and generally a good quality of life [13].

History

T E Starzl performed the first human liver transplant in 1963. Initial attempts of experimental liver transplantation were made in dogs and involved the insertion of a second liver as an auxiliary or heterotopic graft [14 15]. It was thought that the remaining function of the failing liver would provide some reserve in the event of poor performance of the transplanted liver. However, results obtained with auxiliary liver transplantation have been consistently inferior to those obtained with orthotopic liver transplantation [16]. Much of the early experimental work with orthotopic liver transplantation was performed by Cannon [17], and subsequently by Starzl and Calne [18 19]. There was initially a high failure rate due to formidable complexities in surgical technique, as well as poor quality of the implanted organ.

The initial results with clinical liver transplantation in Denver, Boston and Paris were poor. A moratorium was declared to allow further research [20 21 22]. Only after the NIH Health Consensus Development Conference in 1983 did liver transplantation progress from an experimental procedure to an accepted mode of therapy for patients with end-stage liver failure. In the 1980's the one-year survival following liver transplantation improved from about 30% to over 70%.

One of the major reasons for the improvement of these results was the introduction of the immuno-suppressant cyclosporin A. Other factors included improved histological monitoring for early signs of graft damage, refinements in the surgical technique, improved anaesthesia, better post-operative monitoring and the introduction of a close working transplantation team.

Current State

Indications for liver transplantation

Indications for liver transplantation in adults are listed in Table 1.1.

I	**IRREVERSIBLE ADVANCED CHRONIC LIVER DISEASE**
	Predominantly Cholestatic disorders
	Primary biliary cirrhosis
	Primary sclerosing cholangitis
	Chronic drug-induced cholestasis and biliary cirrhosis
	Predominantly hepatocellular disease
	Chronic virus-induced liver disease
	Hepatitis B
	Hepatitis non-A, non-B
	Hepatitis C
	Hepatitis D
	Chronic drug-induced liver disease
	Alcoholic liver disease
	Idiopathic autoimmune chronic active hepatitis and cirrhosis
	Wilson's disease
	Congenital hepatic fibrosis
	Predominantly vascular disease
	Budd-Chiari syndrome
	Veno-occlusive disease
II	**FULMINANT HEPATIC FAILURE**
	Viral Hepatitis
	A,B, D, non-A, non-B, Epstein-Barr virus (EBV), others
	Drug-induced liver disease
	Halothane
	Gold
	Disulfiram
	Acetaminophen
	Anti Tuberculosis Therapy
	Quinidine
	Other agents
	Wilson's Disease
III	**HEPATIC MALIGNANCIES THAT ARE NOT RESECTABLE BUT ARE CONFINED TO THE LIVER**
	Hepatocellular carcinoma
	Cholangiocarcinoma
	Rare non-hepatocellular or bile duct tumors that arise within the hepatic parenchyma
	Isolated hepatic metastatic disease
	Carcinoid
	Others
IV	**INHERITED METABOLIC DISORDERS**
	Hemophilia A
	Type II hypercholesterolemia
	Primary hyperoxaluria type 1

Table 1.1 Indications for liver transplantation in adults

The list of indications continues to expand, so that liver transplantation should now be considered in nearly all patients with advanced chronic liver disease and fulminant hepatic failure. Liver diseases for which liver transplantation has been performed can be divided broadly in 4 groups: I) Advanced, irreversible chronic liver failure, II) Fulminant hepatic failure, III) Hepatic malignancies and IV) Inherited metabolic disorders.

Transplantation for inherited disorders, such as type II hypercholesterolemia, in which the metabolic defect resides in the hepatocyte are rarely applicable in adults, but is becoming increasingly important in the treatment of children. In young patients with primary hyperoxaluria type I, successful combined transplantations of the liver and kidney have been performed. The liver graft provides the missing enzyme (hepatic peroxisomal alanine:glyoxalate aminotransferase) and the kidney transplant corrects the irreversible kidney damage from oxalate urolithiasis and nephrocalcinosis. Similarly, transplantation has been used successfully for the treatment of hemophilia A.

One of the major problems in the management of a patient with liver disease is determining the optimal timing of the liver transplant procedure. Clinical and biochemical indications for liver transplantation are listed in Table 1.2

I	**Acute liver failure**
	Serum bilirubin >10-20 mg/dL and increasing
	Prothrombin time >10 seconds above normal and increasing
	Progressive hepatic encephalopathy (coma)
II	**Chronic Liver Disease**
A	Cholestatic liver disease
	Bilirubin > 10-15 mg/dL
	Intractable pruritus
	Malnutrition
	Recurrent cholangitis
B	Hepatocellular liver disease
	Serum albumin < 2.5 g/dL
	Hepatic encephalopathy
	Prothrombin time > 5 seconds above control values and increasing
C	Factors common to both types of liver disease
	Portal hypertension with bleeding from esophagal varices
	Intractable ascites
	Recurrent spontaneous bacterial peritonitis
	Hepatorenal syndrome
	Recurrent episodes of biliary sepsis
	Development of hepatocellular carcinoma

Table 1.2 Clinical and biochemical indications for liver transplantation

The symptomatology of end stage liver disease and the indications for surgery vary depending on the underlying hepatic disease. The most common clinical indications are intractable ascites, severe encephalopathy, variceal hemorrhage, deteriorating nutritional state or diminishing quality of life.

Cholestatic liver disease patients may be transplanted for intractable pruritis, metabolic liver disease and recurrent or uncontrollable cholangitis. Several inborn errors of metabolism may be cured if the transplanted liver corrects the underlying metabolic defect.

Complications of severe hepatic dysfunction, like new-onset ascites, variceal hemorrhage, hepato-renal syndrome, recurrent spontaneous bacterial peritonitis, spontaneous

encephalopathy and sustained severe jaundice are late manifestations of liver disease and represent an urgent consideration for liver transplantation [11 23].

	I ABSOLUTE CONTRAINDICATIONS
A	Active sepsis outside the hepatobiliary tree
B	Malignancy outside the liver
C	Advanced cardiopulmonary disease (except when heart/liver transplant is an option)
D	AIDS
	II RELATIVE CONTRAINDICATIONS
A	Advanced chronic renal failure (except when liver/kidney transplant is an option)
B	Age greater than 60 years
C	Portal vein thrombosis
D	Cholangiocarcinoma
E	Hypoxemia with intrapulmonary right-to-left shunt
F	HB_sAg and HB_eAg positivity
G	Prior portosystemic shunt surgery
H	Prior complex hepatobiliary surgery
I	HIV positivity without clinical AIDS
J	Chronic alcoholism

Table 1.3 Contraindications to liver transplantation

Contraindications to liver transplantation are listed in Table 1.3. At present, the only absolute contraindications to liver transplantation are AIDS, extrahepatic malignancy, septicaemia and severe underlying cardiopulmonary or systemic disease [24 25]. Relative exclusions are prior extensive abdominal surgery, portal vein thrombosis, multicentric hepatoma or hepatoma bigger than 5 cm, cholangiocarcinoma and HB_sAg and HB_eAg positivity [24].

Surgical Technique

The technique of liver transplantation has remained remarkably similar to the original description of orthotopic liver transplantation by Thomas Starzl in 1963. The complete transplant procedure is composed of four main stages [26]:

1. Donor hepatectomy
2. Recipient hepatectomy
3. Implantation of the graft
4. Hemostasis and bile tract reconstruction

Donor hepatectomy

There are two basic techniques of liver harvesting as part of multiple organ retrieval known as the "standard" or traditional technique and the "rapid" technique [27]. Both techniques are usually performed through a complete, midline sternum-splitting and abdominal incision.

After completion of the preliminary dissection, cannulae are inserted into the distal abdominal aorta and portal vein, via either splenic vein, inferior mesenteric vein or superior mesenteric vein, for the infusion of preservation solution. The liver can be pre-cooled by the infusion of cold Ringer's lactate solution through the splenic vein cannula prior to aortic cross-clamping, or the aorta can be cross-clamped without prior pre-cooling.

Cold preservation solution (University of Wisconsin Solution) is then rapidly infused through the aortic and portal cannulae, while the liver is decompressed by dividing the suprahepatic inferior vena cava in the chest. After cooling, the origin of the hepatic arterial supply is detached from the aorta, the portal vein divided and the suprarenal infrahepatic vena cava is transected with part of the right adrenal gland to allow easy identification and ligation of the right adrenal vein at its entry into the retrohepatic inferior vena cava.

The "standard" technique of liver procurement often requires two or even more hours of preliminary and meticulous dissection of the hepatic hilum. This prolonged manipulation of the liver can inadvertently produce a warm ischemic injury to the liver, and furthermore, such a lengthy procedure may not be tolerated by an unstable donor. In the "rapid" technique there is no preliminary dissection except for the encirclement of the proximal aorta and cannulation of the inferior mesenteric vein and distal aorta. Once the cardiac and renal teams are prepared, the aorta is cross-clamped and rapid infusion of ice cold preservation solution through both cannulae is begun. The heart is usually removed at this stage and the liver allowed to completely flush and cool. Hilar dissection is then safely completed in a bloodless field. The liver can often be removed within 30 - 60 minutes. This technique has increased the practicality of harvesting under various adverse circumstances.

Recipient Hepatectomy

Adequate exposure is usually achieved by a bilateral subcostal incision, with an upper T extension and excision of the xiphoid process.

Previous right upper quadrant abdominal operations can render the removal of the recipient liver very demanding and tedious. The recipient hepatectomy usually represents the most difficult stage of the entire liver transplant procedure. It may be impossible to dissect the structures of the hepatic hilum individually because of prior surgery or because of the massive formation of varices.

Veno-venous bypass

The most critical period of the recipient operation is the anhepatic phase when the native liver has been removed and the inferior vena cava and portal veins have been occluded. This obligatory clamping of the splanchnic and infrahepatic systemic venous systems results in a massive sequestration of blood volume in the mesenteric venous circulation and in the systemic venous circulation of the lower body. Sequelae include severe oedema of the whole gastrointestinal tract, renal venous hypertension and an increase in bleeding from the thin-walled venous collaterals. Other complications include volume overload, hemodynamic

instability, cardiac arrythmias and arrest on reperfusion, due to the accumulation of potassium and other toxic substances in the stagnant venous blood.

These problems were overcome by the introduction of the pump-driven veno-venous bypass system (between the left external iliac and the left axillary vein), without systemic heparinization, during the anhepatic phase of the adult recipient operation. This resulted in a significant reduction in morbidity and mortality [28]. It also made liver transplantation an option in patients, previously assessed as too unstable to qualify for the procedure. Because of the possibility of a longer anhepatic phase, more operative options and numerous modifications in the technique of recipient hepatectomy were developed. Once the portal triad has been encircled, its constituent structures can be individually freed with relatively safety. Early placement of the veno-venous bypass greatly facilitates the mobilization of the liver from the hepatic fossa and the dissection of the vena cava. Appropriate vena caval cuffs and sufficient portal vein length must be developed before the graft is brought into the field for anastomosis.

Graft implantation

After adequate haemostasis of the liver bed has been assured, the supra- and infrahepatic vascular cuffs are prepared. It is much easier to deal with small leaks at this stage, rather than at the moment of revascularization.

After the donor liver is positioned in the recipient hepatic bed, the suprahepatic vena caval anastomosis is performed with polypropylene sutures. Thereafter the infrahepatic caval anastomosis is performed. Prior to completion of the anterior side of the suture line, the liver is flushed with a minimum of 250 ml of a "flush out" solution to wash out the high-potassium containing preservation solution and the air entrapped inside the liver.

The portal veno-venous bypass cannula is then removed and the portal vein is trimmed to the appropriate length. The portal venous anastomosis is then performed, and the portal, infrahepatic and suprahepatic vascular clamps removed, and the liver revascularized. After controlling major leaks with interrupted sutures, the veno-venous bypass is interrupted and the remaining cannulae removed. An "expansion" or "growth factor" is used to prevent suture line stenosis, especially for the portal vein and hepatic artery [29]. The donor hepatic artery is anastomosed to the recipient hepatic artery. Reconstruction of the bile duct is achieved by either a choledocho-choledochostomy or a choledocho-jejunostomy with a Roux-Y loop.

Modifications of the surgical technique

(a) Split and reduced-size liver transplantations

Reduced size liver transplantations were developed in response to the shortage of donor graft organs for paediatric patients. Usually the left lateral segments, the left lobe or the right lobe are transplanted. Patient and graft survival are similar to intact liver transplantation [30].

Success with reduced size liver transplantation led to the development of split-liver technique, in which one liver is split into two functioning units and used for two recipients [31]. This

Success with reduced size liver transplantation led to the development of split-liver technique, in which one liver is split into two functioning units and used for two recipients [31]. This technique is far more challenging, since extensive reconstruction of the segmental vessels and biliary system are required. The initial poor results were due primarily to the selection of poor-risk recipients [32]. It is now performed with good results in selected, experienced centers [33 34].

(b) Living related liver transplantation

Recently, living related liver transplantation has been proposed as a possible solution to the liver donor shortage [35 36 37]. Initially introduced for paediatric patients, this technique is now also used in adults. In some countries like Japan, where brain death laws were not established, most orthotopic liver transplants were from living related donors. Unfortunately, one third of potential living related donors are unsuitable either because of liver problems or due to underlying medical conditions. The risk to the donor is low. The one year survival rates for the recipient are excellent and approach 100% in elective cases [35], while results from procedures performed under emergency circumstances remain poor (50-60% one year survival).

(c) Auxiliary liver transplantation

Heterotopic (auxiliary) liver transplantation has been proposed for fulminant hepatic failure, with the ultimate goal to bridge the patient to allow recovery of the native liver [38]. It has also been performed for end-stage chronic liver disease [39].

A recent modification involves partial resection of the native recipient liver and implantation of part of the donor liver in the orthotopic position, ie auxiliary partial orthotopic liver transplantation (APOLT).

The main advantage of auxiliary liver transplantation is that recovery of the native liver function eliminates the need for chronic immunosuppression. The disadvantages include a higher number of vascular complications.

Immunosuppression

Most liver transplant centers currently use a triple-drug regimen (prednisone, either cyclosporine or tacrolimus, and azathioprine). Some centers use induction immunosuppression with antilymphocyte globulins, such as OKT3 (Muromonab-CD3, Orthodone, Ortho Pharmaceuticals, Raritan, NJ) or Atgam (antilymphocyte globulin(equine), Upjohn Co, Kalamazoo, Mich). The reason for antilymphocyte induction is to avoid the potential nephrotoxicity of either cyclosporine or tacrolimus [2].

Results

Up to 1980 results from human liver transplantations were poor. 1-year survival was approximately 30% and survival beyond 30 months approximately 20%. Improvements in surgical techniques and advances in immunosuppression have been the main reasons for improved patient outcome. At present the average 1- and 3- year's survival rates in the United States of America are 77% and 68%, respectively, and 73% and 65% in Europe [2]. Deaths within the first 6 months are caused by primary non-function of the allograft, hepatic artery thrombosis, infection, multi-organ failure or allograft rejection. Late death is commonly caused by atherosclerotic disease or malignancy.

Future

(a) Problems with donor pool

The major concern for the future is the shortage of donor organ availability. Between 1988 and 1995 the waiting list for liver transplantation increased by almost a 1000% [40 41]. This has fuelled major controversies like the limitation of the number of liver transplantation centres, selection criteria for potential transplant recipients and changes in the present allocation system for donor livers [42].

Improved road safety and initial medical care of severely injured patients have both resulted in a reduced number of potential organ donors [43]. Extending the donor pool using non-heart-beating donors seems unfeasible, until new strategies to attenuate warm ischaemic liver injury still have been developed [44 45].

A key issue is whether the available livers should be given to the sickest patients, despite the lower survival rate or should be used for patients with a better chance.

(b) Xenografting

Xeno-transplantation of the liver in both Pittsburgh and Los Angeles has generated considerable interest, although it has to be stressed that so far, xenograft liver transplantation

has not resulted in long-term survival [46] [47] [48] [49]. The perceived immuno-privileged status of the liver might well open the way for xenografting in the future, especially since the xenograft-rejection mechanism is now better understood and can be therapeutically modulated. Strategies to break through the immunological barrier in xeno-transplantation include modulation of xeno-reactive antibodies by inhibiting their binding by either using soluble ligands or inhibiting complement activation. Donor pigs transgenic for human decay accelerating factor showed a significant attenuated complement activation [50]. Xeno transplantation might well be the only answer to the donor shortage in the long-term.

(c) Hepatocyte transplantation

Transplantation of isolated hepatocytes was initiated in 1970 [51] [52]. Hepatic failure is unlikely to respond to this therapy since it requires large amounts of readily available hepatocytes. However success has been reported with this technique in clinical studies during the bridging period in patients with fulminant hepatic failure [53]. For metabolic diseases, however, this technique is promising. Gene therapy can be applied either in-situ by directly coding in the defective liver or portal vein, or ex-situ by gene manipulation of hepatocytes. Recently, several successful experimental studies have been reported using either isolated hepatocytes or hepatocytes encapsulated in semi-permeable hollow fibers [54] [55].

(d) Extracorporeal whole organ perfusion and liver dialysis

The use of extracorporeal liver perfusion to provide support in patients with liver failure was first studied experimentally by Otto et al in 1958 [56]. Human organs were used for extracorporeal liver perfusion by Fox et al [57]. There were also reports describing attempts to use an extracorporeal pig liver perfusion as a bridge to liver transplantation [58]. Early pig liver dysfunction seems to be the limiting factor. Future modulations of this technique may use transgenic porcine liver perfusion to minimize the immunological damage to the liver grafts.

The concept of liver dialysis has been introduced with the bioartificial liver. In this technique hepatocytes are attached to micro-carriers and placed in a bioreactor, through which the patients blood is circulated. Once a semi-permeable membrane is established, animal hepatocytes can be used without rejection [59]. Several experimental successful studies have been recently reported [60], and a phase I clinical trial of a Bioartificial liver (BAL) system has been recently completed, with promising results [61]. Although this technique promises to keep patients with acute hepatic failure alive, it is as unlikely to offer a definitive solution for chronic hepatic failure as renal dialysis offers a solution for chronic renal failure.

CHAPTER 2

Preservation - Reperfusion Injury - *Review*

Introduction

(a) Preservation Injury

In transplantation, there is a significant delay between the harvesting of the donor organ and the subsequent implantation of the organ into the recipient. The donor organ often has to be transported over significant distances to arrive at the most compatible recipient.

During this time, the donor organ is in a severely unphysiological state and is not being perfused. To decrease the metabolism during this phase, the donor organs are stored on ice [62]. Prior to storage the livers are perfused with specially designed preservation solutions to minimize the damage. The liver is eventually removed from its cold environment, located in the recipient's abdomen, and anastomosed. During this phase, it is impossible to maintain the low temperature of the donor-organ and rewarming takes place. Rewarming invariably leads to increased metabolism, with depletion of the energy stores. It takes a substantial time before the liver is reanastomosed and ready for reperfusion. The injury is thus aggravated by a compulsory anaerobic metabolism with accumulation of toxic products. Functional and structural damage during ischaemia is well documented [63 64 65 66 67 68 69 70 71 72 73 74 75]. This injury is commonly known as the preservation injury.

(b) Reperfusion Injury

It is now well established that reoxygenation of hypoxic tissue can lead to a sequence of events, which is similar to the consequences of prolonged hypoxia, and includes cell injury and necrosis [76]. In fact the injury elicited by reperfusion can be more severe than the injury induced by the ischaemia per se [77]. Although the exact mechanism is still unknown and is most likely to be multifactorial, the tissue injury at reperfusion seems to be mediated by reactive metabolites of molecular oxygen [78 79]. Ischaemic tissues must be exposed to molecular oxygen on reperfusion to manifest tissue injury [80]. Reperfusion therefore seems to initiate a series of cytotoxic events that are associated with the return of oxygenated blood.

There has been an increasing interest in the preservation-reperfusion injury of the transplanted liver, in order to gain further insight into the complicated mechanisms of transplantation, as well as improving the results of liver transplantation. Primary non-function of the transplanted liver still occurs at an unacceptably high rate (up to 20%) and continues to

be a major cause of death after transplantation [81] [82] [83]. There is also clinical evidence indicating that a severe reperfusion injury is associated with an increased incidence of liver graft rejection [84].

HISTORY

(a) Hypothermia

It was recognised early on that the normothermic liver is extremely sensitive to hypoxia [15]. Complete anoxia of only 30 minutes renders the normothermic liver totally unsuitable for transplantation. In 1960, hypothermia was induced by means of whole-body cooling of the donor to 30°C, after which the excised liver was perfused with cold (4°C) Ringers' lactate solution. In this way, the liver could be cooled down to approximately 15°C. These livers were able to sustain life in the recipient dogs, if transplanted within 2 hours [19]. Longer preservation times led to a higher rate of acute failure, due to outflow block of the livers, haemorrhagic diathesis and acute liver failure.

New developments in organ preservation were quickly interchanged between livers and kidneys. Cold preservation solution infusion into the liver rapidly became standard practice for renal cooling [85].

Attempts to cool livers down to -60°C to preserve the liver for longer periods were less successful [86] [87].

(b) Perfusion Techniques

It was also recognised early on that perfusion of the grafts could significantly reduce the time periods of cooling as well as rewarming [88]. Techniques were developed to perfuse the liver in-situ with an extracorporeal heart-lung machine [89] or perfusion of the liver in-situ selectively [90]. The first 15 human liver transplants were all revascularized within 3½ hours after the donor death [91] [92]. In 1967, prolonged storage (24 hours or more) of kidney as well as liver grafts was demonstrated using the technique of extra corporal hypothermia pulsatile perfusion [93] [94]. The results with liver preservation were very variable, most likely due to variations in type and length of the preservation, composition of the perfusion-solution, the applied technique (intermittent, continuous, pulsatile, non-pulsatile) and of course, the individual response of the liver graft to such injury [42].

(c)Preservation Solutions

In 1969 [95], Collins described successful canine kidney preservation with a similar technique of initial perfusion, followed by a 30-hour period of ice-storage. This was adopted for liver transplantation by the Denver/Pittsburgh group [96]. The method of initial perfusion followed by simple hypothermic storage was gradually adopted as the best method.

In 1971, successful experimental liver transplants were reported after simple storage of the graft for 6-8 hours, using balanced salt solutions, to which dextrose was added [97] [98]. Several new preservation solutions were developed, but since the storage time hardly exceeded a 12 hour period, no clear differences in results could be demonstrated [96]. However, in longer preservation times (18 hour storage), there was a clear advantage of Collins' solution over Ringer's lactate, as reported by Benichou et al [96].

The same authors also reported on their clinical experience with human liver preservation, using modified Collins' solution. Subsequently, the role of the modified Collins' solution, or its more modern derivative Euro-Collins, was established, and most centres used it until the introduction of the University of Wisconsin solution in 1988 [99].

Preservation Injury

Pre-preservation Component

The pre-preservation period is defined as the time between injury to the donor and the perfusion of the graft with ice-cold preservation solution. There are numerous donor factors, which can play a major role in the ultimate success of the liver transplantation.

(a) Demographics

Several donor factors have been identified as having a negative effect on the ultimate outcome. The extremes of age have a negative impact on the results of liver transplantation. A prolonged hospital stay, elevated bilirubin and death from a traumatic head injury are also correlated with poor outcome [100]. A prolonged donor stay in the intensive care unit (more than 5 days) should be carefully evaluated, especially if there are other relative risk factors present.

The liver might also be injured subclinically by substance-abuse. Drugs as well as alcohol might have been consumed by the donor chronically or in high amounts just before the event of death. Any illness or injury leading to brain death, such as hypotensive episodes, hypoxia or anaemia, might also result in an injury to the liver.

(b) Metabolic Changes in the Donor

Brain death is associated with profound endocrine alterations, which can lead to hemodynamic instability and other complex metabolic disturbances [101] [102] [103] [104]. Eighty per cent of donors develop diabetes insipidus [105] due to decreased secretion of vasopressin from the posterior pituitary, none of the anterior pituitary hormones show a decline in concentration [103] [106]. An example of endocrine pathology induced in this phase is the euthyroid sick sinus syndrome [101] [107].

The nutritional state of the transplanted liver is also a crucial factor. Glycogen stored in the liver is a substrate for anaerobic glycogenolysis as well as glycolysis, and might protect against ischaemia[108]. Therefore, depleted glycogen stores are likely to predispose the transplanted liver to an increased preservation injury [109 110 111 112]. Livers from fed rats are more resistant to ischemic damage than livers from fasted rats. Experimental studies show that this beneficial effect can be achieved directly, via a higher level of adenosine triphosphate [113], or indirectly, via lowering the intracellular pH as a result of a higher level of lactic acid [114 115 116]. These studies have been confirmed in humans [117 118]. Preservation-reperfusion injury has been diminished in experimental studies in rats, rabbits and pigs, by nutritionally replenishing the liver before transplantation [112 119]. Protection against warm ischaemia has also been shown with nutritional replenishment [120].

Fatty infiltration, which can be found in approximately 20% of donor livers, has a significant impact on the outcome of the transplant [82 121]. The aetiology and natural course of hepatic steatosis has been well described [122 123]. Although certain histological changes have been identified with prolonged preservation times, no objective data for not transplanting livers with hepatic steatosis are available. Several anecdotal reports have described primary non-function of livers with fatty infiltration [124 125], and it has been suggested [126 127 128] that less than 60% fatty cells is acceptable. Hepatic steatosis related to total parenteral nutrition or alcoholic hepatitis [129 130] represents a higher risk for the recipient.

Not all steatosis is related to pre-existing disorders. There is a correlation between physiological stress and steatosis [131 132]. This might indicate that the cause of steatosis is related to events occurring just before and during the phase leading to brain death in the donor.

The exact mechanism of hepatocellular damage by steatosis is unknown. Cooling of the liver might solidify triglyceride droplets resulting in hepatocyte rupture. Extracellular coalescence of fat globules might further disturb the liver architecture and microcirculation, initiating an inflammatory response and resulting in lipid peroxidation [122 124]. Development of strategies to reduce hepatic steatosis in the liver graft could expand the donor pool as well as lead to an improved initial graft function.

Another potential source of injury to the liver graft is the exposure of the organ to circulatory toxins. These can be either chemical or organic. Endotoxemia is a common event in the terminal patient and can injure the liver significantly [133 134 135].

Cold Preservation Injury

The cold preservation injury is the injury which occurs from the time of perfusion with cold preservation solution until the moment the liver is removed from the preservation solution. Hypothermia slows the metabolism, reduces tissue energy requirements and waste production. Cold anoxia, however, induces numerous destructive processes, including energy depletion, pH changes due to anaerobic glycolyses, and electrolyte and water movement across the cell membrane secondary to inhibition of the Na/K ATPase-dependent pump.[66136]

(a)Sinusoidal Lining Cell Injury

During the cold preservation period the sinusoidal lining cells are the first cells to be affected. Hypothermia usually results in injury to all cells, irrespective of their origin [137]. There is however substantial evidence to indicate that in the liver the injury to the sinusoidal cells is of more importance than the injury to the parenchymal cells [138 139 140]. Sinusoidal lining cell injury was first demonstrated in 1984 by means of electron microscopy [141]. The typical sequence of progressive sinusoidal cell injury, as demonstrated by electron microscopy, is swelling, fenestration and eventual detachment of the substratum [142 143 144].

(b)Anaerobic Metabolism

In aerobic cells, the cell integrity is maintained by energy supplied by the mitochondrial cytochrome system using reduction of oxygen to water. ATP is generated in this process, and stored in the cell for use at a later stage. This oxidative phosphorylation ceases at the arrest of oxygen supply. During cold preservation ATP-stores are rapidly exhausted and since ATP is crucial to nearly all energy depending functions of the cell, the ATP shortage quickly leads to impairment of cell functions, progressive cell injury and eventually to cell death [65]. Although the exact pathway from ATP shortage to ultimate cell death is unknown, several morphological changes are well described. These include loss of microvilli and "blebbing". "Blebbing" of the cell surface was first described in 1987 [145]. Rupture of a terminal cell surface bleb seems to be associated with the progression from reversible to irreversible injury, and is thought to be the result of disruption of the cytoskeleton [145]. The F-actin structure, which maintains the cytoskeleton of the cell becomes deranged due to the loss of ATP as an energy substrate. G-actin forms by means of depolymerization and is most likely the initial event in endothelial cell injury [146 147].

(c)The Cytoskeleton

The whole microfilamental and microtubule network, which forms the base of the cytoskeleton, is permanently in an energy requiring, dynamic state of continuous formation and disassembly. It is thus likely to be affected instantly if ATP shortages occur [65]. The rapid loss of energy sources during cooling is therefore likely to affect the normal cytoskeleton. Rounding of endothelial cells has been documented in short cold-stored livers, whereas prolonged periods of cold ischaemia can lead to a total endothelial denudation [143].

Due to absence of the collagenous basement membrane between parenchymal and endothelial layers in the liver [148], retraction of cytoplasmic processes can expose hepatocyte microvilli direct to the sinusoidal lumen [149 150].

Several groups have shown that the majority of sinusoidal lining cells are still alive at the end of the cold storage period [151 152]. Although delayed death can occur after reoxygenation, 93% of the sinusoidal cells remained alive after transplantation and were even able to retract to the substratum [119]. Cold preservation injury with subsequent cell death does not occur secondary to a metabolic derangement, but occurs when the endothelial cells undergo cytoskeleton alteration and lose their substratum connections. Endothelial cell death, therefore, *per se* is not a prerequisite for cold preservation injury. Prolonged periods of cold storage invariably lead to endothelial cell death. However, reperfusion after short periods of cold storage, will supply ATP for the energy dependent cytoskeletal reorganizations with endothelial cell spreading, migration and regeneration.

(d)Biochemical Evidence for the Role of Sinusoidal Lining Cells

Besides the morphological changes described above, there is also biochemical evidence for the role of the sinusoidal lining cells in cold preservation injury. The iso-enzyme creatine kinase BB is solely released by Kupffer cells and hepatic endothelial cells. The length of cold preservation has been shown to correlate with the severity of the endothelial cell damage and creatine kinase BB levels [153 154]. Furthermore, hepatic endothelial cells are the only endothelial cells which can metabolize hyaluronic acid (Ha) and Ha levels have been used as a sensitive indication of hepatic endothelial cell function [155]. An inverse correlation has been shown between Ha uptake and the duration of the cold preservation and the severity of endothelial cell injury [156 157]. Ha levels have also been used as a marker for hepatic endothelial cell function after transplantation [158 159 160].

Rewarming injury

Rewarming injury occurs during the period when the allograft is removed from the preservation solution and implanted into the recipient prior to reperfusion. This is referred to as the second warm ischaemic injury. In liver transplantation, this period lasts approximately from 30 to 90 minutes and has been shown to be an important cause of hepatocellular injury [66 117 161 162 163]. With prolonged rewarming there is an exponential increase in graft injury. In an experimental study, all liver transplants with a warm ischaemia time of longer than 90 minutes resulted in death[44]. Specific morphological changes occur in the liver during warm ischaemia, and include a decrease in the number of hepatocyte microvilli, cytoplasm vacuolization and partial plasma membrane rupture [164]. This is followed by mitochondrial swelling and displacement of the endoplasmatic reticulum fragments and ribosomes. After 2 hours, denaturation and dislocation of cell macromolecules in the hepatocytes takes place[165]. The development of surface "blebs" and loss of microvilli has been associated with the transition of reversible into irreversible ischemic damage [65 166 167 168]. Breakdown of bleb-membranes is

thought to result in cell membrane damage and perforation, leading to an irreversible permeability of organic cations and anions.

The exact nature of rewarming and warm ischaemia is not fully understood. It is likely that an increase in temperature will lead to an increase in anaerobic metabolism, and subsequent loss of energy substrates [169]. It has been demonstrated experimentally that a glycogen-repleted donor liver will have used approximately 70% of its glycogen stores after 1 hour of warm ischaemia [170]. Several studies have indicated that enhanced ATP synthesis and prompt recovery of energy charge after revascularization are prerequisites for normalization of metabolic liver function [171 172 173]. It has also been suggested that normalization of energy charge indirectly reflects the maintenance of mitochondrial integrity during ischaemia which is essential for graft viability [44 174].

Reperfusion Injury

Introduction

In the classic reperfusion injury, the severity of the injury is directly related to the duration and severity of the cold ischaemia as well as the duration of the second warm ischaemic period. Thus, during reperfusion, the injury, which has germinated during the ischaemic hypothermia and the ischaemic rewarming phase is first expressed in full scale. The mediators responsible for the evolution of reperfusion injury can only be produced in sufficient quantity when reperfusion is achieved with oxygenated blood. The severity of the reperfusion injury has been demonstrated to be directly dependent on the severity and duration of the hypothermic period [112 139 175 176 177 178]. Furthermore, there exists a certain point following prolonged (cold) ischaemia, after which the tissues are irreversibly damaged. Subsequent reperfusion is unable to inflict more damage to the tissue.(see Figure 1)

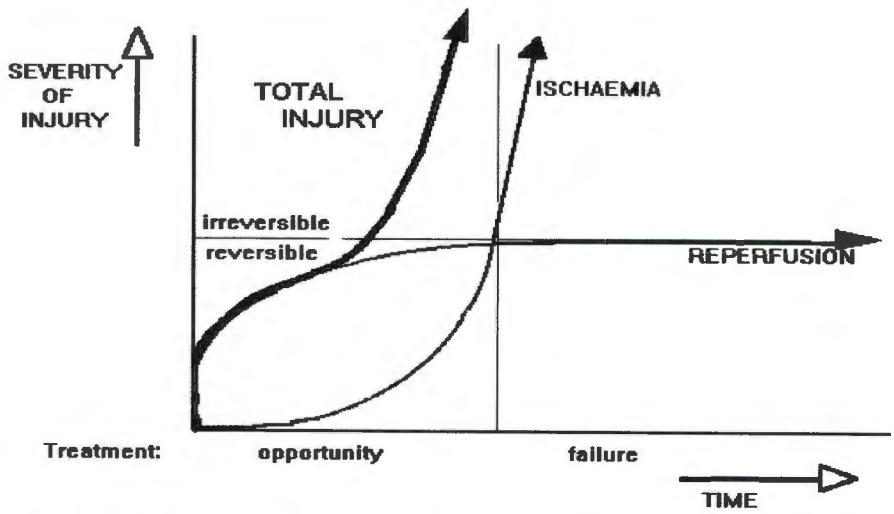


Figure 1. **Diagram of the course of a typical reperfusion injury: at a certain point reperfusion injury can not exceed the ischaemic injury any more.**

The classical understanding of the reperfusion injury is that it is caused by reoxygenation of the ischaemic donor organ. The process is further complicated by a variety of allo-immune reactions between the donor organ and the recipient.

The Role of Various Cell Types in Reperfusion Injury

Hypothermia causes injury to all cell lines. However, several specific cells have been reported to be crucial for the initiation and progression of reperfusion injury. Unlike other solid organs, the parenchymal cells in the liver seem to play only a minor role in PRI. Otto and McKeown [141] [179], were the first to realize the importance of the morphological changes of the sinusoidal lining cells during hypothermia, and since then this has been at the centre of reperfusion research. Other researchers have stressed the role of the Kupffer cells [177] [180] [181] [182] [183], leukocytes and platelets [176] [184] [185] [186]. Although all these cell lines do play a role in the ultimate reperfusion injury, the most likely mechanism of preservation-reperfusion injury is a complicated multifactorial process, occurring in the hepato-sinusoidal complex. The various mechanisms do not follow the easy laws of cause-and-event, but a complicated interplay exists with potentiating of effects.

(a)Sinusoidal lining cells

The importance of the sinusoidal lining cells (SLC) in the pathogenesis of reperfusion injury has been apparent for many years [141] [179]. The major changes in these cells are initiated during the cold ischaemic period. A detailed description of the changes occurring in the SLC can be found on page 14.

(b)Kupffer Cells

Approximately 70% of all reticulo-endothelial cells reside in the liver. These liver macrophages are commonly known as Kupffer cells and represent approximately 30% of all sinusoidal lining cells [187]. At rest, Kupffer cells are flattened and spread out over the endothelial cells to form the inner sinusoidal lining [180] [188]. After cold preservation, these cells increase in volume, with increased vacuolization, increased granular formation inside the vacuoles and polarization of granules towards the luminal surface [177] [178]. Identical changes are noted in macrophage activation [180] [188]. These changes are followed by the release of lysosomal enzymes into the intra-vascular compartment. Evidence for a crucial role of the Kupffer cells in reperfusion injury was reported by Thurman et al.[189] [190] [191], who found that the blockade of the Kupffer cells with either methylpalmitate or nisoldipine resulted in a significant increase in graft survival. They demonstrated activation of Kupffer cells on reperfusion in a low-flow reflow perfusion model. Although it is now well established that Kupffer cells do play a major role in the pathophysiology of preservation-reperfusion injury [177] [180] [192], it remains unclear if this role is primary or secondary [180] [181] [182] [188] [189] [190] [191]. It has been noted in several studies that changes in the endothelial cells precede those in Kupffer cells. It has therefore been assumed that Kupffer cell changes are secondary to endothelial cell damage. Moreover,

Kupffer cell activation occurs usually only after a considerable time (approximately 30 minutes) after reperfusion, which also precludes a primary role [177 193].

Inhibition of the reactive oxygen intermediate production of Kupffer cells does not ameliorate the sinusoidal lining cell injury produced by preservation-reperfusion injury [194]. Initially, nearly all studies concerning the role of the Kupffer cell in preservation-reperfusion-injury (PRI) of the liver were based on morphological signs of the Kupffer cell activation. It has to be stressed that at the time these changes occur, the sinusoidal endothelial cells (SEC) changes have already taken place a substantial time before.

The majority of studies reporting improved graft survival after Kupffer cell blockade with various substances such as nisoldipine, pentoxifylline, adenosine and prostaglandin E, do not refer to several other known and possible unknown effects of these substances, which might well be a major factor in reducing the PRI. Nisoldipine, a dihydro-pyridine calcium channel blocker, has been shown to decrease Kupffer cell activation if added to the preservation solution [195]. Kupffer cell activation might be the consequence of an increase in calcium during the preservation-reperfusion period. However, calcium is known not to be the major factor in hypoxic cell damage.

Like all other macrophages, Kupffer cells synthesize and release a large variety of inflammatory mediators, such as reactive oxygen intermediates (ROI), leucotrienes, cytokines, hydrolytic exzymes, chemotactic substances and tumour necrosis factor alfa (TNFα) [196 197 198 199 200 201]. TNFα, amongst other functions, controls the systemic response to endotoxins and sepsis and also mediates pulmonary leucocyte infiltration after hepatic ischaemia and reperfusion [202 203]. Endotoxin, or lipopolysaccharide (LPS), is able to strongly stimulate TNFα production by Kupffer cells. A number of agents that suppress LPS stimulated TNFα formation by Kupffer cells, also improve graft survival after PRI in experimental models [193]. Nisoldipine, adenosine, pentoxifylline and prostaglandin E all belong to this category. Suppression of Kupffer cell release of TNFα and other proinflammatory cytokines is the possible working mechanism for the beneficial effect of graft recipient treatment with prostaglandin E in clinical liver transplantation and pentoxifyllin in experimental models [81 204 205].

Other mechanisms through which Kupffer cells might influence the severity of PRI, are secretion of leucocyte and macrophage chemotactic and stimulatory substances [196 206] and stimulation of pro-coagulant activity [207]. TNFα might further initiate expansion and synthesis of intercellular adhesion molecules (ICAM's) mediating an increase in leucocyte-sticking [208 209 210].

All these effects result in a major disturbance of the hepatic microcirculation. Kupffer cells also influence hepatocyte function independent of the microcirculatory changes, via formation of glycoprotein, lymphokines and growth factors [188]. Kupffer cell injury might therefore result in a decreased recovery of hepatic parenchymal cells after PRI. In addition, Kupffer cells have also been shown to play an important role in liver regeneration.

Mediators produced by Kupffer cells, and released into the systemic circulation, might contribute significantly to the development of multiple organ failure associated with primary nonfunction of the transplanted liver [201]. Several other Kupffer cell inhibitors, including gandolinium [139 206 211 212] and latex particles [192], have been shown to improve the outcome after PRI, although the exact underlying mechanism has not yet been clarified. The complexity of reactions taking place in the hepatic microcirculation after ischaemia make it unlikely that a single factor is responsible for severity of PRI.

(c)Leucocytes

There are numerous studies about the role of leucocytes in the pathobiology of PRI [80]. Leukocytes are thought to play a major role in PRI. This view is based largely on three lines of evidence: (a) Granulocytes accumulate in the tissues exposed to ischaemia and reperfusion, (b) Depletion of circulating neutrophils significantly reduces the microvascular and parenchymal cell dysfunction associated with PRI and (c) agents that prevent neutrophil activation of leucocyte-endothelial cell adhesion effectively decrease PRI induced tissue injury.

Adhesion of leucocytes on reperfusion suggests that the sinusoidal lining has been altered. Since postischaemic tissues generate large quantities of leucotriene B_4 (LTB_4), platelet activating factor (PAF), C5a and numerous other chemo-attractants, it is of little surprise that these tissues are infiltrated with neutrophils. Lymphocyte adherence to sinusoids occurs within 10 minutes after reperfusion of cold preserved rat livers, and the degree of adherence is related to the duration and intensity of the cold ischaemia [66 176]. Using intra-vital video microscopy, it is possible to quantify the leucocyte-endothelial cell adhesive interactions. After 45 minutes of cold ischaemia approximately 40% of circulating lymphocytes adhere to the sinusoids, while after 30 hours of cold storage, this number is increased to more than 80% [213 214 215].

The leucocyte adherence is also dependent on the grade of inflow occlusion. In low-flow ischaemic models, the adherence is 4-10 fold increased, while in complete arterial occlusion models the adherence is increased 45 fold [80 216]. Several studies have shown a correlation between neutrophil adhesion in the hepatic sinusoids and primary graft function [215 217] in clinical liver transplantation, as well as a correlation between leucocyte adhesion and the extent of the preservation injury [154].

(i)Leucocyte Trapping

Although the leucocyte-endothelial cell adhesion seems to be the major function responsible for leucocyte sequestration, the physical restriction or trapping of leucocytes within capillaries may significantly contribute to the leucocyte accumulation observed in the postischaemic liver [80218 219 220]. Entrapped leucocytes occlude the capillary lumen, and leucocyte-capillary plugging will contribute to the development of the "no-reflow" phenomenon in postischaemic tissues.

There appears to be a strong correlation between the percentage of no-reflow capillaries and the percentage of capillaries containing granulocytes.

This leucocyte-trapping is due to either ischaemia induced leucocyte deformability and / or endothelial cell swelling [221] [222] [223]. Additional factors might be the accumulation of interstitial edema, compressing the microstructure and thereby impeding the movement of blood elements within the capillaries [222] [224]. Compelling evidence for these theories is given by studies which either render the recipient neutropenic [225] [226], or studies in which the recipient receives antibodies affecting the leucocyte-endothelial cell adhesion [226] [227] [228]. In both groups the no-reflow phenomenon is virtually abolished.

Another mechanism of leucocyte accumulation in the postischaemic tissues is a low shear rate, which favours leucocyte-endothelial cell adhesion. Several studies show an increase in the number of rolling and adherent leucocytes if the shear rate was reduced [229] [230] [231]. Adherence of leucocytes by inflammatory mediators is also greatly enhanced by a lower shear rate. Electrostatic cell surface charges can also play a role in leucocyte adherence. Cationic proteins such a lactoferrin and elastase released from activated neutrophils are known to promote neutrophil adhesion [232].

(ii)Adhesion Molecules

It has been well established that the adhesion of leucocytes to endothelial cells is mediated by various coordinately regulated adhesion glycoproteins expressed on the surface of leucocytes, as well as on the surface of endothelial cells [222] [233] [234]. The CD_{11} / CD_{18} glycoprotein complex is rapidly (within 2 minutes) expressed on neutrophils after exposure to inflammatory mediators, like LTB_4 and PAF. L-Selectin, another leucocyte adhesion molecule, is involved in the weak adhesive interactions, manifested as leucocyte-rolling [235].

P- and E-Selectin are adhesion molecules found on activated endothelial cells. These molecules are rapidly mobilized to the cell surface in response to specific stimuli. Histamine, thrombin, and hydrogen peroxide lead to the expression of P-selectin in a few minutes, while E-selectin is only expressed several hours after being induced by cytokines.

Both selectins interact with distinct oligo-saccharides on leucocytes to promote "rolling". Cytokines also induce an increased expression of the endothelial ICAM-1 adhesion molecule. Interaction between endothelial ICAM-1 and leucocyte-CD_{11}/CD_{18} appears to mediate firm adhesion and emigration of leucocytes in post-capillary venules [236]. Several experimental studies have shown a reduction of up to 95% of reperfusion induced leucocyte adherence and leukocyte emigration, after pretreatment with monoclonal antibodies against the adhesion molecules on leucocytes, and up to 50% with monoclonal antibodies against adhesion molecules on the endothelial cells [237].

(iii)Monoclonal antibodies against Adhesion Molecules

The critical role of leucocyte adhesion in PRI is most clearly demonstrated by protection against microvascular and parenchymal cell dysfunction or necrosis by administration of monoclonal antibodies directed against adhesion molecules [238]. Monoclonal antibodies

against leucocyte adhesion molecules appear to offer better protection than antibodies directed at the endothelial adhesion molecules [239]. Although clinical experience is limited, beneficial effects of monoclonal antibodies to ICAM-1 have been reported [240 241].

Reactive oxygen intermediates (R.O.I's) are released by endothelial cells, as well as activated leucocytes, and are able to induce an increase in leucocyte adhesion. Exposure of tissue to exogenous hydrogen peroxide (H_2O_2) or superoxide (O_2), promotes leucocyte-endothelial cell adhesion [236 242 243]. The latter effect of H_2O_2 seems to be mediated via the formation of PAF, while in the superoxide-mediated leucocyte adherence, it seems that nitric oxide might be an important factor in the intermediate phase. Nitric oxide is normally produced in the endothelium, and might protect against ROI mediated PRI [244]. Damaged endothelial cells might produce insufficient nitric oxide and therefore cause an increase in cell adhesion.

It has to be stressed that although adhesion of leucocytes in itself might cause damage due to obstruction and induction of the no-reflow phenomenon, the subsequent activation of adhered leucocytes is the most likely cause of a greatly amplified inflammatory response [245 246 247]. Recent research provides evidence that neutrophil-mediated injury to endothelial cells involves ROI and protease. While ROI's appear to be responsible for the initial injury, proteases seem to be active after this initial period and for a longer duration [246]. The adherence of leukocytes is a prerequisite for the endothelial injury by leucocytes. Adhesion creates an altered micro-environment within the hepatic sinusoids, in which ROI and proteases reach a high concentration and can have their maximum injurious effect.

The neutrophils responsible for the reperfusion injury seem to accumulate predominantly in the sinusoids and in the post-sinusoidal venules. After ischaemia and reperfusion this results in immediate microcirculatory changes. Recent, reports indicate that this microcirculatory disarrangement might be associated with the subsequent permanent hepatocyte damage and mortality [248 249 250].

(d)Platelet & Procoagulant activity

(i)Platelets

Besides leucocytes, platelets also adhere to the hepatic sinusoids immediately after reperfusion of cold preserved livers, and the degree of platelet adherence correlates with the degree of preservation injury [66 186]. Adherence occurs even after heparinization of the perfusate, indicating that the adherence is not dependent on a hypercoaguable state. The beneficial effects of hepatic glycogenation might also be mediated via the mechanism of platelet trapping [119]. In experimental studies, animals fed with glucose preoperatively, showed significantly less platelet trapping than starved animals. Although there are no human studies, recent experimental studies have shown that at least part of the PRI is the result of platelet adhesion to sinusoidal endothelial cells [251]. Furthermore, a significant decrease in the extent of liver injury has been reported after pretreatment of livers and platelets with PGE1, a platelet adhesion inhibitor [251].

(ii)Clotting and Coagulation Factors

Several studies have demonstrated an effect of hypoxia on procoagulant activity [252]. Hypoxia causes suppression of thrombomodulin, as well as synthesis of membrane-associated factor X, which promotes coagulation [253]. Both leukocyte and platelet adherence indicate an alteration of the SLC surface.

Leucocytes secrete heparinase which degrade heparan sulfate [254 255]. Release into the circulation of graft proteases upon reperfusion might further activate plasma factors [256]. Activation of PAF, either by Kupffer cells, leucocytes and/or platelets may further induce platelet adhesion [257] and enhance the procoagulant environment. Although the exact relevance of these mechanisms still have to be elucidated in liver transplantation, a hyper-coagulable state does occur during and after liver transplantation. Fibrin deposition in hepatic sinusoids has been associated with severe primary non-function [66 258].

The Role of Various Mediators in Reperfusion Injury

Several important mediators have been shown to play a role in this complex preservation-reperfusion injury.

(a)Reactive Oxygen Intermediates

The term reactive oxygen intermediate (ROI) refers to any compound derived from molecular oxygen, that has acquired less than four electrons [259 260 261]. The superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-), are considered ROI's because they have been reduced by one, two and three electrons, respectively. ROI's have been implicated in PRI after warm ischaemia in numerous reports [71 247 262 263 264 265 266 267] as well as after cold ischaemia [185 268 269 270].

It is now well established that the reintroduction of molecular oxygen is required to produce the post-ischaemic cellular injury and dysfunction. This supports the view that the PRI is the result of the generation of ROI. The production of oxidant species in postischaemic tissues has been detected by electron spin resonance (ESR), spectroscopy and ESR spin trapping techniques [271 272 273]. Metabolites of oxygen-induced peroxidation of cellular membrane lipids have been demonstrated on numerous occasions in postischaemic tissues [274 275 276 277 278 279 280 281]. Malondialdehyde, lipid hydroperoxides and conjugated dienes are the common markers for reperfusion-injury.

The exposure of normal tissues to exogenous oxidant-generating systems produces structural and functional changes similar to those observed in postischaemic tissues [263 282 283]. Moreover, several agents which limit oxidant production, as well as ROI scavengers, are able to attenuate the PRI [258 284 285].

The production of ROI following PRI is the result of the ischaemia on purine metabolism and the conversion of xanthine dehydrogenase into xanthine oxygenase [286 287 288]. The enzyme

xanthine dehydrogenase, which is found in both parenchymal and endothelial cells, is converted into xanthine oxygenase during ischaemia. Recent reports have indicated a major role for other ROI sources, such as the NADPH-dependent oxidase system, located on the membrane surface of leukocytes and also present in Kupffer cells [262 289 290 291 292]. Inhibition of xanthine oxidase as well as neutrophil depletion are both able to reduce PRI [258 274 275 276]. After the activation of the various enzyme complexes, a respiratory burst occurs upon reperfusion, which consumes 90% of the oxygen in the leukocytes [293] and catalyses the reduction of oxygen to the hydrogen peroxide and superoxide anion [294 295].

ROI causes damage to tissues in several ways. One of the main effects is the oxidation of an entire array of biomolecules which alter cellular functions [296]. Membrane lipid peroxidation, DNA-linking and cross-linking, and degradation of proteins [261 284] are all direct results of oxidant generation. These molecular changes affect cellular functions by interference with structural, contractile and transport proteins, enzymes, receptors, membrane glycolipids, glycosaminoglycans and nucleic acids [80]. Another proposed mechanism is that superoxide radicals may cause the reductive release of iron from ferritin [297 298 299].

There is also accumulating evidence suggesting that ROI attract and activate inflammatory phagocytes [262 300]. Chemotaxis is generated if plasma is treated with an oxidant-generating system. Also, injection of superoxide-treated plasma or Hypoxanthine-Xanthine-Oxidase into the dermis induces massive neutrophil infiltration. Furthermore, oxidant formation contributes to the expression of adhesion molecules on the surface of endothelial cells and neutrophils [301].

(b)Cytokines

Many of the effects of various cytokines are very similar to the effects of PRI, while all the cells involved in the production of cytokines are present in PRI. The main source for cytokines are Kuppfer cells and endothelium adhered leukocytes. Most cytokines act both individually as well as in a network of interrelated and interacting signals [302]. The mechanisms of action of the numerous cytokines are so complicated that discussing them in detail is beyond the scope of this thesis. The proinflammatory cytokine cascade appears to initially involve the release of IL-1 and TNF-α. These cytokines lead to the later production of IL-6 and IL-8, increasing infiltration and activation of leukocytes, and eventually the production of IL-4 and IL-10, which may produce a negative feedback onto the cascade [303 304 305].

(i)Tumor Necrosis Factor

TNF activates endothelial cells in several ways. It increases procoagulant activity [306], increases leukocyte adhesion, by expressing adhesion molecules on both the endothelial cells and the leukocytes [307 308 309 310] and stimulates both ROI release [295] as well as the release of IL-1 and IL-8 [311 312]. The leukocytes are also activated by TNF resulting in ROI generation, aggregation, release of LTB_4, and enhanced phagocytosis [313]. TNF might also

affect the microcirculation via the production of the endothelial-derived relaxing factor, nitric oxide [314].

(ii)The Interleukins

IL-I is a pleiotrophic cytokine with similar effects to TNF. Its main source are the activated monocytes, especially the Kupffer cells [315]. Like TNF-α, it initiates the secretion of other cytokines and activates endothelial cells.

IL-6 is produced by numerous cells, including Kupffer cells and endothelial cells. A major activity of IL-6 involves induction of acute phase proteins in hepatocytes, such as C-reactive proteins, α_1-antitrypsin and fibrinogen [302 316]. IL-8 is produced mainly by hepatocytes under TNF-α and IL-1 stimulation [317]. It causes PMN-chemotaxis, and may mediate degranulation and the respiratory burst in ROI production [312 318]. There is no doubt that cytokines are responsible for controlling and directing various components of the PRI.

(c)Proteases

Proteases are known mediators of PRI in experimental transplant models [319 320]. Proteases are known to facilitate the generation of ROI by proteolytic activation of XO [321 322], and protease inhibitors can inhibit XO, thereby preventing the production of superoxide after reperfusion [321]. Both in pig and rat models, the administration to the recipient of the anti-protease aprotinin had a significant beneficial effect on allograft function and survival. Furthermore, anti-proteases added to the cold preservation solution also yielded a positive effect on survival [154]. Anti-proteases also inhibit coagulation factor activation and the production of platelet activating factor (PAF) [256 323].

(d)Calcium

Intracellular calcium levels increase due to the release of intracellular depots as well as an influx of calcium through the cell membranes. Generally, the cell membrane has a potent function of pumping the calcium out of the cell against a 10.000 fold gradient between the intra-and extra-cellular space. Furthermore, intracellular free calcium is compartmentalized within the mitochondria and the endoplasmatic reticulum [324].

After ischaemia and hypothermia, a tremendous influx of calcium occurs into the cell [325]. The most likely cause for this phenomenon is the failure of the calcium channels and the Na^+ / Ca^{++} exchange systems due to decreased energy stores of ATP. ROI cause destruction of cell membranes by lipid peroxidation. Calcium influx, therefore, may occur through the damaged cell membranes according to the calcium gradient between intra- and extracellular space [277]. Other possible sources for the increase in the resting cytosolic free-calcium concentration are the intracellular organelles, such as the mitochondria [326]. The intrahepatic calcium concentrations correlate with the severity of the liver injury and the survival in experimental models [327].

Calcium channel blockers added to the preservation solution significantly ameliorates the PRI [328] [329]. The beneficial effect of calcium channel blockers on reperfusion injury might be mediated through different pathways. Several calcium channel blockers have anti-oxidant properties [331]. Calcium channel blockers are also well known vasodilators, and this might improve the perfusion of the centrilobular regions of the liver, which appear to be the most sensitive to hypoxic injury [330].

A high calcium concentration will potentiate cell damage from ROI's [331], as well as enhance the conversion of xanthine dehydrogenase (XD) to xanthine oxidase (XO) [332]. Several studies have indicated that the rate of hepatocyte apoptosis post transplantation might be related to an increase in cytosolic calcium concentration [333].

In summary, altered calcium homeostasis appears to be critical in PRI, and recovery from intracellular calcium accumulation seems to be crucial for minimizing cellular injury.

(e)Phospholipase A_2

Although alteration in the calcium homeostasis is strongly implicated in the development of hepatocellular injury, the exact working mechanism is unclear. Several theories have been proposed. One of the effects of an increased cytosolic calcium concentration involves the activation of phospholipase A_1, A_2 and C [334]. Phospholipase A_2 (PLA_2) is a key mediator in various models of inflammation [335]. It is present in both hepatic endothelial cells and Kupffer cells. PLA_2 mediates many of the effects credited to cytokines and also mediates most of the damaging effects of impaired calcium homeostasis [334]. IL-1, IL-6 and PAF also appear to produce several of their effects through PLA_2 activation [336]. PLA_2 triggers a multitude of processes which include activation of proteolytic enzymes, PAF, eicosanoids, ROI and numerous other substances. Experimental studies have shown significantly improved mitochondrial function and graft survival when quinacrine (a phopholipase inhibitor) was added to the cold preservation solution. The precise role of PLA_2 still has to be elucidated.

(f)Eicosanoids

Products of arachidonic acid metabolism are produced through either the cyclo-oxygenase pathway, producing prostaglandins, prostacyclins and thromboxanes, or the lipo-oxygenase pathway, producing leucotrienes. Together these products are called eicosanoids. They are produced mainly from Kupffer cells, play an important role as inflammatory mediators and are released in high concentration after PRI [337]. There is up to a 500-fold increase following OLT, and at these levels they may not only have detrimental effects on the graft, but might also contribute to cardiovascular, haemostatic and immunological derangements after liver transplantation. Not all their effects after OLT are detrimental [81], since PGE-1 has been used to reduce PNF.

The production of eicosanoids appears to be initiated by ROI release, elevation of the intracellular calcium and activation of PLA_2. PLA_2 triggers eicosanoid production by conversion of cell membrane phospholipids into arachidonic acid [325] [338]. PAF, TNF and endotoxin have also been reported to induce eicosanoids production [339] [340]. The main effects

of eicosanoids classically occur through their action on neutrophils. Neutrophils are potently chemo-attracted by LTB_4 and thromboxanes A_2 and B_2. Additionally, thromboxane B_2 is known to activate neutrophil adhesion receptors and has been shown to activate neutrophils to produce more ROI's. Eicosanoids are potent regulators of the blood flow through the microcirculation. They have direct effects on the microvasculature and enhance retraction of endothelial cells [341]. Alteration of blood flow probably results from an imbalance between vasoconstricting and vasodilating eicosanoids [342].

(g)Platelet Activating Factor

Platelet Activating Factor (PAF) is an autacoid phospholipid mediator and is thought to be an important mediator in the pathogenesis of PRI. Important effects of PAF relevant to PRI are the ability to prime a variety of leukocyte and macrophage functions. These include the respiratory oxygen burst and the release of proteolytic enzymes, TNF and the interleukins.[343] PAF is produced by various cell types: macrophages, neutrophils, lymphocytes, monocytes, eosinophils, platelets, endothelial cells and muscle cells [257]. PAF is also a potent activator of inflammatory cells. It mediates direct interactions between platelets and polymorphonuclear leucocytes (PMN) and induces platelet adhesion to endothelial cells if PMN's are present. It also induces platelet aggregation [344]. It appears that the platelet adhesion is caused by activation of PAF-specific membrane receptors on activated PMN's, which then attach the platelet-PMN complexes to the endothelium. PAF also stimulates leucocytes and platelets, resulting in activation, chemotaxis, chemokinesis, adhesion, aggregation and eventually the respiratory oxidant burst [345]. In experimental models, the PAF levels have been found to correlate with the severity of the hepatic injury and the amount of inflammation post-transplantation [346]. The administration of a PAF-antagonist has been shown to attenuate the liver injury and the degree of inflammation [347 348].

(h)Endothelin

Endothelin is a family of 21-amino acid-peptides, initially isolated from the supernatant of cultured porcine endothelial cells [349]. There are several distinct isoforms, which have a wide range of biological effects [350 351]. Endothelin-1 (ET-1) is synthesized *de novo* by endothelial cells and is one of the most potent vasoconstrictors. It acts mainly on the venous and arterial smooth muscle cells. Its potent effect on vascular tone, together with its rapid clearance from the circulation suggests a pivotal role of endothelin in the regulation of regional blood flow [352]. Elevated levels of endothelin have been associated with a variety of pathological conditions, including essential hypertension, congestive cardiac failure and transplantation associated hypertension [353 354].

Several experimental studies have suggested an important role for endothelin in liver bloodflow. Specific endothelin receptors have been demonstrated on liver plasma membranes. Administration of endothelin-1 increases portal hypertension and diminishes sinusoidal bloodflow [355]. Endothelin-1 antibody injected before reperfusion in a partial warm

ischaemia model was able to decrease microcirculatory disturbance, transaminase release and biological damage [356]. The use of an ET-1 receptor antagonist has also been proven to protect against preservation-reperfusion injury in a pig model [357]. There are also a few reports documenting the changes in endothelin-1 levels after human liver transplantation [358] [359]. However the exact pathophysiological role in the clinical situation remains unclear [360]. Clamping of the portal vein during the transplantation procedure results in bacterial translocation [361] and endotoxin is known to stimulate endothelin release, most likely via a TNF-α mediated mechanism [362] [363]. In summary, ET-1 is released from the liver during PRI and participates in local and systemic haemodynamic changes.

(i)Endotoxin

Thus far, all the factors influencing the severity of PRI discussed above are related to the transplanted liver and the duration of cold preservation and warm ischaemia. In contrast, endotoxemia is mainly dependent on recipient factors. Endotoxins are known to contribute to PRI independent of donor characteristics. However, the preservation damage to the liver will be a major determining factor in how the transplanted liver will respond to the endotoxemia.

The clearance of endotoxin is often deficient in liver transplant recipients due to liver failure [364] and might be completely absent during the anhepatic period of liver transplantation. The reticulo-endothelial system plays the main role in detoxification of endotoxin. Kupffer cell dysfunction due to hypothermia results in higher levels of endotoxins, which persist until the donor Kupffer cells are replaced by recipient macrophages. Endotoxin is known to also damage hepatocytes [365], to render macrophages cytotoxic [366] and to induce release of a variety of biologically active mediators [367]. It is also known to enhance the production of ROI by Kupffer cells. These effects are most likely mediated by TNF.

In clinical transplantation, elevated endotoxin levels preoperatively and at the end of the anhepatic period are associated with an increased incidence of graft failure and higher mortality [135] [368] [369]. However, the exact pathogenesis of how endotoxin causes an increase in PRI remains unclear.

Apoptosis

Ischemic injury has been classically described as a coagulative necrosis. In coagulative necrosis (or "accidental cell death") large groups of cells are involved and there is a strong association with a local inflammatory response. It is a passive, energy-independent process [370].

Apoptosis or programmed cell death is a tightly regulated, energy-requiring process involving the activation of *de novo* synthesis of endonuclease, resulting in fragmentation of internucleosomal double-strand chromatin (DNA) [371]. Apoptotic cells are characterized morphologically by blebbing, nuclear and cytoplasmic condensation, subsequent disintegration of the nuclear membrane and the formation of multiple fragments of condensed nuclear material in the cytoplasm; the so called apoptotic bodies. Apoptosis occurs typically in

single cells, which are eliminated by phagocytic cells, without evoking a surrounding inflammatory response [372]. Reactive oxygen intermediates have been recognized as the major mediators of apoptosis [373 374]. The histological and ultrastructural features of apoptosis in experimental porcine liver allograft rejection were already noted in 1974 [375].

Apoptosis has been implicated in cell death following reoxygenation in experimental and human studies [376 377]. Recently, various techniques including the terminal deoxynucleotidyl transferase d-uridine triphosphate nick end labeling, DNA gel electrophoresis and transmission electron microscopy, have been used to show that apoptosis of endothelial cells might be the pivotal mechanism of preservation injury in liver transplantation [378 379].

Implications of the double blood supply of the liver for reperfusion

The liver is unique in that it receives arterial blood from the hepatic artery and venous blood from the portal system. Thus, liver blood flow is determined by hepatic artery vascular resistance, intra hepatic portal venous vascular resistance and intestinal vascular resistance. Each of these factors is variable and dependent on neural and humoral control mechanisms, which makes the study of liver perfusion highly complex [380]. There appears to be reciprocity between the hepatic arterial and portal venous flow in that an increase in blood flow through one circuit leads to an increased resistance in the other, thus maintaining a constant flow through the liver [381 382]. This reciprocity is not only present under physiological circumstances, but also in pathological conditions [383]. In the classical methods of liver transplantation, the portal vein is anastomosed first, and as soon as the anatomosis is completed, the liver is reperfused with portal venous blood only. The hepatic arterial anastomosis is performed thereafter [384 385 386].

Originally, this method has been described to minimize the anhepatic period as well as to limit the warm ischaemic time. However, in recent years, this classical method of liver transplantation has been questioned by several authors. Improved macroscopic perfusion of the livers perfused with arterial blood initially has been reported in porcine liver transplantation [173]. This was confirmed by experimental studies in 1994. In rat liver transplantation, the number of non-perfused acini and non-perfused sinusoids were reduced by 71% and 78% respectively, if the livers were arterialized simultaneously with portal revascularization [387]. Several other indicators of PRI were also reduced, including decreased leucocyte accumulation in sinusoids and postsinusoidal venules by 17% and 64% respectively [387]. Additionally, Kupffer cell activation was attenuated, and improved hepatocellular excretory function was demonstrated after simultaneous arterialization.

In 1995, Sankary et al demonstrated that initial portal revascularization was associated with an increased number of ischaemic biliary strictures and recommended simultaneous revascularization, with the specific aim of avoiding warm bile duct ischaemia [388]. In 1997, a clinical study using initial arterialization reported a decrease in PRI [389]. Although it might be expected that the higher oxygen content of arterial blood would create a greater injury, in fact several experimental studies have demonstrated that the major part of the total blood supply

to the liver is delivered by the portal blood flow, in spite of its lower oxygen content [390] [391]. Furthermore, it was reported that both the mean blood transfusion and antifibrinolytic requirements, as well as the duration of the post-revascularization phase were reduced. The authors concluded that under adequate portal decompression, initial arterialization was a safe option and should be recommended [389]. A recent clinical study showed a statistically significant lower biliary complication rate in simultaneous reperfusion (2%) as compared to initial portal venous reperfusion (34%) [392].

As discussed earlier, endotoxins do play an important role in PRI. It is well known that the endotoxin level of portal venous blood is significantly higher than arterial blood. This difference will be even greater in the setting of a malfunctioning spleno-jugular bypass, leading to an increase in the portal venous pressure, bowel congestion, and an increase in bacterial translocation

Preservation Solutions and Irrigation Solutions

Eurocollins Solution

Studies in the late 1960's demonstrated that kidneys could be safely preserved for 30 hours by simple cold storage only and for up to 72 hours by continuous perfusion [93] [95]. The original paper from Collins [95], in which he showed that cooled kidneys could be preserved for up to 3 times longer if treated with the appropriate preservation solution, rather than with cold blood, compared already seven different preservation solutions. Eurocollins was also used as the preservation solution fluid in liver transplantation [99] [393]. With Eurocollins solution, the livers could be stored for up six hours. The advantages of prolonged storage were rapidly realized and the search for new preservation solutions followed [394] [395] [396] [397].

The University of Wisconsin Solution

Extensive research during the 1980's into the mechanisms underlying reperfusion injury led to the development of the "University of Wisconsin Solution" or "UW-solution". This solution was developed by Folkert Belzer purely on empirical grounds [99]. This solution was first used to preserve the canine pancreas for 72 hours [398]. Subsequently, successful storage of the canine liver for 24 and 48 hours in experimental liver transplantation was reported [399]. This was followed by the report in March of 1988 of the use of UW solution in human liver transplantation. Tolerance for cold ischaemia was significantly improved from 8 hours or less with Collins' solution to over 20 hours [400]. The 12 constituents in UW solution were all added to counter balance specific metabolic processes thought to be responsible for preservation injury.(Table 2.1)

The success of cold storage is primarily related to the prevention of tissue oedema. The most important contributions of UW solution are lactobionate and raffinose as impermeants to suppress hypothermia induced tissue swelling [400]. Lactobionate is a large sized organic anion, which by nature of its size and negative charge, is impermeable to most tissue membranes. Raffinose, a trisaccharide, and hydroxyethyl starch, were both added for additional osmotic support, and to prevent expansion of the extracellular space. The phosphate buffer was added to the solution to prevent tissue acidosis, which is induced by the anaerobic metabolism from the ischaemic cells.

Table 2.1

Composition of University of Wisconsin (UW) Solution

Component	Amount
K+ - lactobionate	100 mM
KH_2PO_4	25 mM
$MgSO_4$	5 mM
Raffinose	30 mM
Adenosine	5 mM
Glutathione	3 mM
Allopurinol	1 mM
Dexamethasone	16 mg
Regular Insulin	40 U/L
Penicillin G	200 000 U/L
Hydroxyethylstarch	50 g/L
Osmolarity	320 mOsm/L
Solution is brought to Ph 7.4 at room temperature with NaOH.	

Adenosine and phosphate were added as precursors for ATP-production as well as to support energy-utilizing reactions [401]. Allopurinol and glutathione were added with the specific goal of preventing toxic oxygen radical related damage. Allopurinol is a well known inhibitor of the xanthine oxidase, while glutathione is known for its toxic oxygen radical scavenging capacity. As stated earlier, the development of UW solution was purely theoretical and it is therefore not surprising that several modifications have not altered its excellent performance. Hydroxyethyl starch has been omitted without negatively influencing the preservation injury [137] [402] [403]. Other substances like raffinose, lactobionate and glutathione can not be omitted without negative effects [404]. The Potassium salts have been replaced successfully by sodium salts [405] [406]. In spite of numerous possible modifications, UW solution remains currently the gold standard cold preservation solution [407] [408] [409] [410].

HTK (Bretschneider) Solution

Originally, Bretschneider developed this solution specifically to provide myocardial protection during the cardioplegic period [411] [412]. He considered the primary goal of any myocardial protection solution to be the reduction of the myocardial energy demand. Since survival of the non-perfused tissue depends on ongoing anaerobic metabolism with resulting tissue acidosis, he postulated that any cardioplegic solution should contain a substance with a high buffering capacity even at low temperatures. He used the physiologic protein buffer histidine to fulfill this role. He also recognized the importance of the delivery of energy substrates in the myocardial protection solutions, and therefore ketoglutarate and tryptophane were added. These substances are able to produce ATP, while inhibiting glycolysis and reducing lactate production and tissue acidosis. Potassium was added to achieve cardiac arrest, while magnesium was added to reduce calcium efflux and stabilize ionic membrane status. Mannitol was chosen as an agent to increase the osmotic gradient and to prevent tissue oedema. Since this solution is based on its substantial buffering capacity and is described as an "equilibrium" solution, high volume flush-outs are used allowing equilibration across the cell membrane [413].This may have the additional advantage of rapid core cooling. Contents of the Bretschneider – HTK4 solution are shown in table 2.2.

After successful introduction in kidney transplantation, HTK solution has also been used in liver transplantation [414] [415]. Several European centers have introduced HTK solution in their liver transplantation program. The interim results of a multicenter trial performed in Germany and Austria show no significant differences between UW and HTK preserved liver transplants [416].

Table 2.2

Composition of Bretschneider HTK4 Solution

Component	Amount
Histidine	180 mM
Histidine-HCl	18 mM
K-Ketoglutarate	1 mM
Tryptophane	2 mM
Potassium	9 mM
Sodium	15 mM
Magnesium	4 mM
Mannitol	30 mM
Osmolarity	300 mOsm/l
pH	7.1

Celsior Solution

In view of the pivotal role of reactive oxygen intermediates in preservation-reperfusion injury, several new solutions have been developed to counteract oxidation. In 1994 a new cardioplegic solution, Celsior, was introduced (Table 2.3) [417] [418]. Experimental evaluation using both isolated heart and heterotopic transplantation, showed that this solution had protective effects. The major feature of Celsior is the presence of three anti-oxidants, reduced glutathione [419] [420], mannitol [421] and histidine [422]. The primary target of Celsior is prevention of free radical injury, which remains an important cause of early graft dysfunction [423] [424] [425] [426] [427]. Reduced glutathione, a powerful anti-oxidant is one of the constituents of Celsior. Unlike other solutions, in which shelf-storage of glutathione will lead to oxidation and development to its potentially cardiotoxic form, Celsior has been specifically designed to allow delivery of a predominantly reduced form of glutathione [420] [428]. Additional antioxidant effects are provided by mannitol and histidine because of their capacity to scavenge hydroxyl radicals [421]. Celsior has mainly been used in cardiac surgery and transplantation. At present there is evidence demonstrating a decrease in reperfusion injury in other solid organ transplantation with Celsior [406] [417] [421] [422] [427] [429].

Table 2.3

Composition of Celsior Solution

Component	Amount
Mannitol	60 mM
Lactobionate	80 mM
Glutamic acid	20 mM
Histidine	30 mM
Reduced glutathione *	3 mM
Potassium	15 mM
Sodium	100 mM
Magnesium	13 mM
Calcium	0.25 mM
Chloride	41.5 mM
pH (at 20°C)	7.3
Osmolarity	360 mOsm/L

* To avoid oxidation of reduced Glutathione, the latter compound is prepared under anaerobic conditions and stored in 5-ml deaerated glass syringes, the content of which is added to the plain Celsior solution immediately before use.

Although a pharmacologic approach to organ preservation solution development might lead to significant improvements in graft function, Southard [430] warns that the use of pharmacologic agents in preservation solutions is fraught with difficulties. These agents are often inactive at

storage temperatures, have poor permeability, and may be rapidly washed away upon reperfusion. Furthermore, PRI involves multiple sites requiring multiple pharmacological agents.

Irrigation Solutions

Because of the high potassium contents as well as the presence of adenosine in the UW solution, it is necessary to rinse out the UW solution from the liver graft prior to revascularization [431]. Ringer's lactate is especially suitable because of its similarity to the extracellular fluid. Several studies have shown that the use of warm Ringer's lactate flush has an advantage over a cold rinse [432 433]. If cold Ringer's lactate is used, it is preferable to reflush with warm portal blood, and to discard the first 300-400 ml of portal blood [434]. The use of 4% serum albumin as a rinse solution has been shown to compare favourably with the use of Ringer's lactate [435].

Carolina Rinse Solution

Recently, a complex rinse solution has been developed with the specific aim of minimizing reperfusion injury and improving graft survival (Table 2.4)[436 437]. The Carolina Rinse Solution consists of 10 empirical components, including adenosine, nisoldipine, desferrioxamine and fructose. It has been shown that adenosine is the most essential component [435 438].

Table 2.4

Composition of Carolina rinse II solution

Component	Amount
NaCl	102 mM
KCl	4 mM
$CaCl_2$	3 mM
Sodium lactate	28 mM
Adenosine	0.1 mM
Allopurinol	1mM
Desferrioxamine	1mM
Glutathione	3mM
Osmolarity	273 mOsm/L
Solution is brought to pH 7.4 at room temperature with NaOH	

Adenosine is able to improve survival by inhibiting platelet-aggregation [439], diminishing Kupffer cell activation [436 438], improving micro circulation [440] and by its conversion to ATP [436 441 442]. This solution has since been modified by the addition of allopurinol, and glutathione to further reduce the sinusoidal reperfusion injury [443]. Clinical trials have demonstrated that

Carolina rinse solution is more effective in reducing cholestatic injury rather than in decreasing hepatocellular damage [444]. The exact role of the rinse solution in human liver transplantation is not yet known, although there are indications that it improves graft function [445]. The most significant benefit of a rinse solution may well be a short rewarming period, just prior to reperfusion, with subsequent improvement of the microcirculation [66 432 433].

CHAPTER 3

Hypothesis and Study Design

The preservation-reperfusion injury (PRI) in liver transplantation has been extensively documented, and has been identified as an important cause of both primary non-function and delayed function of the liver graft. Thus any interventions which minimise the PRI would have a significant impact on the results of liver transplantation.
In addition, the liver is peculiar in that it has dual blood supply, with arterial blood via the hepatic artery and venous blood via the portal vein.

Hypothesis

1. In liver transplantation, the reperfusion injury associated with portal venous revascularization is different from that associated with hepatic artery revascularization.

2. In liver transplantation, early rearterialization of the liver allograft is associated with a lesser reperfusion injury.

3. The use of Celsior as a preservation solution in liver transplantation, because it contains several free radical scavengers, is associated with a lesser reperfusion injury, compared to the University of Wisconsin Solution.

Study Design

Experiment 1: Reperfusion injury associated with portal venous and hepatic arterial reperfusion

Large White X Landrace pigs were subjected to orthotopic liver transplantation and randomly allocated to either portal venous revascularization (PV) 20 minutes before hepatic arterial revascularization (HA), or HA 20 minutes before PV. Blood samples were taken before and after PV, before and after HA, and after total revascularization.

Experiment 2: The effect of early rearterialization of the liver allograft on reperfusion injury, hepatocellular injury and endothelial cell dysfunction

Large White X Landrace pigs were subjected to orthotopic liver transplantation and randomly allocated to the following treatment groups:

Group 1: PV 60 minutes before HA.

Group 2: PV 20 minutes before HA.

Group 3: Simultaneous HA and PV.

Group 4: HA 20 minutes before PV.

Blood samples were taken before and after PV, before and after HA, and after total revascularization.

Experiment 3: Histological assessment after different modes of reperfusion after liver transplantation

Large White X Landrace pigs were subjected to orthotopic liver transplantation and randomly allocated to the groups as described in Experiment 2. Liver biopsies were taken after dissection of the donor liver (base), after the cold storage period , and one hour after completed reperfusion.

Experiment 4: The effect of Celsior, Wisconsin and Eurocollins Solution on the hepatocellular, reperfusion and endothelial cell injury

Large White X Landrace pigs were subjected to orthotopic liver transplantation and the livers stored in the following preservation solutions:

Group 1: Eurocollins solutions.

Group 2: University of Wisconsin Solution.

Group 3: Celsior solution.

Blood samples were taken at various time intervals after total revascularization.

Biochemical analyses

The blood samples were subjected to the following analysis:

Serum AST

Plasma malondialdehyde

Serum vitamin A

Serum hyaluronic acid

Histology

The liver biopsies were subjected to routine histological examination.

CHAPTER 4

Reperfusion Injury Associated with Portal Venous and Hepatic Arterial Perfusion in Liver Transplantation

Summary

Although reperfusion injury in organ transplantation is presently well established, its exact role in liver transplantation still has to be defined.

The aim of this part of the study was to document the reperfusion injury associated with porcine liver transplantation and to evaluate the different components of the reperfusion injury associated with arterial and portal reperfusion.

Large White X Landrace pigs were randomized into 2 groups. Group 1: Initial portal reperfusion and Group 2: initial arterial reperfusion. Several indicators of reperfusion injury, endothelial cell function and hepatocellular damage were assessed.

Malondialdehyde concentrations were lower and Vitamin A concentrations were higher in the animals subjected to initial arterial reperfusion. Serum AST and serum hyaluronic acid concentrations both were higher in the animals subjected to initial portal reperfusion.

Results of this study indicate that the major part of reperfusion injury is constituted during the portal venous reperfusion and that this injury can be, at least partially, attenuated by initial arterial reperfusion.

Introduction

Liver transplantation is now established as the treatment of choice for most patients with chronic end stage liver disease and is performed routinely in most major centres throughout the world. During the transplantation process, the liver allograft may be injured in several ways, including the preservation-reperfusion injury, rejection, infection and drug toxicity. The extent of the preservation-reperfusion injury may vary from mild, causing minimal hepatic dysfunction, to severe, leading to primary non-function.

Despite the major advances in clinical liver transplantation, including the development of the University of Wisconsin cold preservation solution, the preservation-reperfusion injury

continues to be a serious problem and a significant cause of post-transplant hepatic dysfunction [446]. The pathophysiology involved in the preservation-reperfusion injury and the possible preventative modalities have been extensively documented [446 447 448 449]. However, the relative contribution of the portal blood and the hepatic arterial blood to the reperfusion injury has not been investigated previously.

The conventional technique of liver transplantation involves initial reperfusion of the allograft with portal blood followed some time later by hepatic arterial reperfusion (Group I). To evaluate the reperfusion injury associated with hepatic arterial reperfusion, we reversed the order of the anastomoses in Group 2.

The reperfusion injury is thought to be mediated, at least in part, by the formation of reactive oxygen metabolites [262 263]. The latter are responsible for damage to an array of bio-molecules, including membrane lipids, enzymes and receptors. The peroxidation of the membrane lipids results in the formation of malondialdehyde (MDA) and other conjugated dienes. Thus MDA can be used as a marker of the reperfusion injury. Endogenous vitamin A is an important scavenger of reactive oxygen metabolites, and the serum levels can also be used as an indicator of the reperfusion injury.

Hyaluronic acid (Ha) is synthesized mainly by mesenchymal cells and is rapidly removed and degraded primarily by hepatic endothelial cells [157]. Normally, the concentration of Ha is low. However, impaired catabolism by hepatic endothelial cells results in elevation in a porcine liver transplant model [450].

Amino Aspartate Transferase (AST) is a well established marker for hepatocellular injury.

The reperfusion injury of the liver caused by the formation of reactive oxygen species has been extensively documented in experimental systems [66]. There is however, only limited evidence for its occurrence following liver surgery in humans. In addition, the liver is unique in that it has a dual blood supply, and the relative contribution of the portal and hepatic arterial blood to the reperfusion injury has not been investigated previously. Thus, the aim of this study was to document the reperfusion injury associated with liver transplantation in the pig and to evaluate the reperfusion injury associated with portal venous and hepatic arterial reperfusion.

Materials and Methods

This study was approved by the Animal Research Review Committee of the University of Cape Town. Young Large White X Landrace pigs (n=12) of either sex and weighing 20 - 30kg were fasted overnight. Anaesthesia was induced with intravenous thiopentone sodium (2 mg/kg) administered via an ear vein, and maintained with nitrous oxide and oxygen given via an endotracheal tube. Catheters (French 8) were introduced into the common carotid artery, for monitoring of blood pressure and for sampling of arterial blood, and into the jugular vein,

for the infusion of intravenous fluids. The technique of orthotopic liver transplantation was as described previously [451].

Donor Operation

The preparation of the donor liver involved dissection and mobilization of the suprahepatic and infrahepatic vena cavae, the portal vein, the hepatic artery and the bile duct in the hilum of the liver. The animal was heparinized and a catheter inserted into the portal vein for initial flushing of the liver with ice-cold Eurocollins solution. Although the common practice is to use University of Wisconsin solution, we decided to use Eurocollins solution to reduce the costs of the experiments. The liver was excised during the in situ flushing which was continued on the back-table.

The donor liver was stored in ice-cold Eurocollins solution (4 degrees Celsius) for three hours, since prolonged storage time is associated with a high mortality in the pig liver transplantation model.

Recipient Operation

The dissection of the recipient liver was as described above. After heparinization of the animal, the passive splenojugular venous bypass between the splenic vein and the external jugular vein was established. The recipient liver was excised, and the donor liver implanted as follows:

Group 1 (n=6): The anastomosis of the suprahepatic vena cava, the infrahepatic vena cava and the portal vein were completed first; at this stage these vessels were unclamped and the liver perfused with portal blood only; thereafter the hepatic arterial anastomosis was completed and total reperfusion of the liver achieved.

Group 2 (n=6): The suprahepatic vena cava, the infrahepatic vena cava and the hepatic artery were anastomosed first; these vessels were unclamped and the liver perfused with arterial blood only; thereafter the portal venous anastomosis was performed and total reperfusion of the liver achieved.

In both groups, the donor bile duct was anastomosed end-to-end to the recipient bile duct.

The time at which the liver was reperfused with both portal venous blood as well as arterial blood was set as the zero point ("0 minutes") and time was calculated from there to before (negative time scale) and to after completed reperfusion (positive time scale).

Previous studies have shown that the first hour is the most sensitive period to demonstrate alterations in the reperfusion injury markers. Thus, blood samples were taken preoperatively, at 5 minutes before (-25 minutes in group 1 and -5 minutes in group 2) and 5 minutes after portal reperfusion (-15 minutes in group 1 and 5 minutes in group 2), and at 5 minutes before

(-25 minutes in group 2 and -5 minutes in group 1) and 5 minutes after hepatic arterial reperfusion (-15 minutes in group 2 and 5 minutes in group 1), and at 20, 40 and 60 minutes after total reperfusion for assessment of the reperfusion injury markers plasma malondialdehyde and serum vitamin A.

The hepatocellular injury related to the reperfusion injury was determined in the first 24 hours. Blood samples were taken preoperatively and at 1 hour, 2 hours and 4 hours post transplantation for serum AST levels as a marker of the hepatocellular injury.

The marker for endothelial cell function, serum hyaluronic, was also measured in the first hour (at base, at 5, 20, 40 and 60 minutes post reperfusion).

Plasma Malondialdehyde (MDA) levels were determined by the thiobarbituric assay as described by Lepage [452]. 2-Thiobarbituric acid (TBA) was added to deproteinized plasma and the reaction between the MDA and TBA, under conditions of low pH and high temperature, yielded a chromogenic adduct that was detectable by fluorometry.

Serum Vitamin A levels were measured fluorometrically, according to the original method described by Thompson [453]. Vitamin A, which fluoresces in ultraviolet light, was first extracted into water and ethanol, then into hexane and detected by fluorometry.

Serum Hyaluronic Acid (Ha) levels were estimated by radioimmunoassay using a kit purchased from Kabi Pharmacia [454]. 125 I-labeled Hyaluronic Acid binding protein (HABP), isolated from bovine cartilage, was used to bind the Ha in the sample. The unbound 125 I-HABP was quantitated by incubating with Ha covalently coupled to Sepharose particles . Separation was performed by centrifugation and decanting. The radioactivity bound to the particles was read in a gamma counter which was inversely proportional to the concentration of Ha in the sample.

Serum AST levels were determined spectrophotometrically using a continuous monitoring assay.

Results

Plasma malondialdehyde (Figure 4.1).

There was an increase in plasma MDA levels from 2.53 µmol/l preoperatively to 5.64 µmol/l after portal reperfusion in Group 1. Hepatic arterialization in these animals was associated with a slight decrease in MDA levels from 4.73 µmol/l to 4.06 µmol/l. Thereafter plasma MDA levels continued to decrease and were almost back to baseline levels by 60 minutes after total reperfusion.

In the animals in Group 2 which received arterial blood to the allograft first, plasma MDA levels remained unchanged after arterialization and after subsequent portal reperfusion.

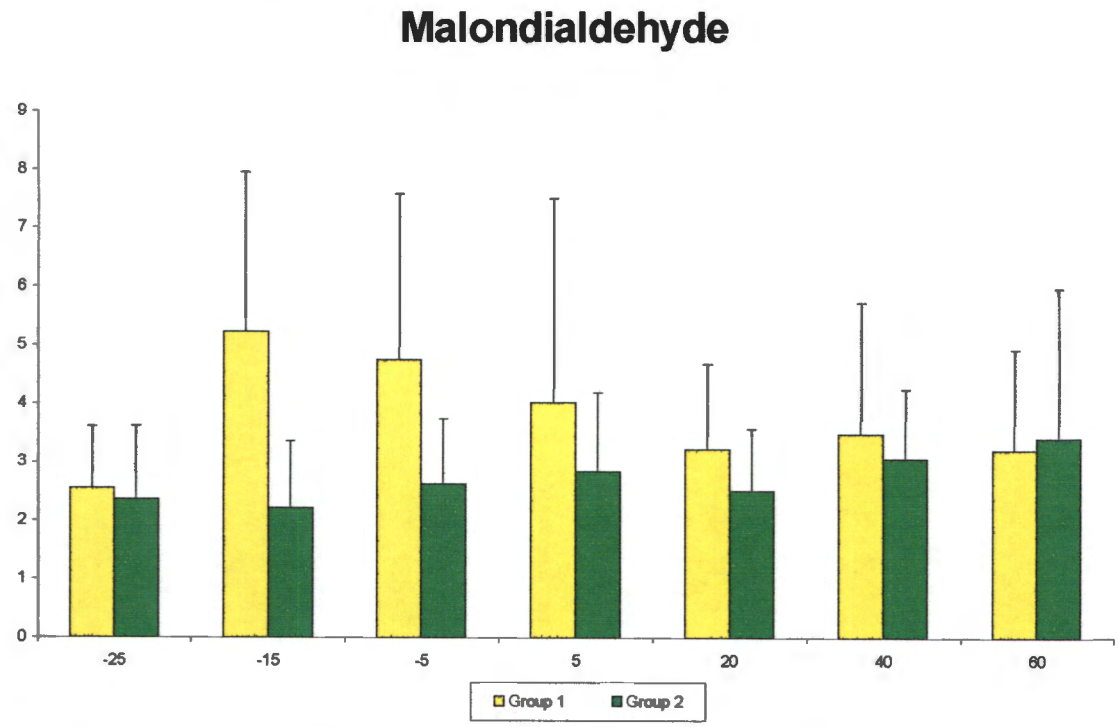


Figure 4.1. Arterial levels of Malondialdehyde (µmol/l) from 25 minutes before completion of revascularization up till 60 minutes after completion.
(Group 1 = initial portal reperfusion; Group 2 = initial arterial reperfusion)

Serum vitamin A (Figure 4.2).

The serum vitamin A levels decreased from 18.7mg/l to 9.6 mg/l after portal reperfusion in the animals in Group 1. Serum vitamin A levels remained unchanged after subsequent arterial reperfusion and remained markedly decreased up to 60 minutes after total reperfusion.

In the animals in Group 2, initial arterial reperfusion was associated with a minimal decrease in serum vitamin A levels. There was a further decrease in vitamin A levels after portal reperfusion in these animals, followed by a trend towards baseline levels by 60 minutes after total reperfusion.

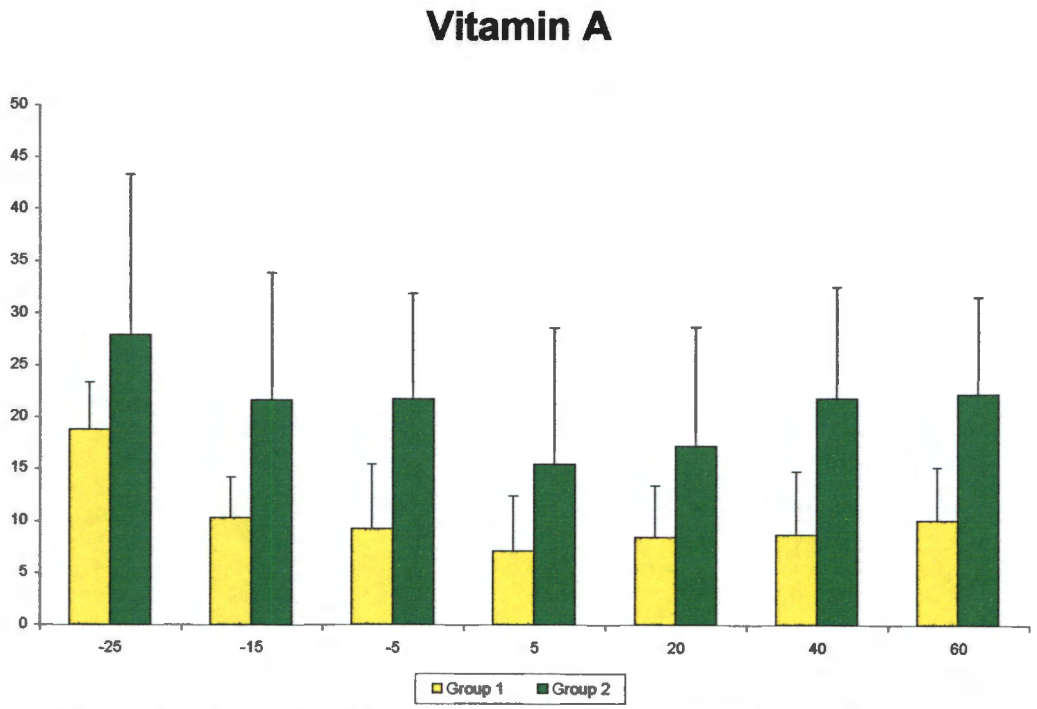


Figure 4.2. Arterial levels of Vitamin A, (mg/l) from 25 minutes before till 60 minutes after completion of revascularization.
(Group 1 = initial portal reperfusion; Group 2 = initial arterial reperfusion)

Serum AST (Figure 4.3).

There was a significant increase in serum AST levels at one hour after total reperfusion in both groups of animals. Thereafter, serum AST levels in the animals in Group I continued to increase up to 4 hours after total reperfusion. In contrast serum AST levels remained unchanged after the first hour in the animals in Group 2. There was no significant difference between the AST levels at 4 hours.

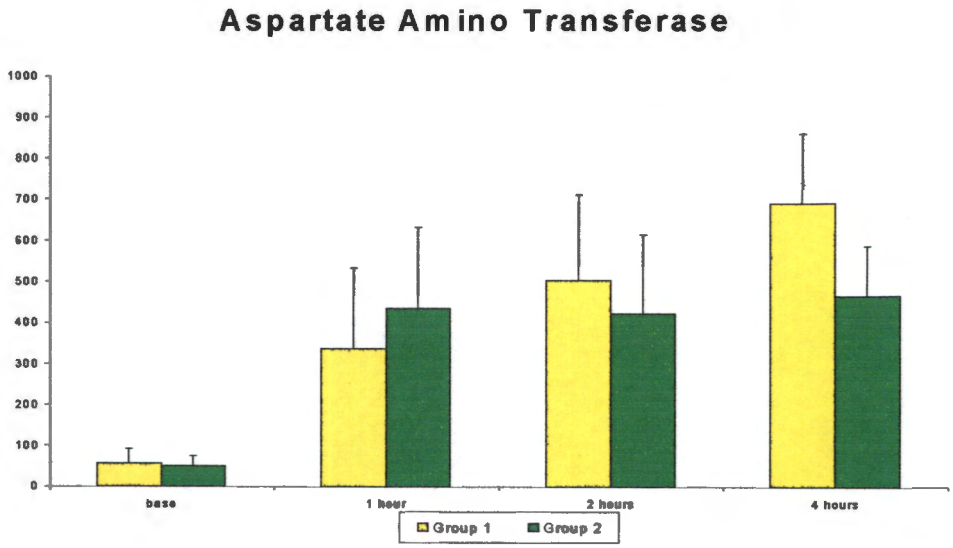


Figure 4.3. Peripheral venous levels of AST (IU) post transplantation at various times. (Group 1 = initial portal reperfusion; Group 2 = initial arterial reperfusion)

Serum Hyaluronic Acid (Figure 4.4).

There was a significant increase in serum Ha levels at 5 minutes after total reperfusion in both groups of animals. The serum Ha levels in the animals in Group 2 were starting to decrease at 60 minutes after total reperfusion, whereas levels were still increasing in the animals in Group 1.

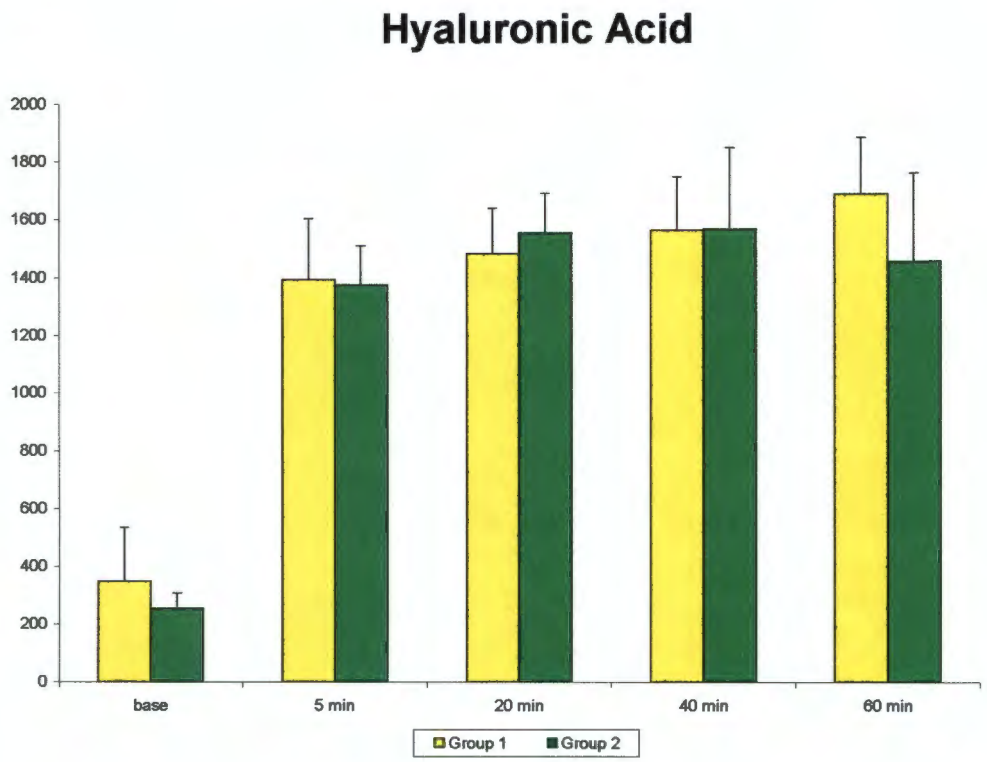


Figure 4.4. Hepatic venous levels of Hyaluronic acid (µg/l) after transplantation at various times.
(Group 1 = initial portal reperfusion; Group 2 = initial arterial reperfusion)

Discussion

In this study, initial reperfusion of the hepatic allograft with portal blood was associated with a marked reperfusion injury, as evidenced by an increase in plasma MDA levels and a decrease in serum vitamin A levels. Subsequent arterialization of the liver in these animals did not attenuate the reperfusion injury, with plasma MDA and serum vitamin A levels remaining unchanged. In contrast, initial reperfusion of the hepatic allograft with arterial blood was not associated with a reperfusion injury, since plasma MDA levels remained unchanged and serum vitamin A levels decreased only slightly. In these animals, subsequent perfusion of the allograft with portal blood did not result in a reperfusion injury (plasma MDA levels unchanged and vitamin A slightly decreased).

In the present study, serum Ha levels continued to increase up to 60 minutes after total reperfusion in the animals perfused with portal blood first and were decreasing by 60 minutes in the animals perfused with arterial blood first. This supports the finding of a greater reperfusion injury in livers perfused with portal blood first.

Serum AST levels, although similar in the first two hours after total reperfusion, were slightly higher at 4 hours in the animals perfused with portal blood first, indicating a greater amount of hepatocellular injury in these animals.

Thus, in summary, there was a significant reperfusion injury associated with portal reperfusion of the allograft, especially if this occurred before arterialization. There was no evidence of a reperfusion injury after arterial reperfusion, irrespective if this occurred before or after portal reperfusion. Furthermore, initial arterial perfusion of the allograft appeared to protect the liver from the reperfusion injury associated with portal reperfusion.

The reasons for the difference in the reperfusion injury associated with portal and arterial perfusion of the allograft remain unclear. One would expect a greater reperfusion injury after arterial perfusion because of its higher oxygen content. However, several studies indicate that the major part of the total oxygen supply to the liver is delivered by the portal blood, even though its oxygen content is lower [455].

The advantage of initial arterial perfusion may be due to the improvement in the micro-circulation of the graft associated with early arterialization, as shown previously in experimental studies [387].

Furthermore, the endotoxin level of portal venous blood is significantly higher, especially if mesenteric venous congestion occurs in the setting of a malfunctioning splenojugular bypass. Endotoxins are known to damage hepatocytes [456], to render Kupffer cells cytotoxic and induce release of a variety of biological active mediators [367]. It is also known to enhance the

production of reactive oxygen intermediates by Kupffer cells. Also, in clinical transplantation elevated endotoxin levels preoperatively and at the end of the anhepatic period have been associated with graft failure and an increased mortality [368] [457].

Conclusion

This study shows that in liver transplantation initial reperfusion of the allograft with portal blood was associated with a marked reperfusion injury and no additional injury after subsequent arterialization.

Furthermore, initial arterialization of the liver allograft was not associated with a marked reperfusion injury and protected the liver against the reperfusion injury following subsequent portal reperfusion.

CHAPTER 5

The Effect of Early Arterialization of the Liver Allograft on Reperfusion Injury, Hepatocellular Injury and Endothelial Cell Dysfunction

Summary

The conventional technique of liver transplantation involves initial perfusion of the graft with portal blood. However, recent evidence suggests that initial arterialization of the graft may be better. The aim of this part of the study is to evaluate the timing of arterialization on the reperfusion injury, hepatocellular injury and endothelial cell function after liver transplantation.

Large White X Landrace pigs (n=24) were subjected to orthotopic liver transplantation. The animals were randomized into 4 groups ranging from late arterialization (60 minutes after portal reperfusion) to early rearterialization (20 minutes before portal reperfusion).

AST levels continued to rise 4 hours post transplant in group 1 (late arterialization), but remained stable after 1 hour post transplant in group 4 (early rearterialization). Levels of malondialdehyde doubled in all groups after portal reperfusion, with the exception of group 4, in which the liver received arterial blood before portal reperfusion. Vitamin A levels decreased in all groups after revascularization, but the decrease was more pronounced and prolonged in group 1 and 2 (late arterialization) as compared to group 3 and 4 (early rearterialization).

Hyaluronic acid levels continued to rise in all groups until 1 hour post transplant, except in group 4, where the level already decreased from 20 minutes post transplantation.

Results of this study show that early rearterialization is associated with less hepatocellular damage, less reperfusion injury and improved liver endothelial cell function. In conclusion, our results indicate that early rearterialization of the graft is beneficial to the transplanted liver.

Introduction

In clinical liver transplantation, reperfusion of the graft occurs after completion of the vena caval and portal venous anastomoses [384] [385]. Originally this was done to keep the portal occlusion time as brief as possible. However, despite the use of the porto-systemic veno-venous bypass to overcome the problems of portal occlusion, the portal vein is still anastomosed first [386]. The rationale for this was to shorten the anhepatic period and to minimize the rewarming of the ischaemic graft. The arterial anastomosis was performed thereafter, leading to a variable period, depending on the difficulty of the dissection of the artery, during which the graft was perfused by portal blood only.

However, in recent years, concerns have been expressed about the delay in performing the arterial anastomosis [388] [458]. Several experimental and clinical studies have shown beneficial effects in liver grafts perfused with arterial blood first [173] [387] [389]. However, quantitative evidence of the advantage of early rearterialization has been limited.

In this study, we investigated the impact of the timing of the arterial anastomosis on the hepatocellular injury, the reperfusion injury, and the endothelial cell function after liver transplantation.

Materials and Methods

The experimental protocol was approved by the Animal Research Review Committee of the University of Cape Town. Twenty-four young Large White X Landrace pigs of either sex and weighing 20-30 kg were fasted overnight and anaesthetized with intravenous sodium thiopentone (2mg/kg). Anaesthesia was maintained with nitrous oxide and oxygen administered through an endotracheal tube. Catheters (Fr8) were inserted into the common carotid artery, for monitoring of blood pressure and for sampling of arterial blood, and into the internal jugular vein for the infusion of intravenous fluids. The animals were subjected to orthotopic liver transplantation using the technique described previously [451].

Donor Operation

The abdomen was explored via a midline incision extending from the xiphisternum to the symphysis pubis. After the hilar dissection and mobilization of the suprahepatic and infrahepatic vena cava, the animal was heparinized and a catheter inserted into the portal

vein for initial flushing. Eurocollins solution was used in stead of University of Wisconsin solution to reduce costs. The liver was then excised during in situ flushing with ice-cold Eurocollins solution through the portal vein. Flushing of the liver with Eurocollins solution was continued on the back-table.

Storage

The donor liver was placed in a plastic bag with Eurocollins solution and stored on ice for three hours, since long storage times are associated with a poor survival in the liver transplantation model.

Recipient Operation

The preparation of the liver was as described above. After the dissection of the hilum of the liver and the suprahepatic and infrahepatic vena cavae, the animal was heparinized and the passive spleno-jugular venous bypass established. The recipient liver was excised, and the donor liver implanted by anastomosing the suprahepatic vena cava, infrahepatic vena cava, portal vein, hepatic artery and bile duct.

The animals were randomly allocated to the following treatments groups according to the order of the vascular anastomoses:

Group 1 (n=6): hepatic arterial perfusion 60 minutes after portal perfusion (PV-60-HA).
Group 2 (n=6): hepatic arterial perfusion 20 minutes after portal perfusion (PV-20-HA).
Group 3 (n=6): hepatic arterial perfusion simultaneous with portal perfusion (HA-PV).
Group 4 (n=6): hepatic arterial perfusion 20 minutes before portal perfusion (HA-20-PV).

Perioperative Management

During the operation the animals received an infusion of Plasmalyte B with added dextrose and 300 ml of donor blood to maintain haemodynamic stability. Postoperatively the pigs were returned to warmed cages and intravenous infusion continued for two days.

Blood sampling and assays

The moment of completed venous and arterial blood supply and the start of reperfusion through the liver through both vessels was set a moment zero, and times of sampling were calculated from there. (Negative values before reperfusion, positive values after revascularization.) The transplantations were performed in such a fashion, that this moment "zero" was exactly 3 hours post storage, to reduce an error in results due to variable ischemic times. Details about the various sampling times are stated in Table 5.1.

	Group 1	Group 2	Group 3	Group 4
-65 minutes	Sample			
-60 minutes	**Portal Reperfusion**			
-55 minutes	Sample			
-25 minutes	Sample	Sample		Sample
-20 minutes		**Portal Reperfusion**		**Arterial Reperfusion**
-15 minutes	Sample	Sample		Sample
-5 minutes	Sample	Sample	Sample	Sample
Zero	**Arterial Reperfusion**	**Arterial Reperfusion**	**Arterial and Portal Reperfusion**	**Portal Reperfusion**
+5 minutes	Sample	Sample	Sample	Sample
+20 minutes	Sample	Sample	Sample	Sample
+40 minutes	Sample	Sample	Sample	Sample
+60 minutes	Sample	Sample	Sample	Sample
+ 2 hours	Sample	Sample	Sample	Sample
+ 4 hours	Sample	Sample	Sample	Sample

Table 5.1 Blood sampling times in the four different groups.

Blood samples for AST's were taken from catheter in a peripheral vein, at baseline, 1 hour, 2 hours and 4 hours after completed transplantation. Blood samples for assessment of Hyaluronic acid were taken from a catheter in the hepatic vein, at baseline, 5, 20, 40 and 60 minutes after completion. Assessment of Malondialdehyde and Vitamin A was performed on

arterial blood samples, taken at 5 minutes before and after portal venous reperfusion, as well as 5 minutes before and after arterial reperfusion.

Plasma Malondialdehyde (MDA) levels were determined by the thiobarbituric assay as described by Lepage [452]. 2-Thiobarbituric acid (TBA) was added to deproteinized plasma and the reaction between the MDA and TBA, under conditions of low pH and high temperature, yielded a chromogenic adduct that was detectable by fluorometry.

Serum Vitamin A levels were measured fluorometrically, according to the original method described by Thompson [453]. Vitamin A, which fluoresces in ultraviolet light, was first extracted into water and ethanol, then into hexane and detected by fluorometry.

Serum Hyaluronic Acid (Ha) levels were estimated by radioimmunoassay using a kit purchased from Kabi Pharmacia [454]. 125 I-labeled Hyaluronic Acid binding protein (HABP), isolated from bovine cartilage, was used to bind the HA in the sample. The unbound 125 I-HABP was quantitated by incubating with HA covalently coupled to Sepharose particles. Separation was performed by centrifugation and decanting. The radioactivity bound to the particles was read in a gamma counter which was inversely proportional to the concentration of Ha in the sample.

Serum AST levels were determined spectrophotometrically using a continuous monitoring assay.

Results

The mean AST levels pre-operatively were similar in all four groups. There were significant increases in serum AST at one hour after total reperfusion, from 61±36 to 469±118 iu in group 1, 55±34 to 336±196 iu in group 2, 72±37 to 361±94 iu in group 3, and from 48±24 to 434±198 iu in group 4. The serum AST levels at 4 hours were highest in group 1 (925±234 iu) and lowest in group 4 (464±120 iu). This difference at 4 hours between the group 1 and the other groups was significant ($p<0.001$).

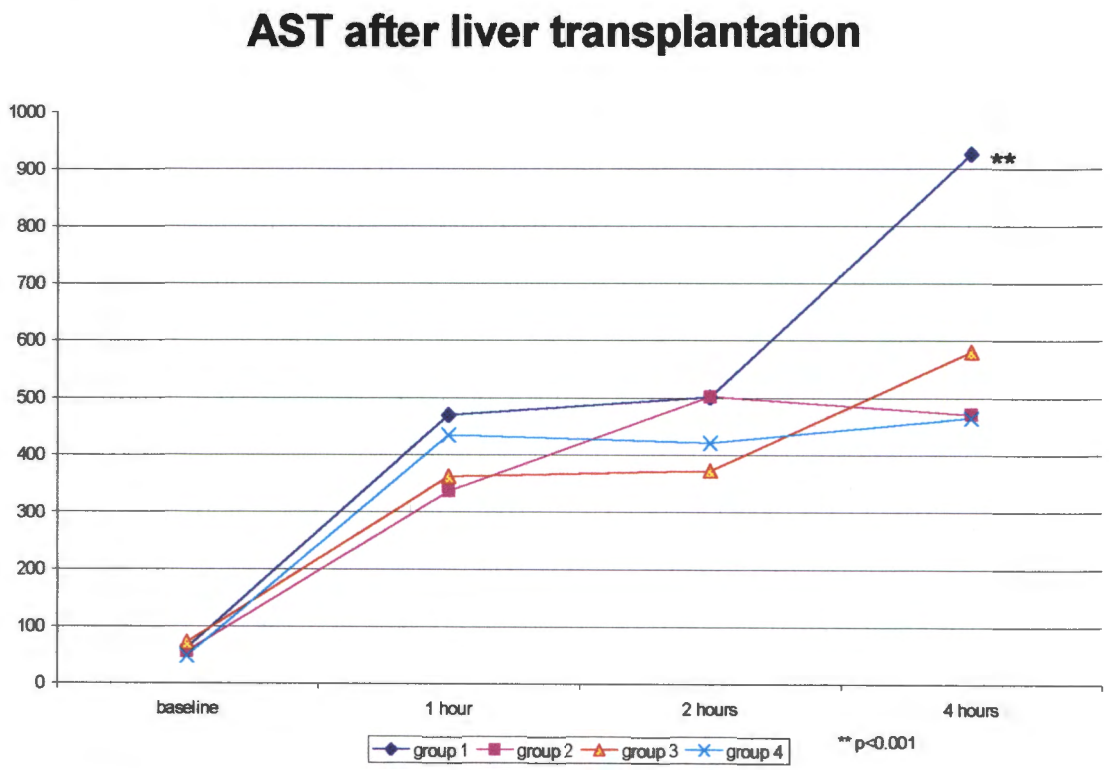


Figure 5.1 Arterial levels of serum AST at base, 1, 2 and 4 hours post transplantation in international units at various times.

The preoperative plasma malondialdehyde levels were similar in all 4 groups. There was a marked, but not statistically significant, increase in plasma malondialdehyde levels associated with portal reperfusion in the animals in groups 1, 2 and 3. However, plasma malondialdehyde levels remained unchanged in the animals in group 4 which received arterial blood 20 minutes before portal reperfusion. There was no further rise in malondialdehyde levels after portal revascularization. At 60 minutes after total reperfusion plasma MDA levels were similar in all four groups.

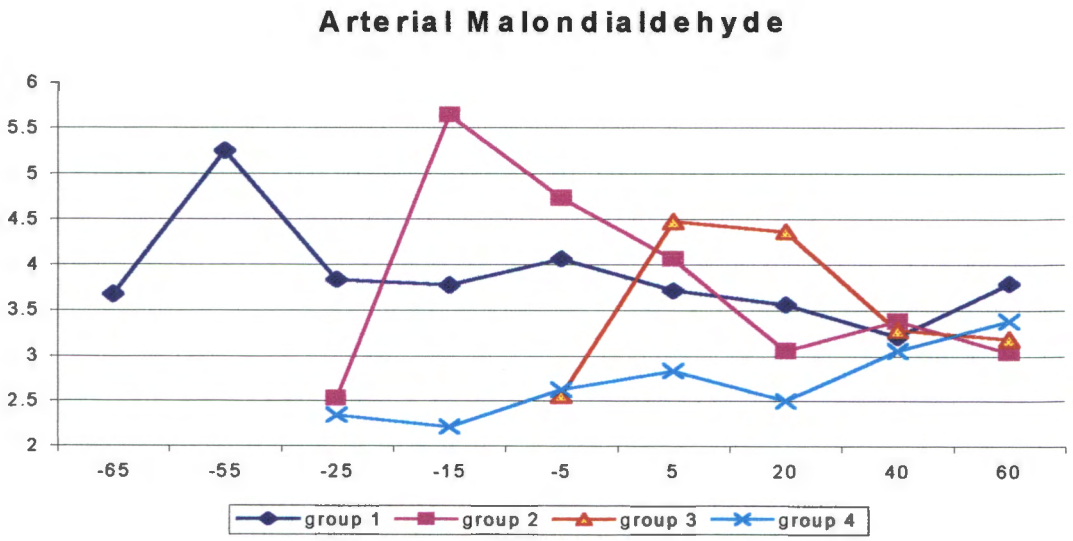


Figure 5.2 Arterial Malondialdehyde concentrations during and after liver transplantation in micromols/liter at various times in minutes.

	Group 1	Group 2	Group 3	Group 4
- 60 minutes	Venous reperfusion			
- 20 minutes		Venous reperfusion		Arterial reperfusion
zero	Arterial reperfusion	Arterial reperfusion	Arterial and venous reperfusion	Venous reperfusion

Table 5.2 Arterial and venous reperfusion times in the various groups.

In the animals in groups 1 and 2, which received portal blood before arterial blood, portal reperfusion was associated with a decrease in serum vitamin A levels and levels remained low for the duration of the study. In contrast the vitamin A levels in the animals in groups 3 and 4, which received arterial blood before or simultaneous with portal reperfusion, remained elevated for the duration of the study. Vitamin A levels were significantly higher after portal reperfusion in the animals in groups 3 and 4, compared to those in groups 1 and 2.

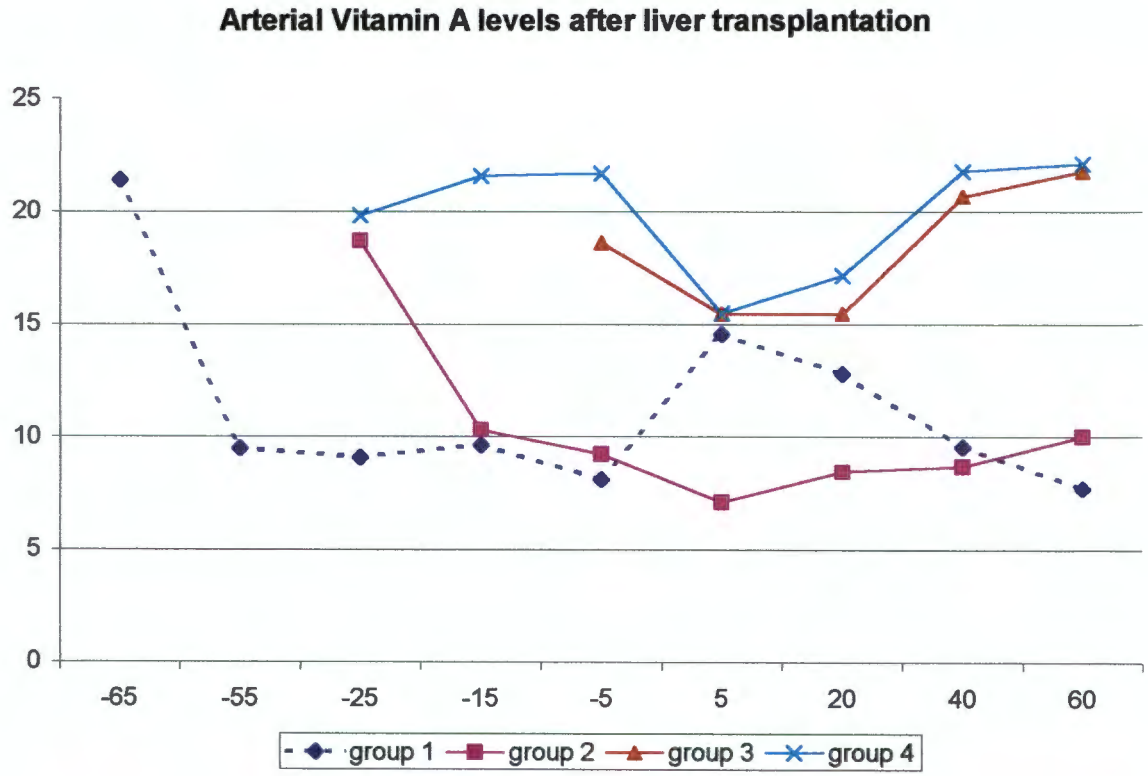


Figure 5.3 Arterial Vitamin A levels from base till 60 minutes after liver transplantation in mg/l at various times in minutes.

	Group 1	Group 2	Group 3	Group 4
- 60 minutes	Venous reperfusion			
- 20 minutes		Venous reperfusion		Arterial reperfusion
zero	Arterial reperfusion	Arterial reperfusion	Arterial and venous reperfusion	Venous reperfusion

Table 5.3 Arterial and venous reperfusion times in the various groups.

The baseline serum hyaluronic acid (Ha) levels were similar in all four groups. There was a significant increase in Ha levels in all four groups of animals ($p<.00001$). There were no significant differences between Ha levels of the various groups.

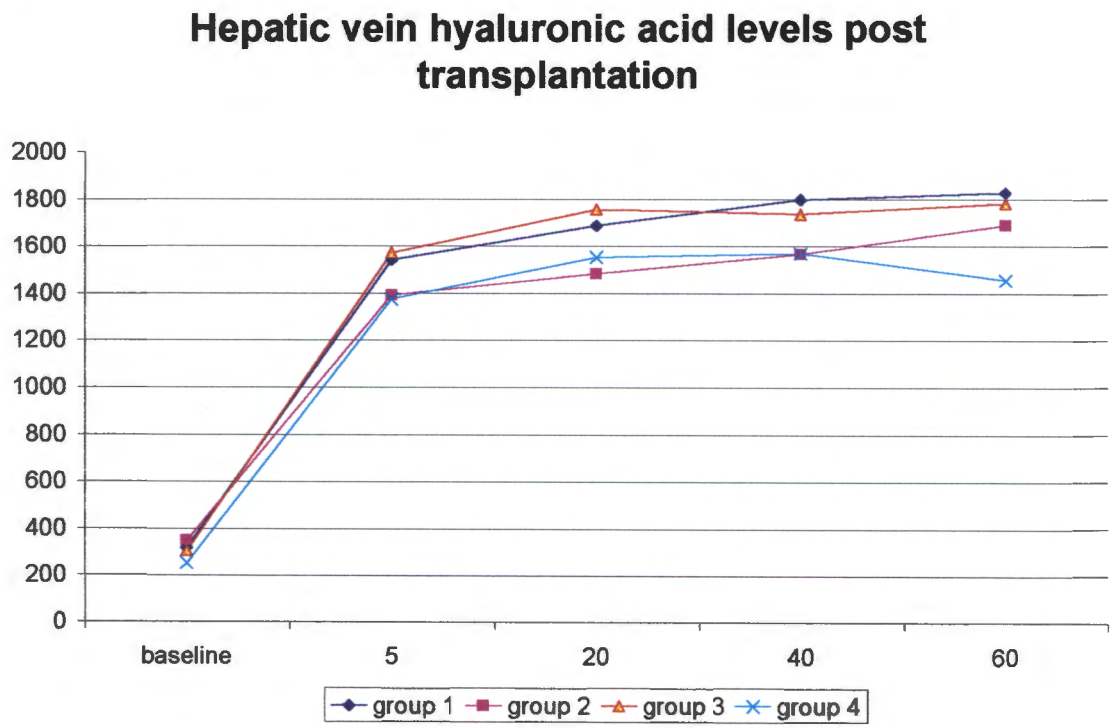


Figure 5.4 Serum Hyaluronic acid levels at base up to 60 minutes post transplantation in microgram/l at various times in minutes.

Discussion

The standard technique of liver transplantation involves initial reperfusion of the graft with portal blood followed by arterial perfusion. The delay in arterialization of the graft could vary from about 20 minutes to beyond one hour, depending on the difficulty of the arterial dissection or whether an arterial conduit is needed. This order of revascularization has been questioned in recent years. One clinical study comparing liver allografts rearterialized soon after portal reperfusion with delayed rearterialization failed to show any difference in liver function and patient and graft survival. However, several clinical and experimental studies have shown an advantage for liver allografts rearterialized before portal reperfusion [173 387 388 389]. In this study we investigated the impact of the timing of rearterialization in liver transplantation using more sensitive parameters of injury, including markers of hepatocellular injury, reperfusion injury and endothelial cell function.

In this study liver allografts rearterialized 20 minutes after portal reperfusion (PV-20-HA), which represents a standard uncomplicated transplant, were compared with rearterialization 60 minutes after portal reperfusion (PV-60-HA), which represents the difficult hepatic artery dissection, and rearterialization simultaneous with (HA-PV) or prior to (HA-20-PV) portal reperfusion, representing the new trend in clinical liver transplantation.

All transplanted livers undergo preservation-reperfusion injury. The pathophysiology involved in this preservation-reperfusion injury and possible preventative modalities have been extensively reviewed [66]. The effect of the timing of rearterialization of the liver allograft on the reperfusion injury has not been documented previously. The reperfusion injury is known to be mediated, at least in part, by the formation of reactive oxygen metabolites, which damage a spectrum of bio-molecules found in tissues, including membrane lipids. Peroxidation of these membrane lipids results in the formation of malondialhyde, which can thus be used as a marker of reperfusion injury. Vitamin A is a potent endogenous scavenger of reactive oxygen metabolites, and can also be used as a parameter of the reperfusion injury.

In this study, there was a marked increase in MDA levels after portal reperfusion in the animals which received arterial blood after or simultaneous with portal reperfusion. In the animals which received arterial blood before portal reperfusion, MDA levels remained unchanged. This indicates that, using MDA as an indicator, there was no detectable reperfusion injury associated with portal reperfusion in the animals which received arterial blood before portal reperfusion.

This abrogation of the reperfusion injury by early arterialization was confirmed by the vitamin A levels remaining elevated in the animals which received arterial blood before portal

reperfusion. The nearly identical patterns of vitamin A levels in group 3 and 4 add further support for the theory that primarily portal reperfusion is responsible for reperfusion injury, rather than arterial reperfusion. The decrease in vitamin A levels in the animals with portal reperfusion before arterialization also confirmed the reperfusion injury in these animals.

Interestingly, there appeared to be evidence of reperfusion injury after portal reperfusion but not after arterialization. This may be related to the difference in the oxygen content between the arterial and portal blood. Although the arterial blood has a higher oxygen content, the major part of the total oxygen delivery to the liver is from the portal vein [455].
Serum AST levels were the highest in group 1, indicating that delayed arterial reperfusion resulted in a significant increase of hepato-cellular damage.

Hyaluronic acid (Ha) is removed primarily by hepatic endothelial cells [459]. Increased Ha levels have been demonstrated in several types of liver disease [460]. Since hepatic endothelial cell dysfunction is associated with increased Ha levels, Ha is considered to be a potential marker of hepatic endothelial cell function. In this study, there were no statistical differences between Ha levels of the four groups.

Conclusion

This study showed that early arterialization prior to portal reperfusion after liver transplantation was associated with less reperfusion injury and less hepatocellular injury. There was no demonstrable effect of earlier HA revascularization on endothelial dysfunction. These findings would support previous suggestions that in liver transplantation, the allograft should be rearterialized before portal reperfusion.

CHAPTER 6

Histological Assessment After Different Methods of Reperfusion after Liver Transplantation

Summary

Preservation – reperfusion injury is a major cause for graft failure after liver transplantation. This injury refers to a variety of insults after reperfusion of the graft, independent from technical errors, vascular problems, immunological reactions or infection. Significant injuries occur during the period of cold preservation. Loss of sinusoidal endothelial attachments to the underlying extracellular matrix results in loss of the normal antithrombogenic milieu. Reperfusion with recipient blood results in platelet aggregation and neutrophil sludging in all areas of denudation, preventing adequate reoxygenation. Early histopathological findings in biopsy specimens can predict poor graft outcome. The aim of this study is to analyze the effect of early rearterialization on histological findings of the liver biopsies after liver transplantation.

Twenty young Large White X Landrace pigs (weight 22-28 kg) were subjected to orthototopic liver transplantation. Livers were stored in Eurocollins solution for three hours on ice and the animals were randomized in four different groups of increasing earlier arterialization. Groups were aligned from delayed rearterialization (group 1) to early rearterialization (group 4), Biopsies were taken before and after cold storage, as well as 1 hour post transplantation.

Results show that both hepatocyte vacuolization and neutrophil infiltration were significantly reduced in group 4 (early rearterialization), as compared to group 1, 2 and 3.

Single cell necrosis and group cell necrosis of the hepatocytes were both significantly reduced in group 3 and 4 as compared to group 1 and 2 (early venous reperfusion).

In this study it is demonstrated that an early rearterialization is associated with a decrease in early histopathological changes in the transplanted liver, indicating a lesser amount of primary dysfunction at a later state.

Introduction

The majority of allografts which fail early after liver transplantation are due to preservation - reperfusion injury, vascular thrombosis, humoral rejection or a combination of these [461 462 463]. Preservation - reperfusion injury refers to a variety of pathological processes resulting in allograft dysfunction that begin immediately after transplantation and are not readily explained on the basis of a technical or vascular problem, alloimmunological reaction or infection [458 464 465 466 467].

Clinical and experimental animal studies show that one of the most significant injuries occurs during the period of cold preservation, when the donor organ is stored in a physiologically compatible preservation solution [66 468 469]. Loss of sinusoidal endothelial attachments to the underlying extracellular matrix results in loss of the normal antithrombogenic milieu. Reperfusion with recipient blood results in platelet aggregation and neutrophil sludging in all areas of denudation. Microvascular thrombosis and localized activation of neutrophils with release of oxidative enzymes act together to diminish bloodflow and prevent adequate reoxygenation.

It is well known that routine histopathological findings in biopsy specimens taken within hours after completed revascularization can, with reasonable accuracy, predict poor allograft function during the first few postoperative weeks [464 465].

Specific indicators of severe preservation injury and reperfusion injury include zonal or confluent coagulative necrosis, particularly if it is periportal or bridging, and severe neutrophilic exudation [464 465 466 467]. The subscapsular parenchyma is the most susceptible to damage in the peri-operative period [470].

Histopathological changes of less severe hepatic cellular ischemic injury, usually reversible, include microvesicular steatosis and hepatocellular cytoaggregation. Cytoaggregation is a reversible form of cell injury manifest morphologically by a "rounding-up" of the hepatocytes, so that the cell assumes a rounded appearance instead of the normal polygonal configuration.

The aim of this study is to assess the impact of early rearterialization on the histology of open liver biopsies after liver transplantation.

Materials and Methods

Twenty young Large White X Landrace pigs (weight 22-28 kg) were subjected to orthototopic liver transplantation. Intravenous Sodium Thiopentone was used for induction of the anaesthesia, while anaesthesia was maintained with nitrous oxide and oxygen administered through an endotracheal tube.

Donor operation: A midline laparotomy was performed and the liver mobilized, starting from the suprahepatic inferior vena cava downwards, followed by hilar dissection and dissection of the infrahepatic inferior vena cava. After this dissection the first liver biopsy was taken. All liver biopsies were taken as a 1 cm by 1 cm wedge from the anterior border of the right lobe of the liver. The liver surface was secured with chromic catgut (2.0). The liver was than flushed with ice-cold Eurocollins Solution through the portal vein. Flushing was continued on the backtable, where both the hepatic artery and the bile duct were flushed.

Storage: The donor livers were placed in a plastic container with Eurocollins solution and stored for three hours on ice. After this storage period the second liver biopsy was taken.

Recipient operation: The animals were randomized in four different groups of increasing earlier arterialization. In group 1 the hepatic artery was perfused only one hour after portal vein perfusion, while in group 2 the hepatic artery was perfused 20 minutes after portal vein perfusion. In group 3 the hepatic artery was reperfused simultaneously with the portal vein, while in group 4 the hepatic artery was perfused initially, 20 minutes before the portal vein perfusion. The third liver biopsy was taken 1 hour after completed revascularization.

Technique: Tissues were fixed in a buffered 10% formalin solution. Following fixing the specimens were processed automatically in a tissue processor overnight , through alcohol 70%, 96% and absolute alcohol, xylol to paraffin wax. The tissues were then embedded in paraffin wax for cutting. Sections were cut with the aid of a microtome 2 micrometer thick, floated onto glass slides and fixed onto the slide with aid of heat (55-60 degrees Celsius). The sections were stained using Mayer's haematoxylin eosin method [471].

The sections of the liver were assessed for the following features: 1. Hepatocyte vacuolization; 2. Single cell necrosis of the hepatocytes; 3. Group cell necrosis of the hepatocytes; 4. Infiltration of neutrophils. Morphometric analysis of the histological indicators was performed on a semi-quantitative assessment of changes based on a score from 0 to 5, in which zero represents normal tissue, and the score increases gradually from 1 (representing minimal changes) to 5 (representing severe alteration from normal tissue). All sections were assessed blindly by two independent pathologists with experience in the transplantation field.

All statistics were calculated according to the method of the least significance difference (General ANOVA) as decribed by George Snedecor and William Cochran [472].

Results

Results of the hepatic biopsies at base, post storage and one hour after full revascularization are shown.

The results of hepatocyte vacuolization are shown in figure 6.1. The mean value of the five animals is shown in each group. Hepatocyte vacuolization appeared to be the highest in group 2 and 3, while it was the lowest in group 4. Vacuolization was less in group 1, compared to group 2 and 3, but more than group 4. This difference was significant ($p<0.03$).

A typical example of hepatocyte vacuolization is shown in picture 6.1 (Original magnification 100X) and picture 6.2 (Original magnification 400X).

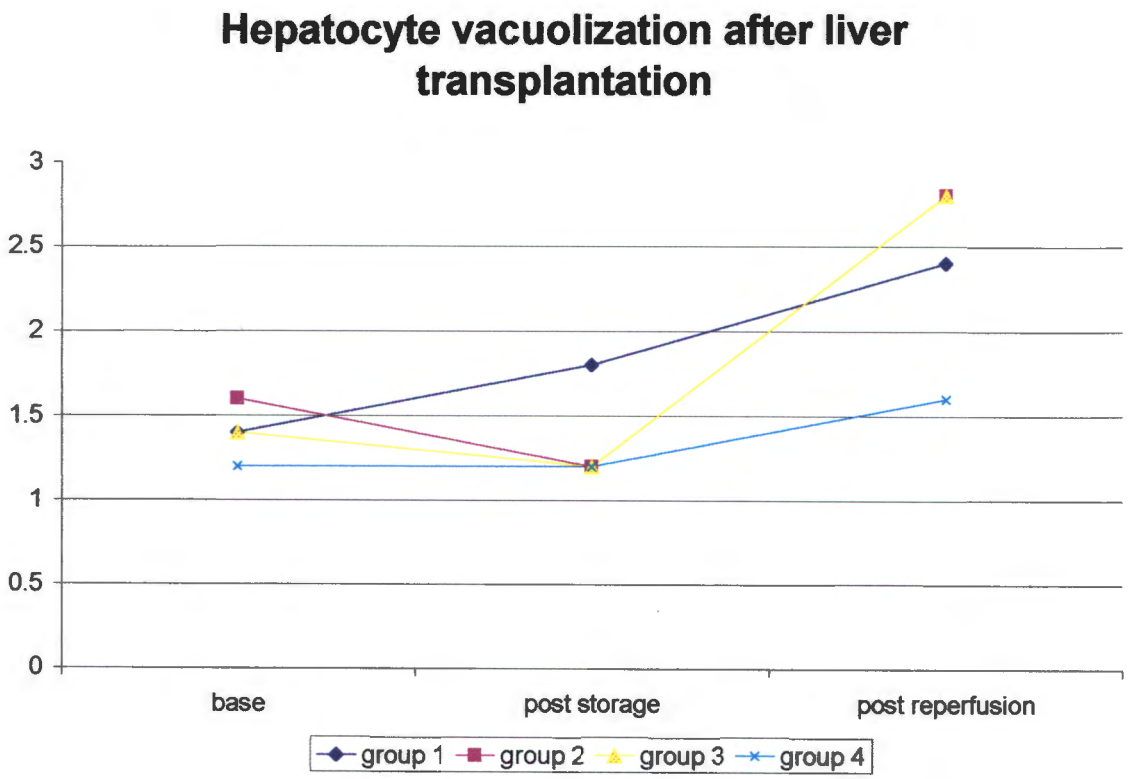
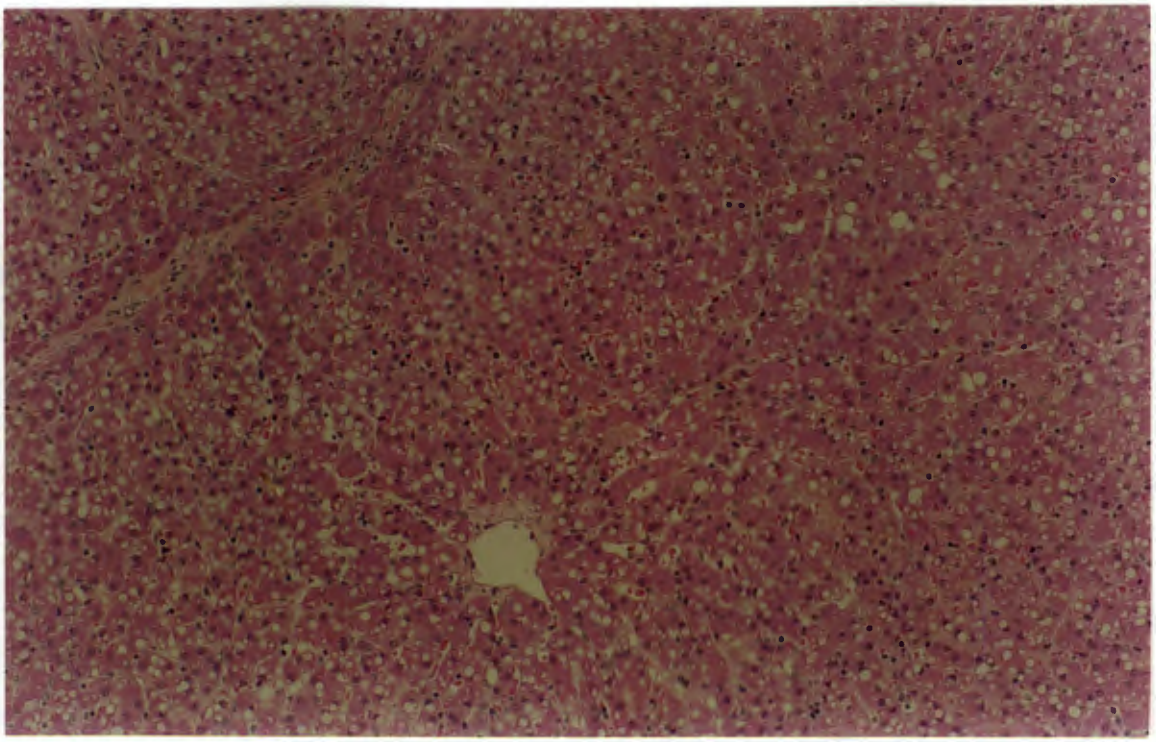
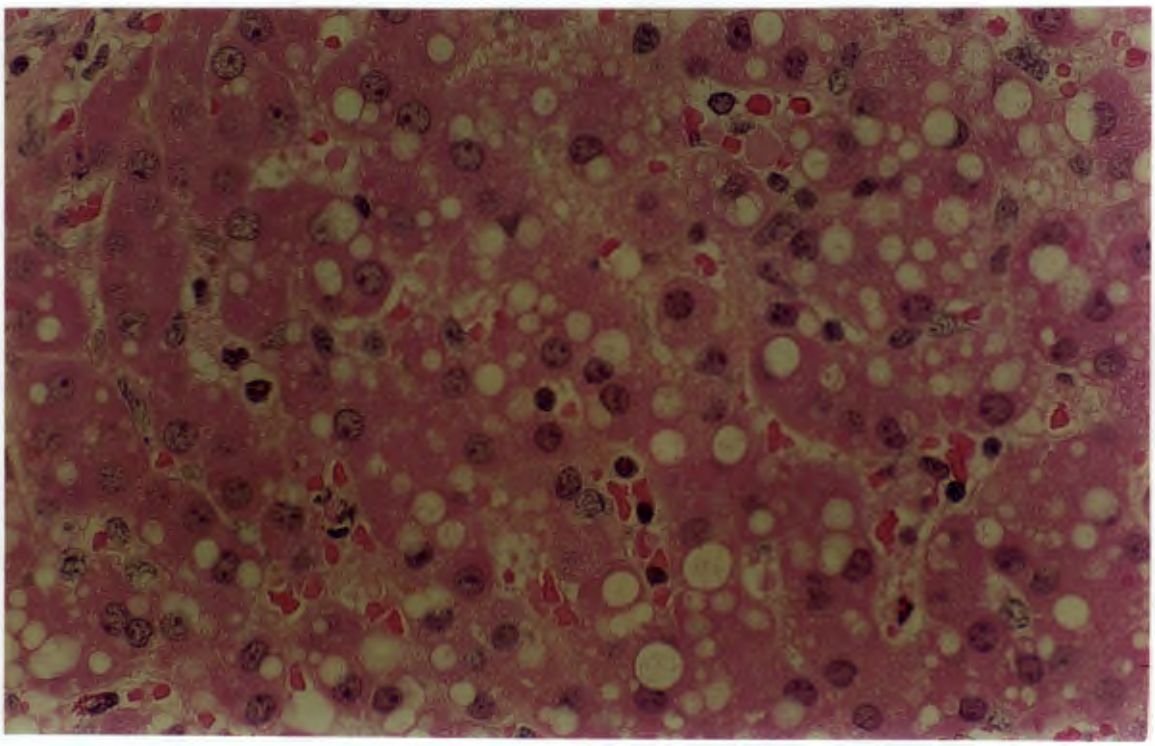


Figure 6.1 Mean hepatocyte vacuolization after liver transplantation in the four groups in a semi-quantitative score from 0 to 5.

Picture 6.1 Hepatocyte vacuolization. Original magnification 100X.



Picture 6.2 Hepatocyte vacuolization. Original magnification 400X.



Single cell necrosis is shown in figure 6.2. In group 1 single hepatocyte cell necrosis was the highest, followed by group 2. Single cell necrosis was significantly less in group 3 and 4 compared to group 1 and 2 ($p<0.001$). There was also a statistical difference between group 3 and 4 ($p<0.05$).

A typical example of hepatocyte cell necrosis can be seen in picture 6.3 (original magnification 200X) and picture 6.4 (original magnification 400X).

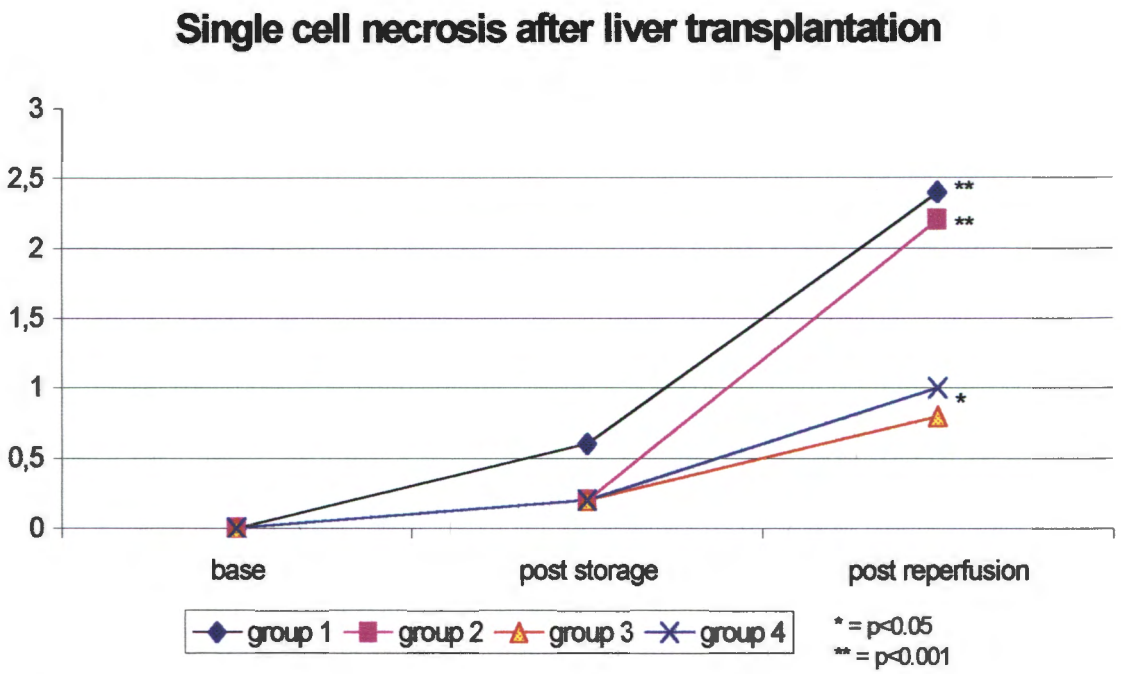
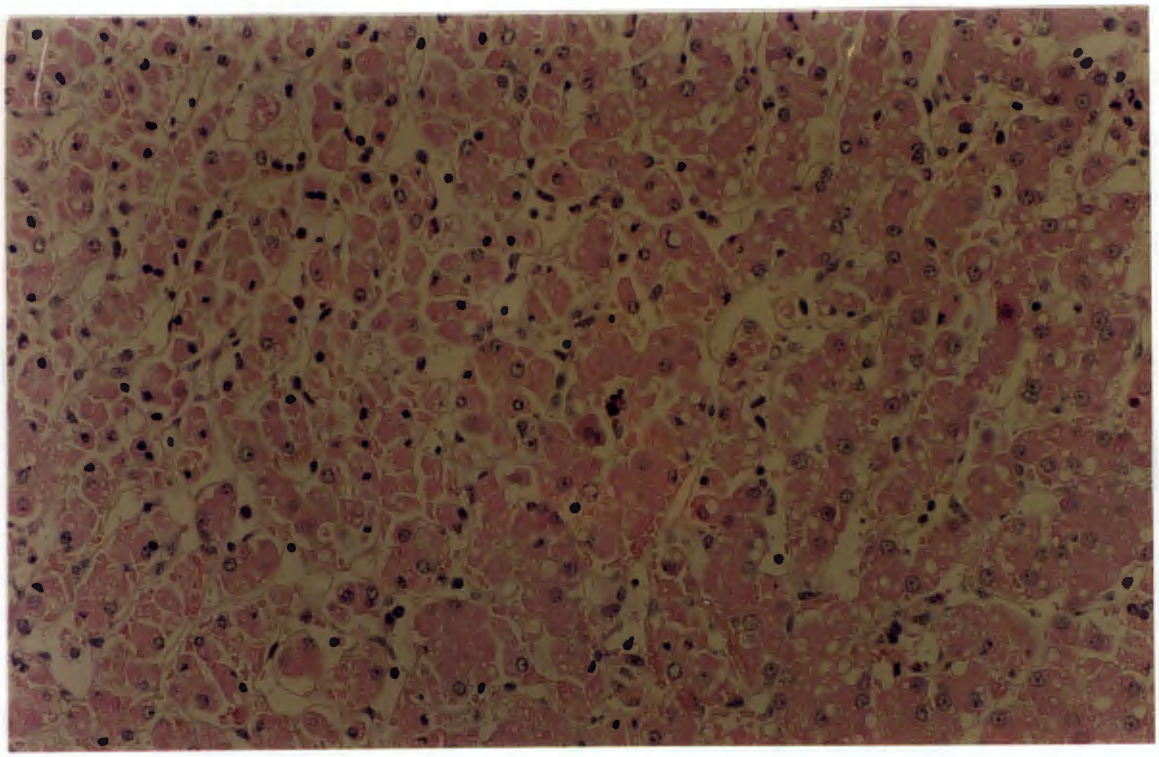
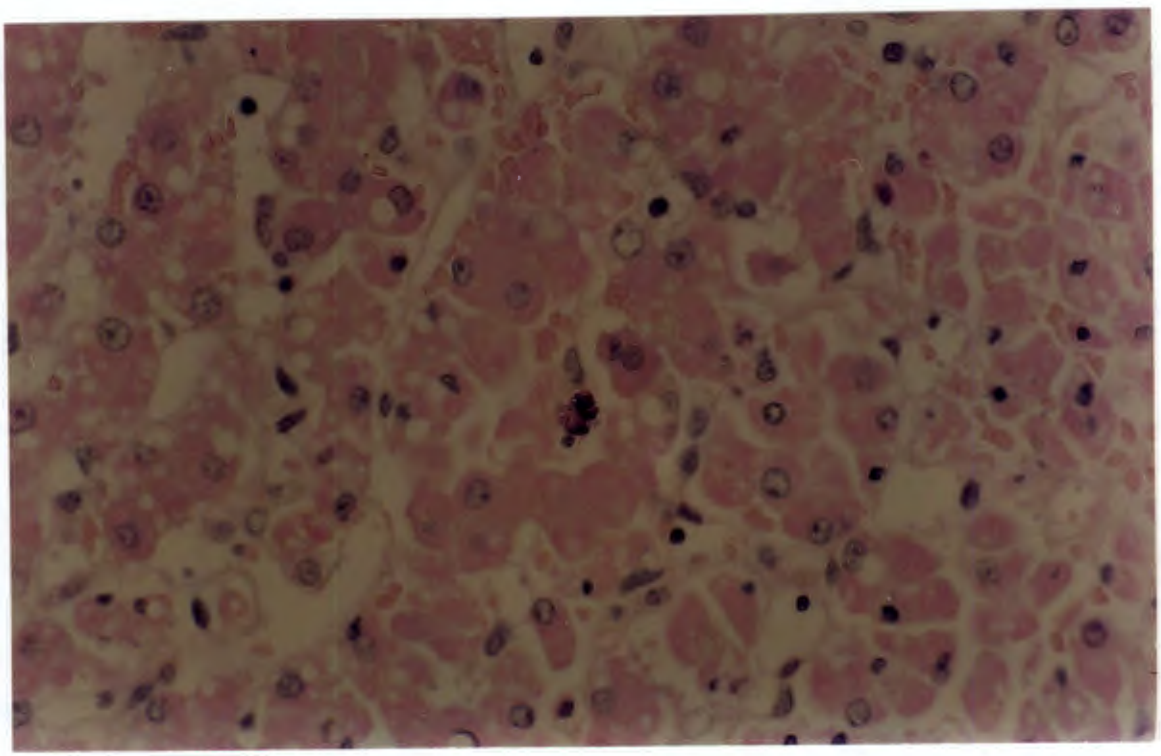


Figure 6.2 Mean single cell necrosis after liver transplantation in the four groups in after liver transplantation

Picture 6.3 Single cell necrosis of hepatocytes. Original magnification 200X.



Picture 6.4 Single cell necrosis of hepatocytes. Original magnification 400X.



Group cell necrosis of hepatocytes is demonstrated in figure 6.3. Again, necrosis is the highest in group 1, followed by group 2, while there is minimal group cell necrosis in group 3 and 4. The difference between the groups was statistically significant ($p<0.001$).

A typical example of group cell necrosis can be seen in picture 6.5 (original magnification 200X) and picture 6.6 (original magnification 400X).

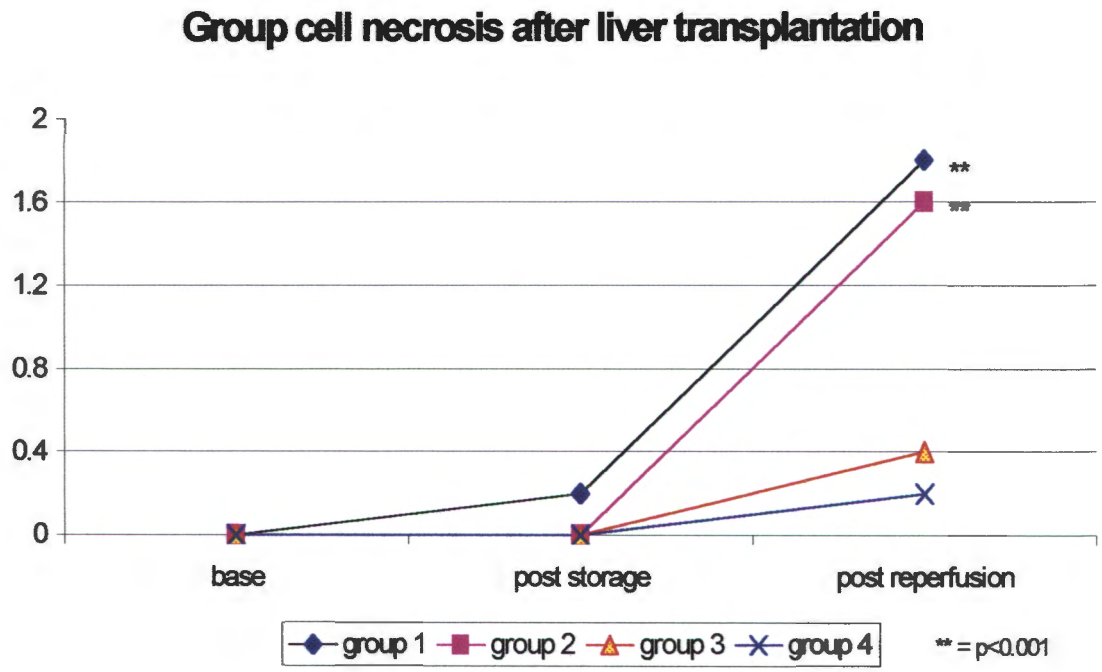
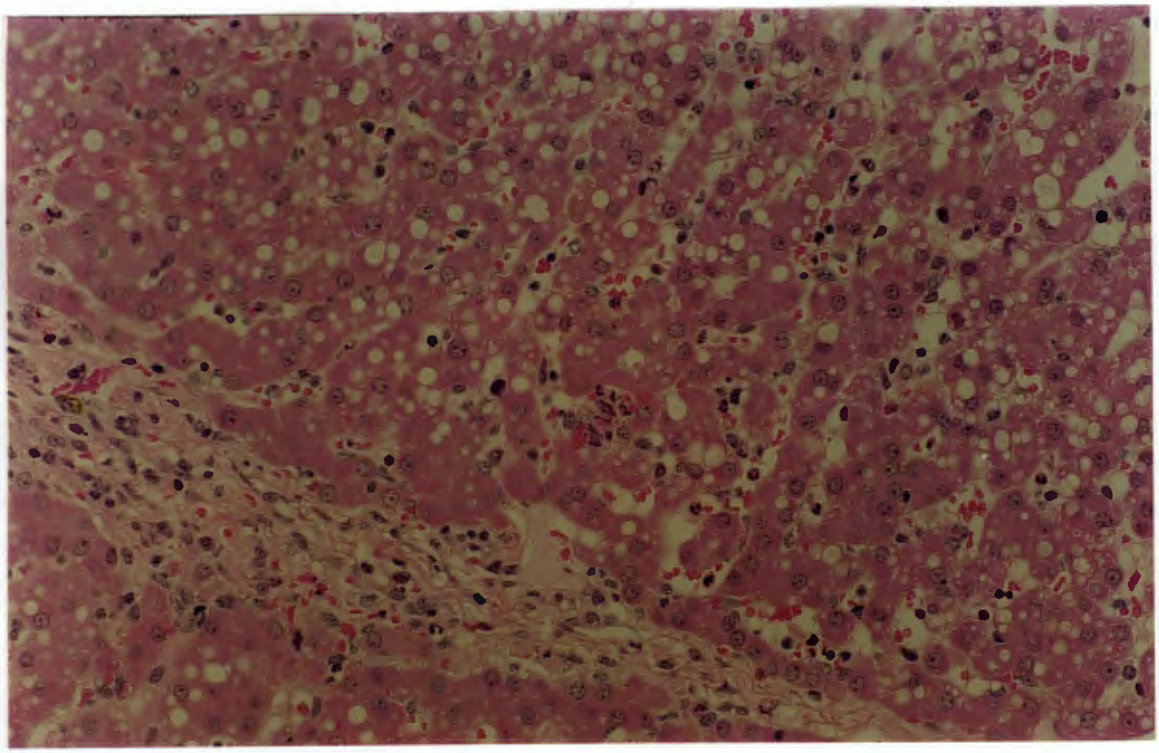
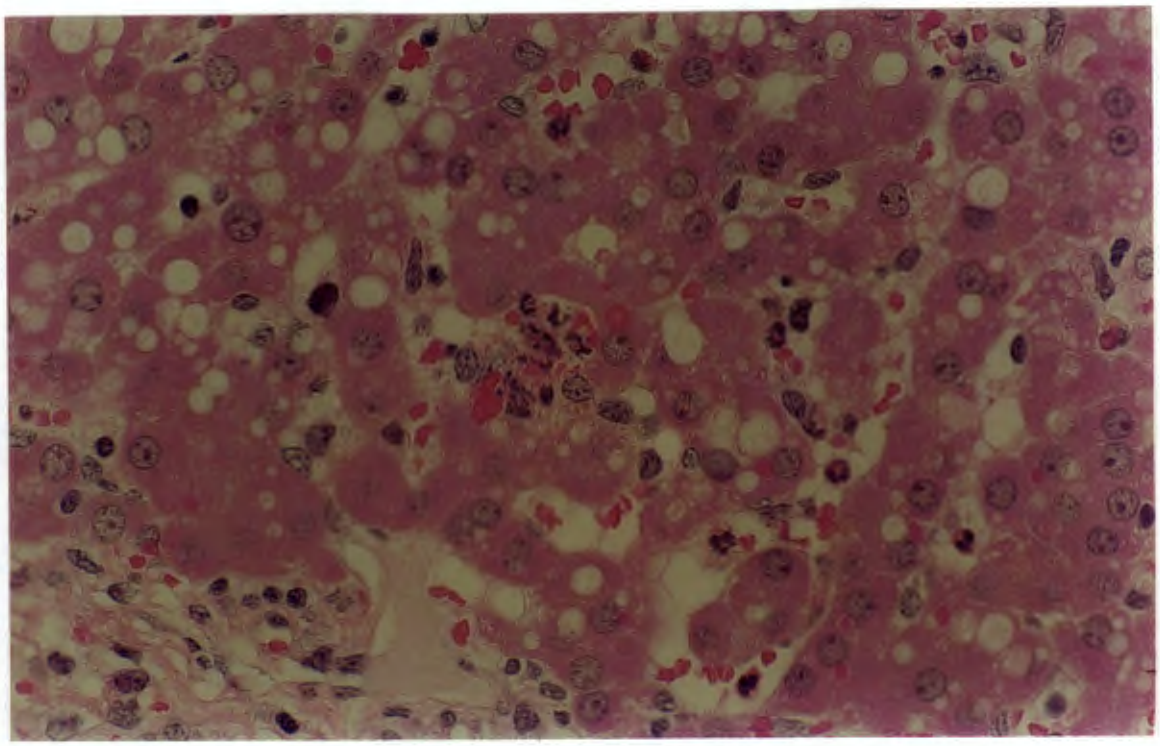


Figure 6.3 Mean group cell necrosis in the four groups after liver transplantation in a semi-quantitative score.

Picture 6.5 Group cell necrosis of hepatocytes. Original magnification 200X.



Picture 6.6 Group cell necrosis of hepatocytes. Original magnification 400X.



Infiltration of the hepatic tissue with neutrophils is demonstrated in figure 6.4. Infiltration of neutrophils was significantly raised after transplantation in group 1, 2 and 3 ($p<0.005$, $p<0.02$ and $p<0.02$), but remained low in group 4.

A typical example of neutrophil infiltration is shown in picture 6.7 (original magnification 200X) and picture 6.8 (original magnification 400X).

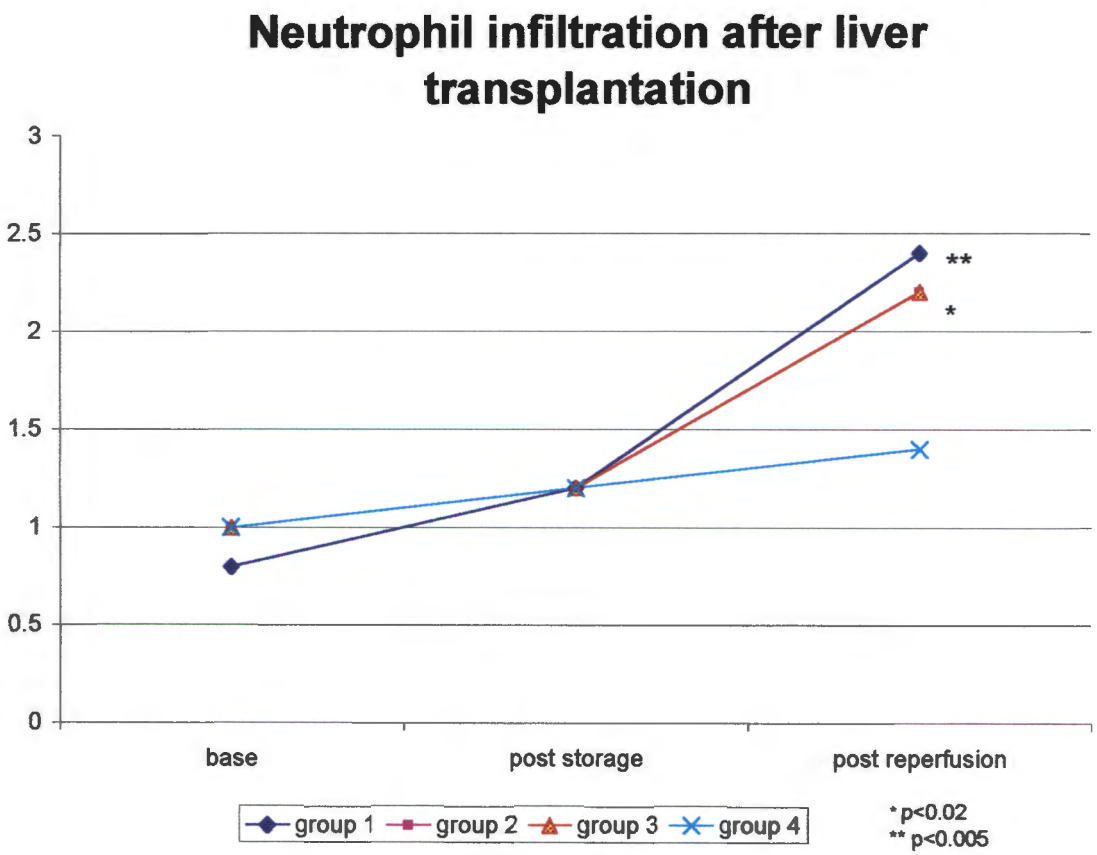
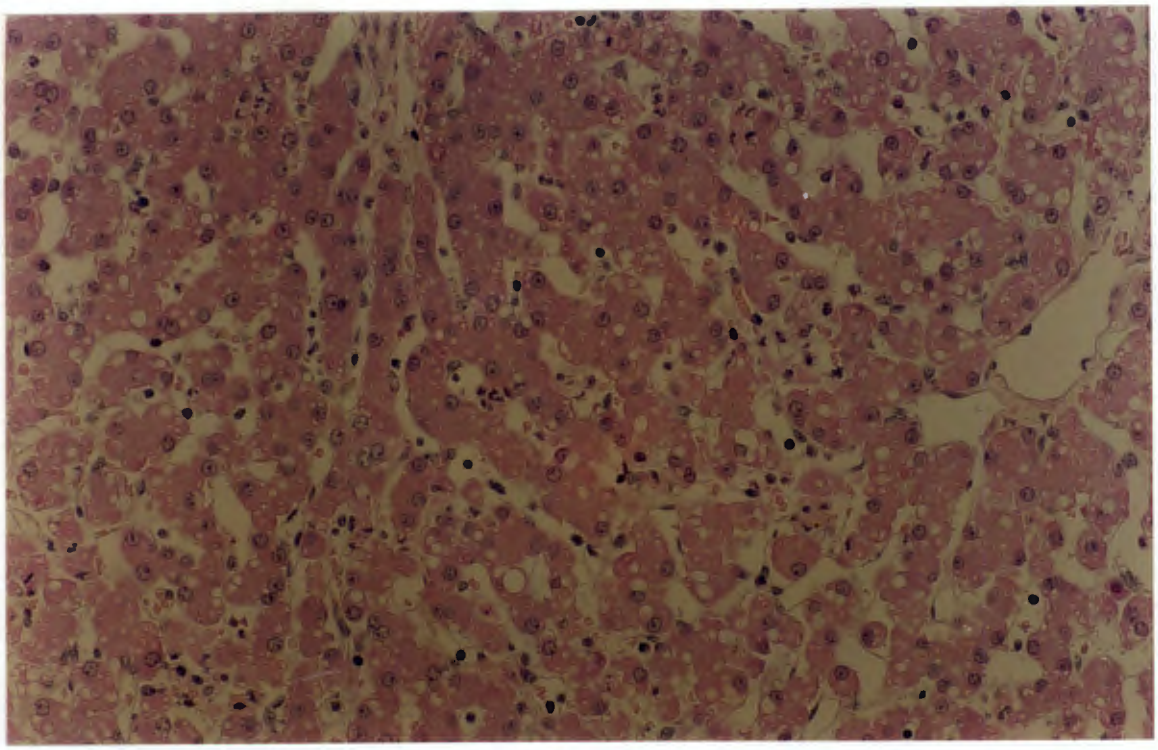
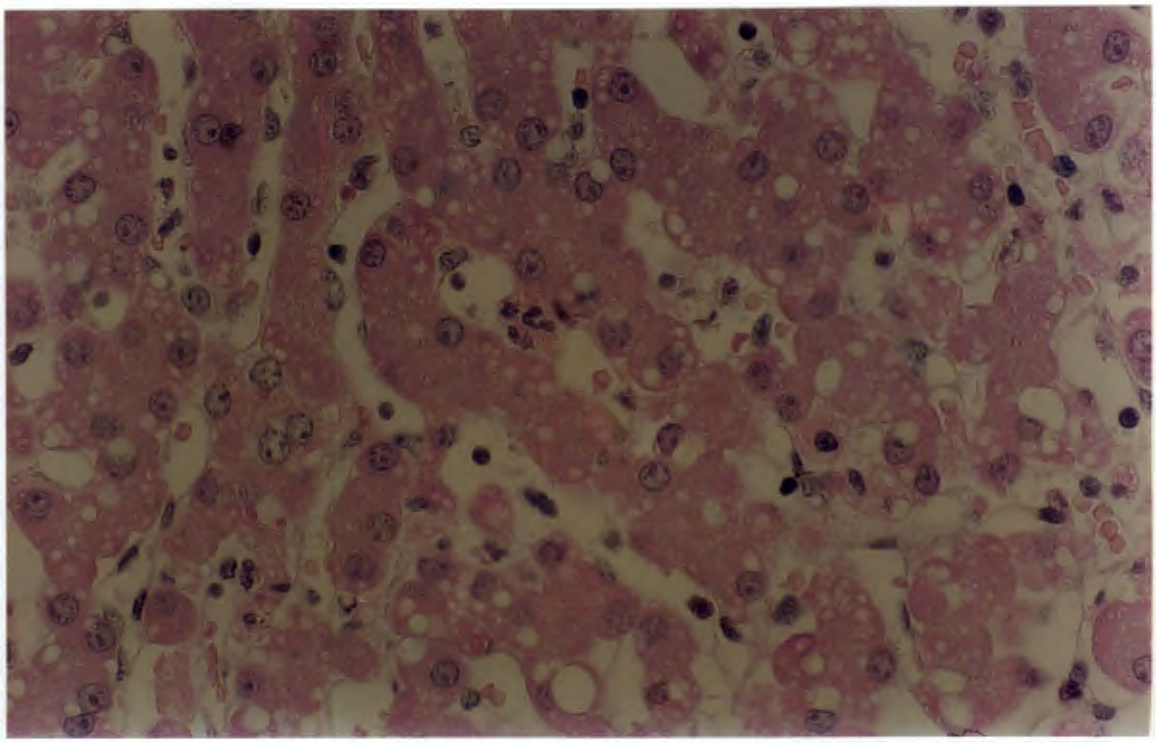


Figure 6.4 Mean neutrophil infiltration after liver transplantation in the four groups in a semi-quantitative score.

Picture 6.7 Neutrophil infiltration in the transplanted liver. Original magnification 200X.



Picture 6.8 Neutrophil infiltration in the transplanted liver. Original magnification 400X.



Discussion

In normal liver transplantation, arterialization of the hepatic artery takes place after portal venous reperfusion. This leads to variable intervals between the reperfusion of the two vessels, dependent on the sometimes technically difficult arterial anastomosis [385] [386]. Recently, there are various reports of the advantage of early rearterialization of the liver after orthotopic liver allograft [387] [388] [389]. Although there are several publications looking at a variety of biochemical indicators and clinical factors, there has been no objective histological measurements.

In this study we looked specifically at the histopathology of transplanted livers after various modes of reperfusion. Early rearterialization was associated with less histological evidence of injury in all four categories looked at. In fact, our results show a correlation between the degree of injury and the time of rearterialization.

As indicated earlier by Kakizoe and Yanaga [458], most animal models evaluating preservation - reperfusion injury allow a precisely controlled analysis but ignore the contribution of the arterial blood flow and the metabolic derangements caused by a poorly functioning native liver. In humans, arterial flow plays a more vital role. The time sequence between reperfusion of the venous and arterial systems may vary considerably, particularly when difficulties are encountered with the arterial anastomosis or when an arterial graft has to be used. During this time, the liver is reperfused and warmed by relatively hypoxic , endotoxemic portal blood, which may contribute to an extension of the preservation - reperfusion injury.

Conclusion

It is clearly demonstrated that an early rearterialization is associated with a decrease in early histopathological changes in the native liver.

CHAPTER 7

The Effect of Celsior, Wisconsin and Eurocollins Solution on the Hepatocellular, Reperfusion and Endothelial Cell Injury after Porcine Liver Transplantation

Summary

Initially, preservation solutions were developed to maintain cell function of the transplanted organs. However, recently developed preservation solutions also contain a variety of substances to reduce the reperfusion injury. In this study, we investigated the effect of three different preservation solutions, on the liver cell injury, endothelial cell function and reperfusion injury after liver transplantation.

Large White X Landrace pigs of either sex were subjected to orthotopic liver transplantation. Donor livers were flushed and stored in University of Wisconsin Solution (UW), Eurocollins Solution (EU) or Celsior Solution (CEL) for 3 hours. Blood samples were taken at various times post transplantation for assessment of aspartate aminotransferase (AST), hyaluronic acid (Ha), malondialdehyde (MDA) and Vitamin A (Vit A) levels.

Serum AST levels were lower in the livers preserved in the UW solution. Plasma MDA levels were lower and serum Vit A levels were higher in the livers preserved in CEL solution. Serum HA levels increased after liver transplantation but were similar with all three solutions.

These studies show that there was less hepatocellular injury in the livers preserved in UW Solution and less reperfusion injury with the CEL solution. The endothelial cell injury was similar with all three solutions.

Introduction

Clinical liver transplantation is now performed routinely in the treatment of end-stage liver disease. Although much progress has been made in the field of organ preservation, it remains a popular topic of research, as a result of the increasing demand for better-functioning donor organs.

In 1969 Collins described a cold storage solution for kidney preservation [95]. This solution was used initially in clinical liver transplantation as well, with preservation times limited to 6-8 hours. The University of Wisconsin solution was introduced into clinical liver transplantation in the 1980's and resulted in not only an extension of the preservation time to 18-24 hours but also an improvement in the quality of the donor organ. Celsior was originally developed as a cardioplegic and cardiac preservation solution. Recently efforts have been made to establish Celsior as a liver and kidney preservation solution. One of the advantages of Celsior would be that a single solution could be used for the preservation of all organs.

It is becoming increasingly apparent that the preservation of the liver is extremely complex and involves many forms of injury, besides ischaemia, to both parenchymal and non-parenchymal cells. Thus new preservation solutions not only have to prolong the duration of preservation and improve the quality of preservation, but should also modify the various types of injury. In this study, we investigated the effect of UW solution, Celsior and Eurocollins solution on the hepatocellular injury, reperfusion injury and endothelial cell injury after liver transplantation.

Materials and Methods

The study was approved by the University of Cape Town Animal Research Review Committee. 15 Large White X Landrace pigs weighing 20-30kg were anaesthetized with intravenous thiopentone sodium (2mg/kg) and anaesthesia maintained with nitrous oxide and oxygen delivered via an endotracheal tube. Catheters (Fr8) were inserted into the internal jugular vein for the infusion of intravenous fluids and into the carotid artery for blood sampling and blood pressure monitoring. The abdomen was explored via a midline incision extending from the xiphisternum to the symphysis pubis.

Donor Operation

The ligaments attached to the liver were divided and the porta hepatis dissected to isolate the portal vein, hepatic artery and bile duct. The supra- and infra-hepatic vena cavae were also isolated. At this stage the animals were heparinized and a perfusion cannula placed in the portal vein. The vessels were clamped and the liver perfused in situ with ice-cold preservation solution (according to the randomization below) via the portal vein. The liver was excised and perfusion with ice-cold preservation solution continued ex situ via the portal vein, hepatic artery and bile duct. The portal vein was perfused with 700 ml, the hepatic artery with 200 ml and the bileduct with 100 ml of the particular solution. Thereafter the liver was secured in a plastic bag with the remaining cold storage solution and stored on ice for three hours.

Preservation Solution

The donor livers were randomly allocated to the following treatment groups, and perfused and preserved as follows:

Group 1 (n=5): Eurocollins solution

Group 2 (n=4): University of Wisconsin solution (UW)

Group 3 (n=6): Celsior solution

All livers were preserved for three hours prior to transplantation.

Recipient Operation

The preparation of the recipient liver was similar to the donor liver as described above. After heparinization, the non-pulsatile veno-venous bypass from the splenic vein to the external jugular vein was established. The bile duct was divided, the vessels clamped and the recipient liver excised.

Implantation of the donor liver was achieved by anastomosing the suprahepatic vena cava, the infrahepatic vena cava and the portal vein. These vessels were unclamped and the liver perfused with portal blood. Thereafter the hepatic arterial anastomosis was completed. Finally, the biliary anastomosis was performed and the veno-venous bypass removed.

postoperatively. The blood samples were used to evaluate the serum AST, plasma malondialdehyde, serum vitamin A and serum hyaluronic acid levels.

Sampling and Assays

Blood samples were taken at various time intervals. From previous studies it has been shown that the first hour is the most sensitive to alterations in reperfusion injury markers. The reperfusion injury markers (Malondialdehyde and Vitamin A) were measured at base, before portal reperfusion (pre-port), after portal reperfusion (post-port), before arterial reperfusion (pre-art), after arterial reperfusion (post-art) and 5, 20, 40 and 60 minutes after completed reperfusion. Hepatocellular injury related to reperfusion injury was determined in the first 24 hours. Blood samples were taken preoperatively and at 1 hour, 2 hours and 4 hours post transplantation for analysis of the hepatocellular injury marker, serum AST.

The marker for endothelial cell function, serum hyaluronic acid, was also measured in the first hour (at base, at 5 and 60 minutes post reperfusion), which is the most crucial time for the endothelial cell function post reperfusion.

Plasma Malondialdehyde (MDA) levels were determined by the thiobarbituric assay as described by Lepage [452]. 2-Thiobarbituric acid (TBA) was added to deproteinized plasma and the reaction between the MDA and TBA, under conditions of low pH and high temperature, yielded a chromogenic adduct that was detectable by fluorometry.

Serum Vitamin A levels were measured fluorometrically, according to the original method described by Thompson [453]. Vitamin A, which fluoresces in ultraviolet light, was first extracted into water and ethanol, than into hexane and detected by fluorometry.

Serum Hyaluronic Acid (Ha) levels were estimated by radioimmunoassay using a kit purchased from Kabi Pharmacia [454]. 125 I-labeled Hyaluronic Acid binding protein (HABP), isolated from bovine cartilage, was used to bind the Ha in the sample. The unbound 125 I-HABP was quantitated by incubating with Ha covalently coupled to Sepharose particles. Separation is performed by centrifugation and decanting. The radioactivity bound to the particles is read in a gamma counter which is inversely proportional to the concentration of Ha in the sample.

Serum AST levels were determined spectrophotometrically using a continuous monitoring assay.

Results

The changes in serum AST levels after liver transplantation in the three groups of animals are shown in Figure 7.1. There was a significant increase in serum AST levels after liver transplantation in all three groups.($p<0.001$) Serum AST levels were lower on the second postoperative day in the UW livers compared to the Eurocollins($p<0.19$) and Celsior($p<0.02$) preserved livers.

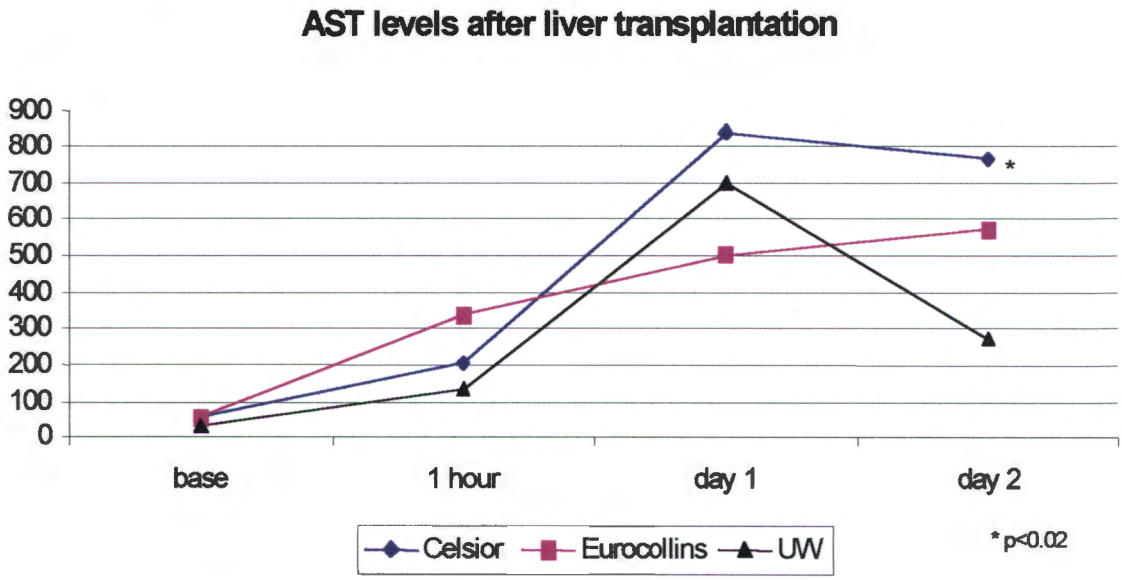


Figure 7.1: Mean Serum AST levels after liver transplantation in the 3 groups in international units.

The changes in plasma malondialdehyde levels are shown in Figure 7.2. There was a significant increase in plasma MDA levels after portal reperfusion ($p<0.04$) in the animals with livers preserved in Eurocollins, Plasma MDA levels in the animals with UW preserved livers remained unchanged after liver transplantation. Plasma MDA levels were lowest in the recipients of livers stored in Celsior. Plasma MDA levels were back to baseline levels by 20 minutes after transplantation.

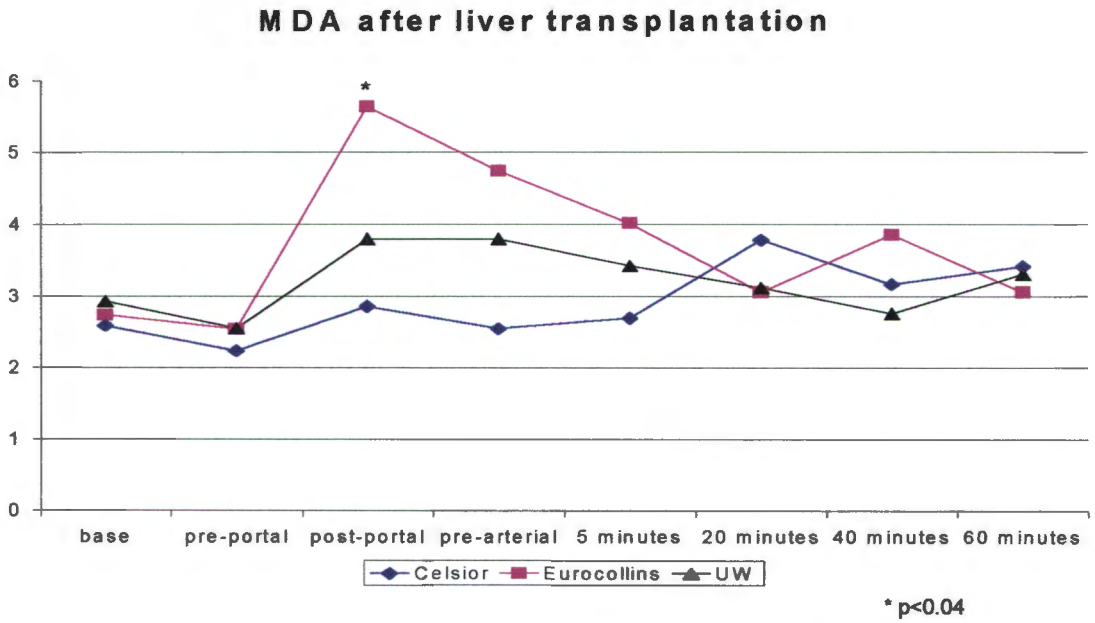


Figure 7.2: Arterial levels of Malondialdehyde during and after liver transplantation in µmol/l.

The serum vitamin A levels, as shown in Figure 7.3, were highest in the Celsior group and lowest in the recipients of livers which were preserved in Eurocollins solution. There was a decrease in serum vitamin A levels after portal reperfusion in all three groups of animals, only the decrease in the Eurocollins group being statistically significant ($p<0.04$).

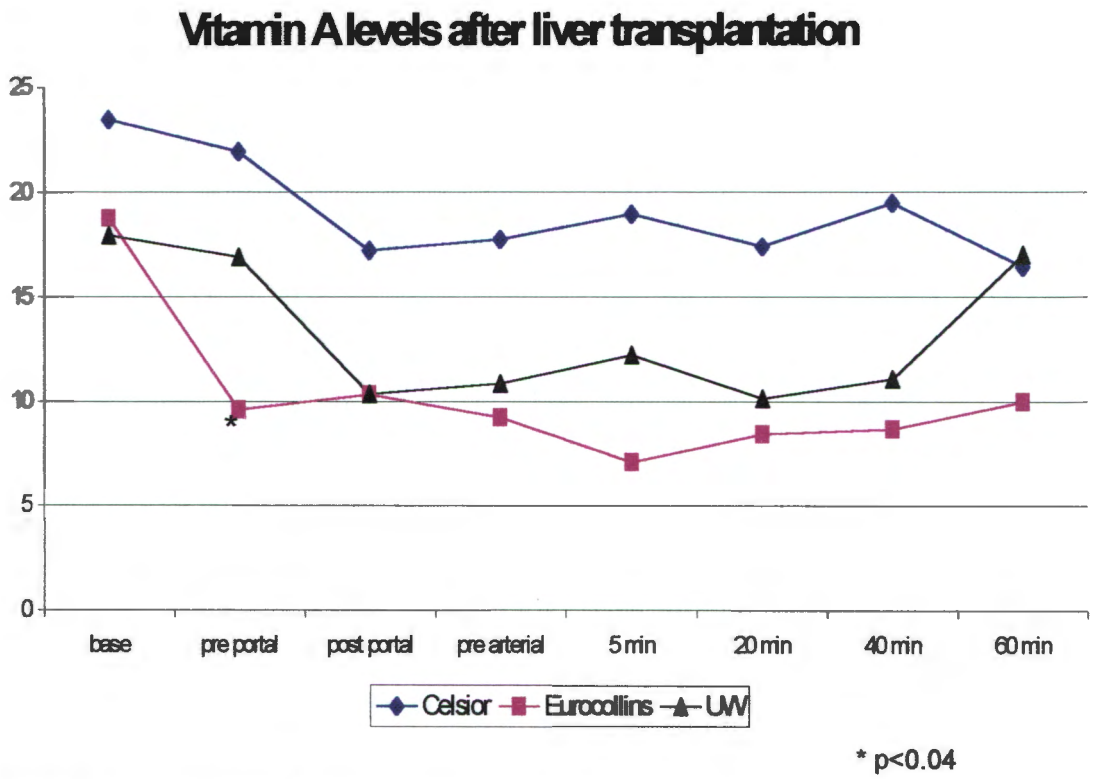


Figure 7.3 Arterial levels of Vitamin A at various times during and after liver transplantation in mg/l.

The changes in serum hyaluronic acid levels after liver transplantation are shown in Figure 7.4. Serum Ha levels increased after transplantation in all three groups of animals. However, there was no difference in serum Ha levels between the groups of animals with different preservation solutions.($p<0.30$)

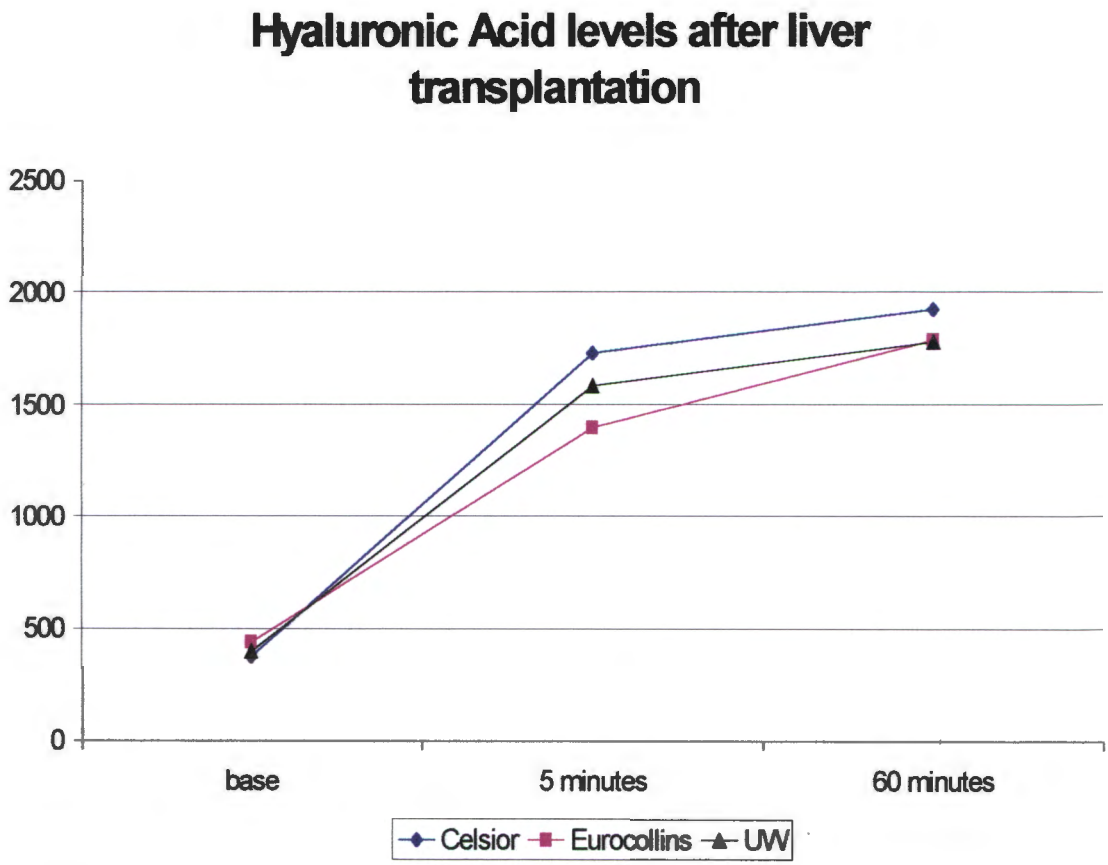


Figure 7.4 Hepatic venous levels of hyaluronic acid after completion of liver transplantation in µg/l.

Discussion

Improvements in the duration and quality of preservation of the livers for clinical transplantation are of paramount importance in order to both increase the number of transplants performed and the function of the donor organs. With Eurocollins solution preservation of the liver was limited to 6-8 hours of cold ischaemia. The introduction of the UW solution not only resulted in the extension of the preservation period beyond 24 hours, but there was also an improvement in the function of the liver allograft. Celsior has been used as a cardioplegic and cardiac preservation solution in the past. The use of Celsior as a liver preservation solution has not been documented previously.

These preservation solutions contain many substances and the importance of each of the individual constituents for simple cold storage is sometimes not clear. The UW solution contains lactobionate and raffinose as impermeants to suppress hypothermia-induced cell swelling, reduced glutathione as an antioxidant, adenosine as a precursor for synthesis of adenine nucleotides, allopurinol as a free radical scavenger, and hydroxyethyl starch as a colloid for oncotic support. Celsior is similar to UW solution in that it contains lactobionate as an impermeant and reduced glutathione as an antioxidant. However, Celsior also contains mannitol because of its dual capacity to behave as an osmotic agent and a free radical scavenger, histidine for its buffering properties, and glutamic acid for its capacity to yield ATP.

It is becoming increasingly apparent that the preservation of organs for clinical transplantation using simple cold storage is complex and involves more than the prevention of the anoxic damage to the parenchymal cells. For example, the reperfusion injury may be responsible for a greater amount of damage than the anoxic injury [66]. There is also a difference in the susceptibility of the different types of cells in the liver to the various forms of injury [179].

The pathophysiology involved in the preservation-reperfusion injury has been extensively documented [76]. Reactive oxygen metabolites are responsible for the reperfusion injury by damaging a spectrum of bio-molecules found in tissues, including membrane lipids. Peroxidation of these membrane lipids results in the formation of malondialdehyde. The level of MDA in the blood correlates with the degree of reperfusion injury. Vitamin A is a potent endogenous scavenger of reactive oxygen metabolites and serum vitamin A levels are also an indication of the extent of the reperfusion injury.

One of the particular problems in this study are the small numbers, making it difficult to reach statistical differences between the groups. The reason for the small number of animals used was cost-saving.

The plasma MDA levels after portal reperfusion in this study were the highest in the animals with livers preserved in Eurocollins solution and lowest in the animals with livers preserved in Celsior. Similarly, serum vitamin A levels were the highest in the Celsior animals and lowest in the Eurocollins group. Thus, there appears to be the greatest amount of reperfusion injury in Eurocollins group and the least in the Celsior group.

The serum AST levels were used as a marker of hepatocyte injury. Serum AST levels on day 2 postoperatively, were the lowest in the animals with livers preserved with UW. Serum AST levels were similar in the Celsior and Eurocollins groups of animals. Thus, the least amount of hepatocyte injury was found in the animals with livers preserved with UW solution.

New refinements in preservation solutions will have to address the effect of the preservation-ischaemia-reperfusion injury on other non-parenchymal cell-types. For example, significant hepatic endothelial cell dysfunction may have a significant impact on the outcome and incidence of septic complications after liver transplantation. The effect of the different preservation solutions on hepatic endothelial cell function has not been documented previously. Hyaluronic acid (Ha) is removed primarily by hepatic endothelial cells and an increase in Ha levels is associated with hepatic endothelial cell dysfunction. Thus, in this study, Ha was used as a marker of endothelial cell function. There was a significant increase in Ha levels after liver transplantation, indicating marked endothelial cell dysfunction. This injury was not modified by any of the preservation solutions.

In future, new preservation solutions will have to be evaluated not only by its ability to prolong preservation time and improve survival, but also by its effect on the various types of cell injury. For example, both UW solution and Celsior contain antioxidants and free radical scavengers to minimize the reperfusion injury.

Conclusion

Livers preserved in UW solution had less hepatocellular injury and more reperfusion injury compared to those preserved in Celsior. The degree of endothelial cell injury was similar in UW solution and Celsior preserved livers.

CHAPTER 8

Conclusions

This thesis was primarily directed at defining new methods to decrease the preservation reperfusion injury after orthotopic liver transplantation.

1. There is a significant reperfusion injury associated with portal venous revascularization (PV) during liver transplantation and not with hepatic arterial revascularization (HA). Furthermore, HA before PV prevented the reperfusion injury associated with PV.

2. Delayed revascularization of the liver during liver transplantation was associated with significant reperfusion injury and hepatocellular injury.

3. Early rearterialization of the liver during liver transplantation prevented the reperfusion injury and was associated with less hepatocellular injury.

4. Early rearterialization of the liver during transplantation was associated with histological evidence of less hepatocellular injury when compared to delayed rearterialization.

5. The use of Celsior as a preservation solution in orthotopic liver transplantation was associated with a lesser reperfusion injury, but a greater hepatocellular injury, when compared to the University of Wisconsin Solution.

Addendum

Abbreviations

ANOVA= Analysis of variance

Df = Degrees of freedom

Ms = Mean sqare

F = f-statistic

p-level = level of significance

Analysis of variance tables

All means were compared by the method of the least significance difference (General ANOVA) as decribed by George Snedecor and William Cochran [472].

ANOVA	1-group, 2-time					
Effect	Df Effect	Ms Effect	Df Error	Ms Error	F	p-level
1	3	2.373362	77	6.137996	.386667	.762907
2	4	1.231139	77	6.137996	.200577	.937312
1&2	12	1.638839	77	6.137996	.266999	.992637

Table 4.1 Malondialdehyde

ANOVA	1-group, 2-time					
Effect	Df Effect	Ms Effect	Df Error	Ms Error	F	p-level
1	3*	532.5300*	60*	75.64205*	7.040132*	.000392*
2	6	15.9746	60	75.64205	.211186	.931246
1&2	18	33.2849	60	75.64205	.440031	.940331

Table 4.2 Vitamin A

ANOVA	1-group, 2-time					
Effect	Df Effect	Ms Effect	Df Error	Ms Error	F	p-level
1	3*	71903.*	70*	21724.45*	3.30978*	.024976*
2	4*	1007649.*	70*	21724.45*	46.38315*	.000000*
1&2	12	31350.	70	21724.45	1.44308	.167689

Table 4.3 Aspartate amino transferase

ANOVA	1-group, 2-time					
Effect	Df Effect	Ms Effect	Df Error	Ms Error	F	p-level
1	3	169395.	53	68228.64	2.48275	.070829
2	4*	4576059.*	53*	68228.64*	67.06947*	.000000*
1&2	12	19412.	53	68228.64	.28452	.989452

Table 4.4 Hyaluronic acid

Analysis of variance tables

All means were compared by the method of the least significance difference (General ANOVA) as decribed by George Snedecor and William Cochran [472].

ANOVA	1-group, 2-time					
Effect	Df Effect	Ms Effect	Df Error	Ms Error	F	p-level
1	3*	71903.*	70*	21724.45*	3.30978*	.024976*
2	4*	1007649.*	70*	21724.45*	46.38315*	.000000*
1&2	12	31350.	70	21724.45	1.44308	.167689

Table 5.1 Aspartate amino transferase

ANOVA	1-group, 2-time					
Effect	Df Effect	Ms Effect	Df Error	Ms Error	F	p-level
1	3	2.373362	77	6.137996	.386667	.762907
2	4	1.231139	77	6.137996	.200577	.937312
1&2	12	1.638839	77	6.137996	.266999	.992637

Table 5.2 Malondialdehyde

ANOVA	1-group, 2-time					
Effect	Df Effect	Ms Effect	Df Error	Ms Error	F	p-level
1	3*	532.5300*	60*	75.64205*	7.040132*	.000392*
2	6	15.9746	60	75.64205	.211186	.931246
1&2	18	33.2849	60	75.64205	.440031	.940331

Table 5.3 Vitamin A

ANOVA	1-group, 2-time					
Effect	Df Effect	Ms Effect	Df Error	Ms Error	F	p-level
1	3	169395.	53	68228.64	2.48275	.070829
2	4*	4576059.*	53*	68228.64*	67.06947*	.000000*
1&2	12	19412.	53	68228.64	.28452	.989452

Table 5.4 Hyaluronic Acid

Analysis of variance tables

All means were compared by the method of the least significance difference (General ANOVA) as decribed by George Snedecor and William Cochran [472].

ANOVA	1-group, 2-time					
Effect	Df Effect	Ms Effect	Df Error	Ms Error	F	p-level
1	3	.950000	48	.500000	1.90000	.142194
2	2*	5.716667*	48*	.500000*	11.43333*	.000087*
1&2	6	.450000	48	.500000	.90000	.502762

Table 6.1 Hepatocyte vacuolization

ANOVA	1-group, 2-time					
Effect	Df Effect	Ms Effect	Df Error	Ms Error	F	p-level
1	3*	1.30556*	48*	.308333*	4.23423*	.009821*
2	2*	17.51667*	48*	.308333*	56.81081*	.000000*
1&2	6*	.87222*	48*	.308333*	2.82883*	.019380*

Table 6.2 Single cell necrosis

ANOVA	1-group, 2-time					
Effect	Df Effect	Ms Effect	Df Error	Ms Error	F	p-level
1	3*	1.444444*	48*	.258333*	5.59140*	.002265*
2	2*	7.016667*	48*	.258333*	27.16129*	.000000*
1&2	6*	1.194444*	48*	.258333*	4.62366*	.000881*

Table 6.3 Group cell necrosis

ANOVA	1-group, 2-time					
Effect	Df Effect	Ms Effect	Df Error	Ms Error	F	p-level
1	3	.266667	48	.291667	.91429	.441117
2	2*	6.650000*	48*	.291667*	22.80000*	.000000*
1&2	6	.383333	48	.291667	1.31429	.269101

Table 6.4 Neutrophil infiltration

Analysis of variance tables

All means were compared by the method of the least significance difference (General ANOVA) as decribed by George Snedecor and William Cochran [472].

ANOVA	1-group, 2-time					
Effect	Df Effect	Ms Effect	Df Error	Ms Error	F	p-level
1	2	108430.3	33	49056.05	2.21033	.125643
2	3*	901396.6*	33*	49056.05*	18.37483*	.000000*
1&2	6	81322.1	33	49056.05	1.65774	.162691

Table 7.1 Aspartate amino transferase

ANOVA	1-group, 2-time					
Effect	Df Effect	Ms Effect	Df Error	Ms Error	F	p-level
1	2	5.283679	88	6.442274	.820158	.443700
2	7	3.618097	88	6.442274	.561618	.785060
1&2	14	2.018104	88	6.442274	.313260	.990980

Table 7.2 Malondialdehyde

ANOVA	1-group, 2-time					
Effect	Df Effect	Ms Effect	Df Error	Ms Error	F	p-level
1	2*	674.7001*	76*	58.04640*	11.62346*	.000039*
2	7	78.6456	76	58.04640	1.35487	.236855
1&2	14	16.4544	76	58.04640	.28347	.994371

Table 7.3 Vitamin A

ANOVA	1-group, 2-time					
Effect	Df Effect	Ms Effect	Df Error	Ms Error	F	p-level
1	2	46052.	21	36112.97	1.2752	.300135
2	2*	56369335.*	21*	36112.97*	156.0917*	.000000*
1&2	4	367787.	21	36112.97	1.0187	.420444

Table 7.4 Hyaluronic acid

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