PHARMACOLOGICAL MODIFICATION TO PREVENT REPERFUSION INJURY FOLLOWING LIVER TRANSPLANTATION

A thesis presented to
The University of Manitoba
in partial fulfillment of the requirements for the degree of
Masters of Science in Surgery

by:
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PHARMACOLOGICAL MODIFICATION TO PREVENT REPERFUSION

INJURY FOLLOWING LIVER TRANSPLANTATION

BY

ARI J. COHEN

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University

of Manitoba in partial fulfillment of the requirements of the degree

of

MASTER OF SCIENCE

Ari J. Cohen   1997 (c)

Table of Contents

Abstract

The principle objectives of this study were to determine whether hepatocyte membrane potentials (PD) are altered during liver transplantation and whether such changes may be of pathophysiologic importance in ischemia/reperfusion injury and graft survival.

Livers of adult male Sprague-Dawley rats (N=3-4/group) were impaled with intracellular microelectrodes prior to and at various time periods for six hours following complete hepatic resection. Just prior to resection each liver was perfused with preservation solutions associated with high (normal saline, NS), moderate (Eurocollins, EC) and low (University of Wisconsin solution, UW) risks of reperfusion injury. Baseline (in situ) PD's were similar in all groups (-37±4 mV, mean ± SD). Ten minutes post resection hepatic PD's were as follows; NS; -23.8±3.5, EC; -11.4±0.4, and UW; -8.7±0.3 mV (p<0.01 for all groups). Maximum depolarization occurred at 6 hrs post resection (NS; -8.1±1.1, EC; -7.7±1.3 and UW; -8.6±1.0 mV).

To determine whether these changes are of pathophysiologic importance, the NS solution was modified (addition of 0.1% ethanol) to achieve similar PD changes as those observed with UW. Liver transplants were then performed using a nonarterialized, cuff technique in adult Lewis rats where the donor livers had been perfused and preserved for six hours with either NS or the modified NS, (MNS) solution. Post transplant (10 d) survival was 1/6 (17%) in the NS group and 4/6 (67%) in the MNS group (p < 0.05). The second series of transplants compared MNS to UW with a 36 hour preservation time. There were no long term survivors in either the MNS or UW groups with a 36 hour preservation time.

Regarding the effects of PD changes on ionic flux, intracellular calcium levels were documented by fluorescence video microscopy using Fura-2 in isolated hepatocytes exposed to NS, UW and MNS solutions. UW and MNS exposed hepatocytes had significantly lower intracellular calcium levels than NS exposed hepatocytes (p<0.005).

In conclusion, the results of this study indicate that hepatocytes undergo prompt and marked depolarization following hepatic resection and that the extent of the depolarization correlates with graft survival and intracellular calcium levels following transplantation.

Acknowledgments

I would like to acknowledge my supervisor, Dr. G.Y. Minuk, for his encouragement and support both during my research year and in my life endeavors. I would like to thank Drs. J. Lipschitz, F. J. Burczynski and B.G. Rosser for their assistance and contribution to my research. I am indebted to all the members of the Liver Diseases Unit and thank them for their friendship. This work was supported in part by the Dr. Paul Thorlakson Foundation.

List of Abbreviations

AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
CyA	Cyclosporine A
DVFM	Digitized fluorescence videomicroscopy
EC	Euro-collins solution
Fura-2-AM	Fura-2-acetoxymethylester
GABA	Gamma-aminobutyric acid
ICU	Intensive care unit
MNS	Modified normal saline
NS	Normal saline
PD	Membrane potential
UNOS	United Network for Organ Sharing
UW	University of Wisconsin solution

List of Figures

List of Tables

Background

During the past fifteen years, liver transplantation has progressed to become the treatment of choice for end stage liver disease . The first successful human liver transplant was carried out in 1967 in a child with a hepatic malignancy, who survived only 400 days[1]. By 1980, 330 liver transplants had been performed with a survival rate of 28%[1]. Currently, over 3000 liver transplants are performed yearly (U.S. data) with 5 year survival rates at 70%[2].

Although improved care of the donor, better patient selection, improved anesthesia, standardization of the surgical procedure, blood banking and improved postoperative care are all noted to have positively impacted on the survival of liver transplant patients, the major advances came with the introduction of immunosuppressive agents and the development of modern organ preservation solutions. Specifically, the introduction of Cyclosporine A (CyA) as the primary form of immunosuppression resulted in an immediate and dramatic effect on the survival of patients undergoing liver transplantation with the one year survival rates increasing from 30% with azathioprine and steroids to over 65% with CyA and steroids[1,3]. The development of the University of Wisconsin solution (table 1) also resulted in significant advances in liver transplantation by providing longer preservation times.[4] Despite the above improvements, approximately 25% of patients continue to die on the liver transplant waiting list as a result of limited donor organ availability[5]. One method of reducing this mortality rate is to minimize organ wastage, and further increase organ availability and sharing.

History of Modern Preservation Solutions

There are multiple purposes for an effective preservation solution. The most important objective is for a preservation solution to store an organ such that it will be effective postoperatively in an uneventful and cost effective manner. Other goals include providing sufficient storage time to transport organs from distant locations to permit greater sharing of organs, allowing time for biopsing and interpreting the results of the donor liver, and alleviating the need for overlapping the donor and recipient operations. Finally, proper organ preservation provides sufficient time to adequately prepare the recipient prior to surgery.

Prior to 1988, Euro-Collins solution was utilized as the principle preservation solution with varying results for storage times depending on the organ preserved. Euro-Collins solution was able to store livers for six to ten hours, however the incidence of preservation related injuries remained significant[6]. The era of modern preservation solutions was heralded by the introduction of the University of Wisconsin solution[4].

The recognized complications of hypothermic organ preservation lead to the development of UW solution by Drs. Belzer and Southard[4]. These complications include hypothermic cellular/tissue edema, intracellular acidosis, development of oxygen-derived free radicals and the depletion of energy compounds used during reperfusion. Each of these complications was addressed by specific ingredients in the UW solution. The components included in UW at the time of it's creation were partly based on theoretical concerns. For example, lactobionate and raffinose were included as large molecular weight compounds to prevent tissue edema. Hydroxyethyl starch was included to prevent the expansion of the extracellular space. These compounds replace the glucose and mannitol which had been used in the Euro-Collins solution. The removal of glucose serves a secondary purpose as it was felt it might stimulate the production of lactic acid and hydrogen ions, contributing to intracellular acidosis. Phosphate was included in UW to serve as a buffer to prevent tissue acidosis. Allopurinol and glutathione were included as free radical scavengers. Adenosine was added as a precursor to ATP synthesis. Magnesium was used as a membrane stabilizing compound[4].

The theoretical inclusion of the components of UW have been challenged in multiple animal studies[7,8,9,10]. These studies have shown that omission of hydroxylethyl starch[10],

adenosine[10], allopurinol[10], bactrim[9], insulin[9], raffinose[10], $MgSO_4$[10] and the phosphate buffer[10] does not result in changes in survival. The reversal of the sodium and potassium concentration may in fact improve survival[8,9]. Despite the suggestions that a simpler UW solution may be utilized, there have been no modifications to date of the clinically used UW solution.

Other considerations associated with the current usage of the University of Wisconsin solution is cost ($250/Liter U.S., Dupont Pharma). Since most donor operations currently are for multiple organs which require flushing and then storage in separate containers of preservation solution, the total volume of preservation solution has increased in recent years. This cost adds to the already costly endeavor of performing transplant surgery. Therefore, a preservation solution which is cheaper and as effective would be ideal.

The introduction of UW solution allows for the extended storage of donor livers up to 12 hours[11,12]. This solution has been shown to be clearly superior to the previously used Euro-Collins solution in both preservation times and preservation related injuries[4,11,12]. Despite the fact that it closely approximates the goals for an ideal preservation solution, due to the severe shortage of donor organs and an increasingly growing waiting list, the incidence of preservation related injuries is still unacceptably high[11,12].

Preservation-Reperfusion Injuries

All livers undergo some degree of injury during transplantation. Livers which receive severe damage (primary nonfunction) are generally unable to sustain life and often result in death or re-transplantation. Other livers show borderline function initially post transplant (primary dysfunction) and result in prolonged patient stays in the ICU and hospital. The incidence of primary nonfunction and primary dysfunction following liver transplantation is approximately 6% and 22% respectively[13]. Although the precise cause of these conditions is likely multifactorial, preservation injuries have frequently been implicated. Preservation injuries may also result in an increased frequency of allograft rejection[14].

Preservation-reperfusion injuries can be grouped into four major categories which include pre-preservation injuries, cold preservation injuries, rewarming injuries and reperfusion injuries.

Pre-preservation injuries are injuries to the liver prior to flushing the donor liver with a preservation solution. The donor can have pre-existing unrecognized problems with their liver prior to brain death. Following brain death, hemodynamic instability, hypoxia, anemia and endocrinologic complications may all result in damage to the donor liver[15]. Furthermore, the nutritional status of the donor liver has been shown to affect the outcome in the recipient[16,17].

The mechanism of injury in cold preservation injuries is multifactorial. Hypothermic preservation is critical for preserving organs for periods longer than one hour[18,19]. Hypothermia decreases the metabolic rate and slows the rate of intracellular enzymes. This unfortunately results in anaerobic metabolism and lactate accumulation resulting in intracellular acidosis[20]. ATP has been shown to become depleted[17,21] and intracellular calcium levels rise resulting in activation of proteases. Na/K ATPase is inhibited resulting in increased intracellular sodium with a net result of cell swelling[22].

Rewarming injuries occur from the time of removing the liver from the preservation solution until the liver is reperfused through the portal vein and hepatic artery. Prolonged rewarming times have been shown to have negative impacts on the final outcome of the transplanted liver[23].

Complex models detailing preservation injuries describe the intimate relationship

between cold preservation injuries, rewarming and subsequent reperfusion injuries[24]. These models involve the activation of sinsuiodal cells[25,26], white cells[27] and platelets[19] resulting in adhesion and increased coagulation[28]. The mediators for this process include the release and activation of eicosanoids[29], reactive oxygen intermediates[30,31], cytokines[32] and proteases[33]. The damage occurring to the donor liver at the reperfusion stage is felt to be the common final pathway for the events which occurred prior to this stage. The ingredients of UW solution are aimed at prevention of reperfusion damage by avoiding the initial damage during the preservation period.

Hepatocyte Membrane Potentials

Hepatocyte membrane potentials (PD) help regulate cell function and viability by altering nutrient uptake and intracellular enzyme activity[34]. Specifically, PD changes have been shown to be involved in the regulation and transport of fatty acids, amino acids and bile acids[35,36,37]. They also appear to influence the transport of anions and cations across the hepatocyte membrane, some of which thereafter activate or suppress intracellular enzyme activity[36]. Furthermore, changes in hepatocyte PD have been shown to be associated with partial hepatectomy and implicated in regulating recovery from cell injury and hepatic regenerative activity[38].

Minuk et al has shown that GABA and GABA receptor agonists hyperpolarize the hepatocyte membrane and ethanol causes a depolarization of the hepatocyte membrane potential[39]. The specific mechanism whereby ethanol causes membrane potential changes has not been elucidated.

Hypothermia has been shown to alter the hepatocyte membrane potentials as well as alter the influx of calcium into hepatocytes[40].

There are no studies which document changes in hepatocyte membrane potentials during liver transplantation. Moreover, no studies have examined the effects of different preservation solutions on the hepatocyte membrane potential in either isolated cells or whole excised livers.

Calcium and Hepatocellular Injury

The role that calcium plays as an intracellular hepatocyte mediator of preservation injury has not been fully elucidated. Extracellular fluid in comparison to intracellular fluid is rich in calcium and there is a large electrochemical gradient to drive calcium into cells. This gradient is maintained by the impermeability of the plasma membrane to calcium and the active extrusion of calcium by the cell[41].

Hypothermia has been shown to alter intracellular calcium levels by releasing intracellular depots and calcium influx through the plasma membrane[40,42,43]. During the preservation period, ATP stores become depleted and intracellular acidosis increases thereby preventing or reducing the amount of calcium which can be extruded[40].

Increases in intracellular free calcium in other conditions are associated with biochemical and histological evidence of cell death likely due to the activation of phospholipases and protein kinases[44,45,46,47]. Elevated intracellular calcium has also been shown to potentiate the effects of free radical scavengers in causing cell death[48].

The role of calcium in preservation related injuries is further strengthened by studies which have added calcium channel blockers to preservation solutions which resulted in improved survival following transplantation[49]. On the other hand, the site of action of these calcium channel blockers has not been determined and calcium channel blockers have cytoprotective effects which are separate from their effects on intracellular calcium[48].

To date, the effects of different preservation solutions on hepatocyte intracellular calcium levels has not been reported.

Hypothesis

Hepatocyte PD changes are of pathophysiologic relevance and not merely a consequence of preservation injury. The PD changes associated with more effective preservation solutions likely contribute to protecting hepatocytes from preservation injury by preventing rises in intracellular calcium.

Aim

To elucidate the protective effects the PD has on hepatocytes, we propose to demonstrate that different preservation solutions alter the hepatocyte membrane potential differently. Thereafter, by mimicking the PD changes associated with the most effective preservation solution presently available (University of Wisconsin solution) and documenting similar effects on hepatocyte preservation and changes in intracellular calcium, we hope to establish that PD changes are of pathophysiologic importance.

Specific Objectives

Employing an animal model of liver transplantation, the objectives of this study were to;

1) Describe the changes in PD associated with different preservation solutions and determine if a correlation exists between hepatocyte PD and the established clinical effectiveness of the preservation solution.

2) Alter the PD of the least effective preservation solution [normal saline (NS)] to match the PD of the most effective preservation solution (UW).

3) Determine if the altered preservation solution [modified normal saline (MNS)] results in improved survival when compared to NS.

4) Determine if MNS is as effective as UW.

5) Determine if MNS alters intracellular free calcium levels in a manner dissimilar to NS and similar to UW.

Materials and Methods

Animals: All animals used in this study were cared for in accordance with the guidelines of the Canadian Council on Animal Care and the University of Manitoba Animal Care Protocol. The study protocol was approved by the University of Manitoba Animal Ethics Committee

Methods: After being trained in each of the involved laboratories, the candidate performed each of the following methods which are subsequently described with the exception of the cell isolation methods. These cells were provided by Dr. Rosser's Laboratory which the candidate used to study intracellular calcium levels. The candidate is solely responsible for the generation of all of the data shown in the results section.

Membrane potential studies: Adult male Sprague-Dawley rats (325-350g) were housed in an animal holding area and allowed free access to food and water until the morning of surgery when food but not water was withdrawn.

Surgery was performed in the morning of experimental days while the animals were anesthetized with sodium pentobarbital (50mg/kg) which has previously been documented not to alter hepatic PD values[38]. Under anesthesia, laparotomy with cannulation of the portal vein and ligation of the inferior vena cava (above the adrenal veins) was performed. The livers were then perfused using either sterile normal saline (NS), Euro-Collins Solution (EC) (Fresenius AG) (table 2), University of Wisconsin solution (UW) (Dupont Pharma), or modified normal saline (MNS) (table 3) at 4°C. This method resembles the human liver harvesting conditions. Following perfusion, the livers were removed and placed in baths of the perfusates at 4°C for varying periods of time as indicated.

PD Measurements: The PD of hepatocytes in intact anesthetized rats were measured using intracellular microelectrodes[50]. Single barreled microelectrodes were drawn on a horizontal micropipette puller (Brown-Flaming, model P-87, Sutter Instruments, Novato, CA) from Omega-Dot Borosilicate tubing (1.0mm-OD, 0.5mm-ID, Sutter Instruments), filled with 0.5M potassium chloride and beveled to a 30° angle to a tip resistance of 120micro-ohms . Electrical signals were conducted via a Ag-AgCl electrode connected to an Axoprobe 1A amplifier (Axon Instrument, Frost City, CA). Data acquisition was controlled by interfacing the

amplifier to an 80486 computer via a TL-1 DMA (Axon Instrument) and using the computer program Axo Tape (Version 2.0, Axon Instruments). The reference electrode was placed in the bath solution surrounding the liver. Multiple PD measurements were performed at each time point in randomly placed sites in the liver. The results represent the mean of at least three PD determinations. Baseline PD's in situ, prior to perfusion were obtained from preliminary experiments performed in the laboratory.

Liver transplant methods:

Model Selection: Several requirements are needed in an animal model of liver transplantation in order to study preservation injuries. The time required to perform the surgery must be brief and standardized such that the times are reproducible. The surgery needs to be as simple as possible to avoid injury to the liver during the surgery, which postoperatively could be confused with preservation injuries. Since preservation injuries manifest within the first few days post transplant, the long term consequences of a model need not be considered. On this basis, the Kamada model of liver transplantation was chosen[51,52]. It is a nonarterialized orthotopic model of liver transplantation which is simple, quick and results in minimal damage to the donor liver. This model is the standrard model used for studying preservation injuries[7,8,9,10.] Although, there are long term problems associated with this model (alterations in connective tissue, hepatocyte volume and bile duct proliferation[53,54]), they do not affect animal survival within the first ten days post transplant[53,54,55].

Establishment of Liver transplant model: The candidate was initially trained by taking the microvascular anastomosis training course at the University of Alberta. The Kamada model for liver transplantation in the rat was acquired through an intensive one month training period with Dr. L. Zhu, a recognized world expert in microvascular multi-organ transplant surgery. A six month period at the University of Manitoba was used for establishing and proving that the model could be performed with essentially no mortality under non-experimental conditions.

Animal Selection and Size: To eliminate the role of rejection, male Lewis rats (inbred) weighing 180-250 grams were used as both recipients and donors. Male rats were chosen as the penile vein provides easy intravenous access. Heavier rats have an increased amount of retroperitoneal fat which lengthens both the donor and recipient operations. The size of the

portal vein in smaller animals makes the surgery technically more difficult.

Animal Nutrition: Rat chow was withheld for 12 hours prior to surgery. A 5% dextrose in water solution was available ad lib to the time of surgery. Transplant recipients were allowed free access to rat chow until sacrificed by exsanguination.

Donor Operation: The abdominal cavity was entered through a midline incision. The phrenic vein was ligated with 7-0 silk. The hepatic artery was ligated and then divided. Donor bile duct cannulation was performed with a polyethylene stent (PE50, I.D. 0.58 mm, O.D. 0.965 mm, Intramedic, Becton Dickinson and Company, Sparks MD) which was inserted into the distal bile duct and secured with 7-0 silk (figure 1). The pyloric and suprarenal veins were ligated. The right renal artery and veins were ligated and divided. Rats were injected with 1 ml of Ringer's lactate solution containing 100 units of heparin via the penile vein. The infrahepatic vena cava was clamped at the level of the left renal vein and the portal vein was clamped at the level of the splenic vein. The liver was perfused with 4 ml of perfusion solution (figure 3). The portal and infrahepatic veins were divided at the level of the clamps and the suprahepatic vena cava was excised at the level of the diaphragm. The livers were then stored in their perfusion solution at 4°C.

Cuff Preparation: Cuffs were prepared in the perfusion solution at 4°C. Cuffs were fashioned for both the portal vein and infrahepatic vena cava (figure 2). The ends of the veins were passed through the lumen of the polyethylene tubing (Portal vein: Polyethylene tubing PE240, I.D. 1.67 mm, O.D. 2.42mm, Inferior vena cava: Polyethylene tubing PE260, I.D. 1.77 mm, O.D. 2.80 mm, Intramedic, Becton Dickinson and Company, Sparks MD), everted over the cuff and secured with 5-0 silk.

Recipient Operation: Through a midline incision, the phrenic vein was ligated. The hepatic artery was then ligated and divided. The bile duct was ligated proximally at the level of the first bifurcation and then divided (figure 4). The right suprarenal vein was ligated. Prior to removal of the liver, 2ml of 5% dextrose was injected i.v. via the penile vein. Microvessel clips were applied to the infrahepatic vena cava and portal vein. A Cooley clamp was applied to the suprahepatic vena cava. The native liver was then excised. Prior to implantation, the donor liver was flushed via the portal vein with 10ml of Ringer's lactate, because the UW solution is high

in potassium. The suprahepatic vena cava anastomosis was performed with a continuous 7-0 prolene suture. Both portal vein and infrahepatic vena cava were passed over the cuffs of their respective veins and secured with a 7-0 silk (figure 5). All vascular anastomoses were flushed prior to completion to prevent air embolism. The bile duct stent was inserted into the recipient bile duct and secured with 7-0 silk (figure 6). Omentum was wrapped around the biliary anastomosis. The abdomen was closed with a 3-0 dexon suture.

Postoperative Care: Postoperative analgesia consisted of Banamine s.c. 2.5 mg/Kg, which was given immediately postoperatively and every 12 hours for the first 3 days, then as needed. Blood samples on surviving animals were obtained by cardiac puncture every other day post-operatively. No animals died within 4 hours of cardiac puncture.

Survival rates were determined at 10 days. All dead animals in the post-operative period underwent a post-mortem examination. Deaths without positive findings at autopsy were attributed to severe preservation injury.

Intracellular calcium measurements:

Hepatocyte Isolation and Culture Hepatocytes were isolated and cultured from fasted male Sprague-Dawley rats (250-300g) using the standard technique of collagenase perfusion, differential centrifugation and culture on collagen coated coverslips[56]. Animals were anesthetized with pentobarbital and the abdominal cavity was opened. The portal veins were cannulated and the animals euthanized by exsanguination. Livers were perfused using a recirculating technique with calcium and magnesium free Hanks buffer (pH 7.4) for 5 minutes. The perfusates were then changed to standard Hanks buffer containing 2 mM Calcium, 0.5 mM Magnesium, 0.05% low trypsin collagenase (Boehringer Mannheim) and 0.1 mg/ml soybean trypsin inhibitor. Livers were perfused for 20 minutes then the tissues were mechanically dissociated in fresh Hanks buffer and filtered through sterile gauze and 100 um tissue sieves. The resulting cell suspensions were centrifuged at 60 g for 2 minutes. The supernatants were discarded and the cell pellets resuspended in fresh Hanks buffer. Centrifugation was then repeated and the cell pellets resuspended in fresh Hanks buffer. Cell viability was assessed using the trypan blue exclusion technique. Isolates with viability greater than 90% were subsequently cultured on collagen coated coverslips at a concentration of 2.5×10^5 cells per ml in

sterile Waymouths medium supplemented with 10% fetal calf serum, 100 nM beef insulin, 50 mg/ml gentamicin, 200 ug/ml streptomycin and 200 units/ml penicillin. A microprocessor controlled CO_2 incubator (95% O_2: 5% CO_2) was used for culture of cells at 37°C.

Digitized Fluorescence Videomicroscopy (DVFM) The general scheme for evaluation of fluorescent probes in isolated cultured hepatocytes using DVFM is as follows[57]. Hepatocytes cultured on collagen coated coverslips were mounted in buffer on a heated microscope stage, loaded with the fluorescent dye of interest and exposed to excitation light of an appropriate wavelength. The emission wavelength of interest was selected and the light from isolated hepatocytes passed on to a camera collecting device (CCD) with on camera digitizing capabilities. The signal was analyzed using computer imaging software (Axon Imaging Workbench Software, Axon Corporation) and a pseudocolor image was generated for monitoring and data analysis. The system consisted of the following: a Leitz DM-IRB inverted fluorescence microscope (Leica Corporation) with appropriate fluorescence microscopy objectives; a 75 watt mercury lamp source; a high speed shutter and filter wheel (Leundl Instruments); appropriate excitation filters, dichroic mirrors, and emission filters (Omega Optical Corporation); a Variocam Intensified CCD camera with on camera digitizing capabilities (Optikon Corporation); a 486DX2 computer with a one gigabyte hard drive and a data storage system consisting of a Fujitsu rewritable optical disc drive system.

Measurement of Ca^{++}_i using Fura-2-AM: Hepatocytes cultured on collagen coated coverslips were loaded at 37°C with 5 uM Fura-2-acetoxymethylester (Fura-2-AM) for 30 minutes. Cover slips were mounted on an inverted fluorescence microscope in physiologic buffer (Krebs-Ringers-Hepes buffer with 2 mM calcium). Excitation light was provided from a 75 watt mercury lamp source. Ratio imaging of cell fluorescence was performed with excitation wavelengths 340 nm and 380 nm using a dichroic cut-off of 510 nm for emission spectra. Ratio values were converted to cytosolic calcium concentrations using an in vitro calibration technique and a Kd of 224 nM.

Enzyme Analysis: AST levels were determined using a Sigma diagnostics kit (Sigma Chemical Co., St. Louis, MO).

Statistical Analysis

Statistical analyses were performed by a Kruskal-Wallis test and a U test (Mann-Whitney) for nonparametric data. Results represent mean $\pm$ SD. P values < 0.05 were considered significant. Analyses were performed by Winstat Statistics for Windows (Version 3.0, Kalmia Co. Inc.).

Table 1: Contents of the University of Wisconsin solution.

Component	Concentration
K lactobionate	100 mmol/L
Raffinose	30 mmol/L
Pentafraction (hydroxyethyl starch)	50 g/L
KH2PO4	25 mmol/L
Glutathione (GSH)	3 mmol/L
Adenosine	5 mmol/L
Allopurinol	1 mmol/L
MgSO4	4 mmol/L
Penicillin	200,000 U/L
Insulin	40 U/L
Dexamethasone	16 mg/L
Na	25 mmol/L
K	125 mmol/L
pH	7.4
Osmolality	320 mOsm/L

Table 2: Contents of Euro-Collins Solution

Glucose	38.5 grams
K	115 mmol/L
Na	10 mmol/L
Cl	15 mmol/L
HCO3	10 mmol/L
H2PO4	15 mmol/L
HPO4	42.5 mmol/L
pH	7.25
Osmolality	375 mOsm/L

Table 3: Contents of the Modified Normal Saline Solution

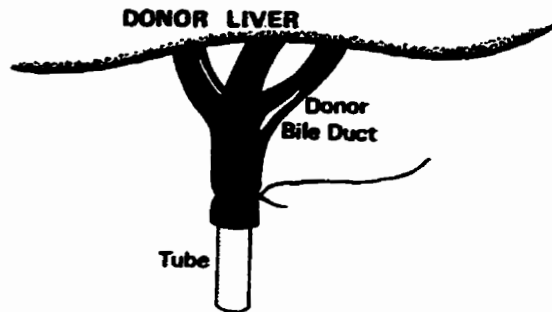| Na	154 mmol/L
Cl	154 mmol/L
Ethanol	0.1% vol/vol
pH	5.7
Osmolality	308 mOsm/L

Figures 1-3: Donor Liver Preparation[51]

FIGURE 1. Donor bile duct preparation. A tube is inserted into the lumen and secured with a circumferential suture. One suture end is left long.

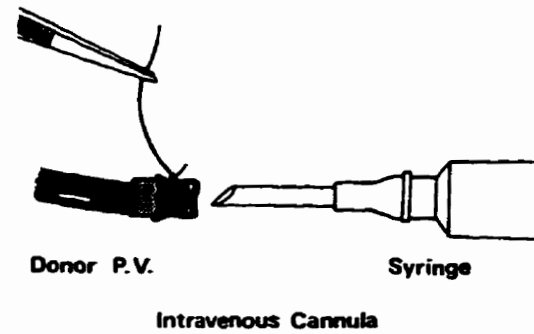FIGURE 3. Liver perfusion via portal vein cuff. The cuff is steadied by grasping the suture ends and a cannula is gently inserted.
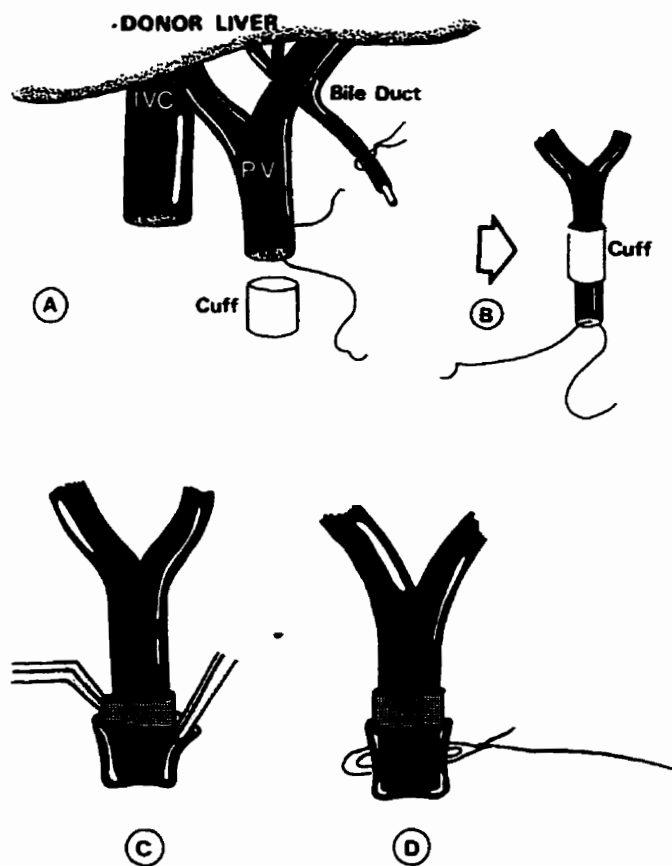

FIGURE 2. Cuff preparation of the donor portal vein. A suture is passed through the portal vein (A) and the cuff is slipped over the vein (B). The divided end of the vein is everted over the cuff (C) and a circumferential suture secures the cuff (D). The suture ends are left long.

Figures 4-6: Recipient Liver surgery[51]
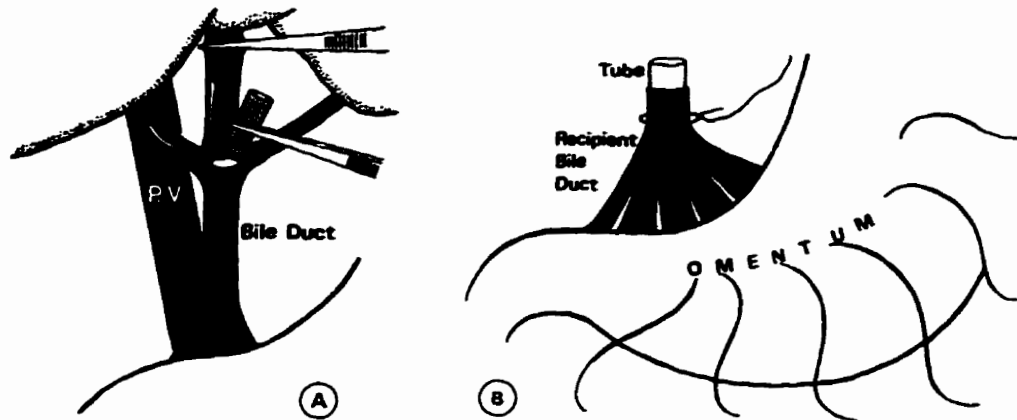
FIGURE 4. Recipient bile duct preparation. A choledochotomy is made in the anterior wall of the duct and a tube is inserted (A). A suture :ures the tube and the duct is then transected (B). Suture ends are left long.

FIGURE 6. Bile duct anastomosis. The donor bile duct tube is telescoped into the recipient duct tube and the securing sutures are tied together to complete the anastomosis.

FIGURE 5. Portal vein anastomosis. Sutures in the right and left ranches apply tension to recipient vein. A venotomy is made in the iterior wall (A). The donor portal vein cuff is inserted into the ·notomy and the anastomosis is completed with a circumferential iture (B).

Experimental Protocols

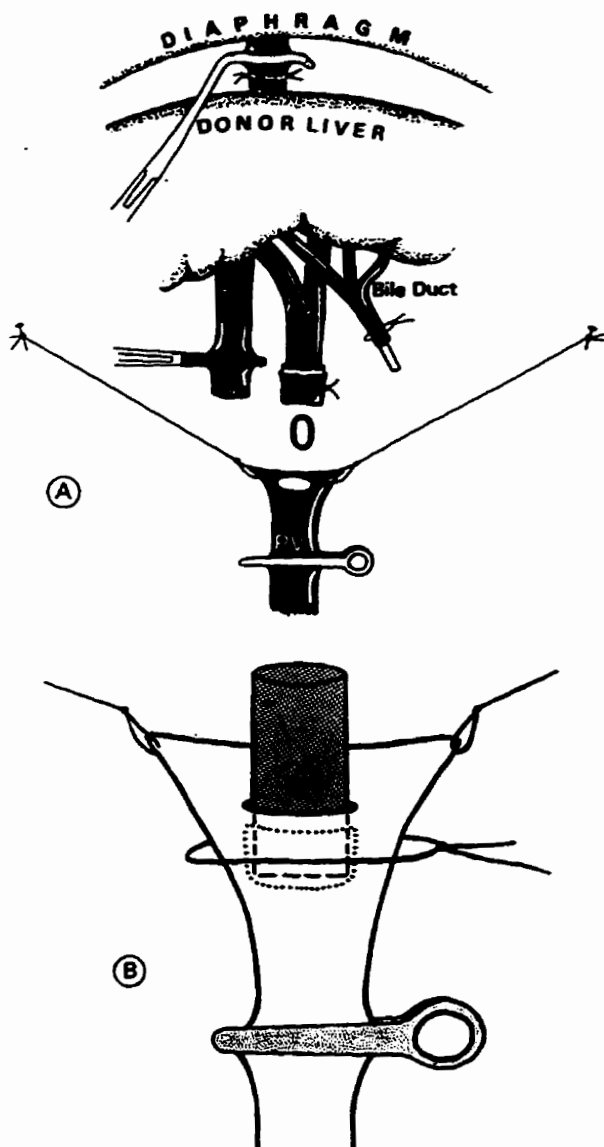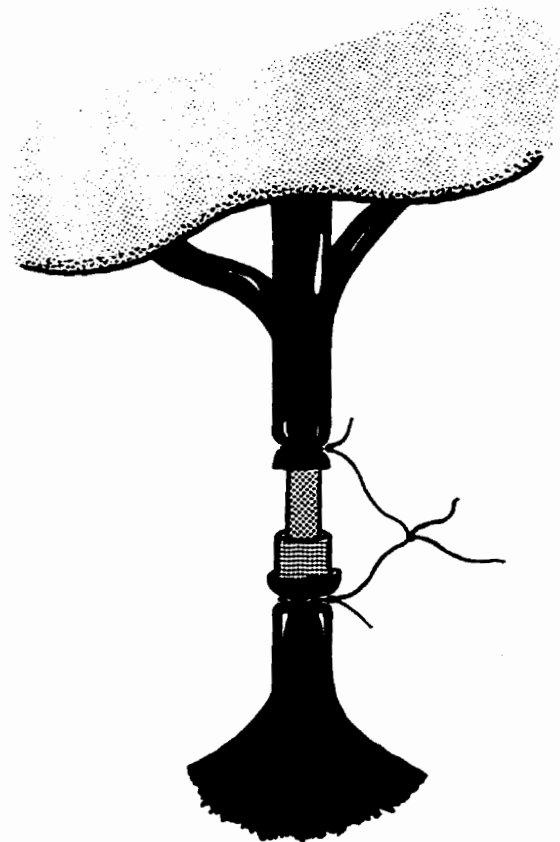*Membrane potential studies:* The initial experiments were designed to document PD changes associated with established preservation solutions following excision of the liver. Fourteen rats were divided into three groups (N=4-5/group). Five rat livers were perfused and bathed with either NS or EC while 4 rat livers were perfused and bathed in UW. Each rat liver underwent perfusion at time 0 followed by excision and placement of the liver in the respective perfusion bath at 4°C. PD determinations were done at 10 minutes, then hourly for 6 hours.

The second series of experiments was designed to identify the modifications of NS required to affect the same PD changes as those observed with UW. Previous studies had indicated that ethanol modifies hepatocyte PD values[39]. Thus to determine the appropriate composition of ethanol in the MNS solution, livers were perfused with either 0.03%, 0.06%, or 0.1% ethanol in normal saline. The perfusion and surgery were carried out in an identical method to the first series of experiments. PD determinations in this series were limited to 10 minutes and 1 hour.

Samples of the perfusion baths from both series of experiments were collected with each PD reading and stored at -70°C until analyzed for AST levels. The results were compared to results obtained from three livers perfused and maintained with warm saline (room temperature) in a manner similar to the above described method.

Liver transplant studies: The initial series of transplant experiments were designed to test whether the MNS solution improved survival when compared to NS. The surgeon was blinded to the preservation solution and the solutions were randomized. These experiments compared MNS to NS with a six hour preservation time (N=6/group). The second series of transplants compared MNS to UW with a 36 hour preservation time (N=6/group). These experiments were not blinded as the solutions were visibly different. The solutions were used in a random order.

Intracellular calcium measurements: Baseline calcium measurements were obtained for each sample of cultured hepatocytes. The cells were then suspended in either NS, MNS, or UW and measurements taken every 30 seconds for a 15 minute period (N= 3-4/group).

Results

Membrane Potential Studies

The PD in the left, right and caudate lobes were similar (See figure 7) indicating that PD values were not location dependent[38]. The mean in situ baseline PD value was -37±4 mV. Initial (10 minute) ex vivo PD values for NS, EC, and UW were -23.8±3.5, -11.4±0.4 and -8.7±0.3 mV respectively. Each group was statistically different from the others (p < 0.01) (figure 8). Subsequent hepatic depolarization was most marked in the NS group (PD at 6 hrs; -8.1±1.1). The process was more gradual and limited but complete in the EC group (PD at 6 hrs; -7.7±1.3 mV). The UW group showed no change in PD values throughout the study period (final PD -8.6±1.0 mV). As shown in figure 9, differences in the rates of change (slope of decline) in PD values over one and six hours between each group were also significant (p < 0.01).

The initial PD for the MNS (0.03%, 0.06% and 0.1% ethanol) solutions were -16.7, -12.0, and -9.2 mV respectively. Final PD's at one hour were -13.4, -11.7 and -8.7 mV respectively. Only the 0.1% MNS solution's PD's were similar to the PD values of the UW solution (figure 10). PD's for MNS were measured for six hours but had reached a plateau after one hour.

Enzymes

The initial AST levels at 10 minutes in all bath solutions were zero. The six hour AST levels in the bath solution for NS, EC, and UW were 10.1±7.5, 29±19.4, and 4.26 ±5.9 I.U. respectively. The AST level in the warm saline (control livers) at six hours was 41.8±22.2 I.U. Only the UW livers were significantly different at six hours when compared to the control livers (p < 0.05) (figure 11).

The mean AST level for the MNS group at six hours was 9.5±13 I.U. This was not different from either the control (P=0.14), NS group (P=0.29) or the UW (P=0.39) group. Both the MNS and UW group were statistically different from the EC group (P < 0.05).

Figure 7: PD in Different Liver Lobes
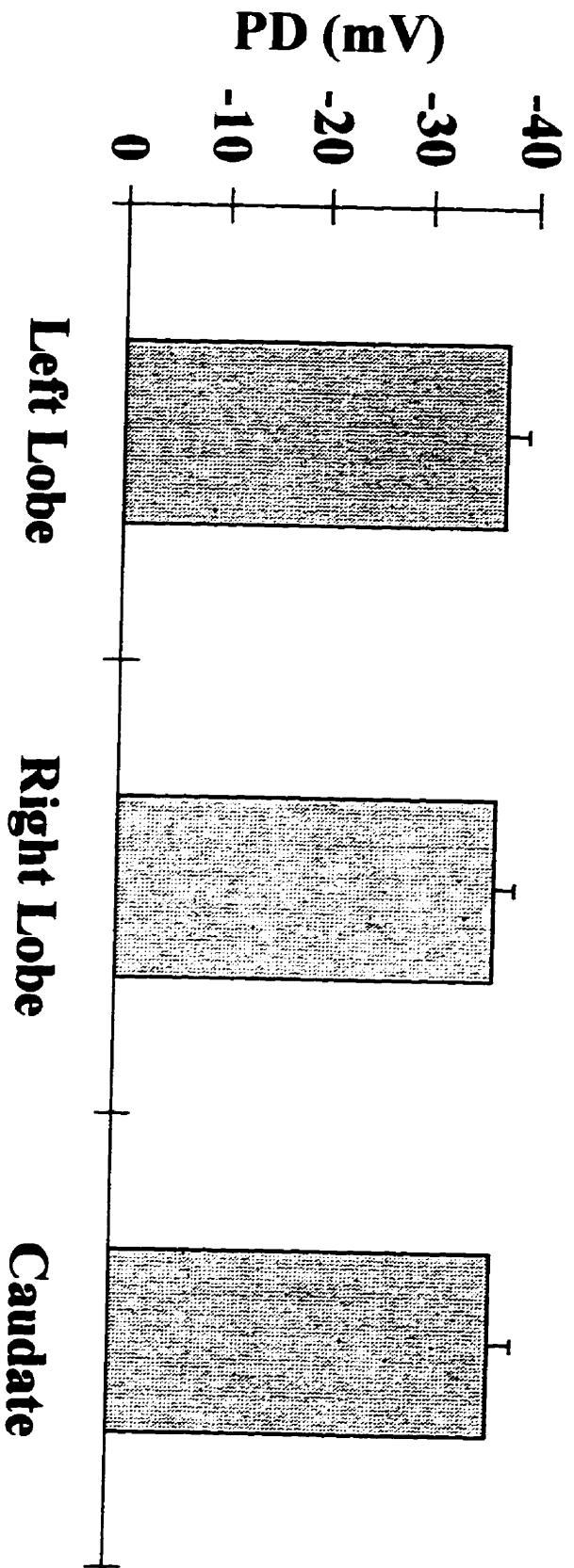
Figure 8: Membrane Potentials 10 minutes post resection
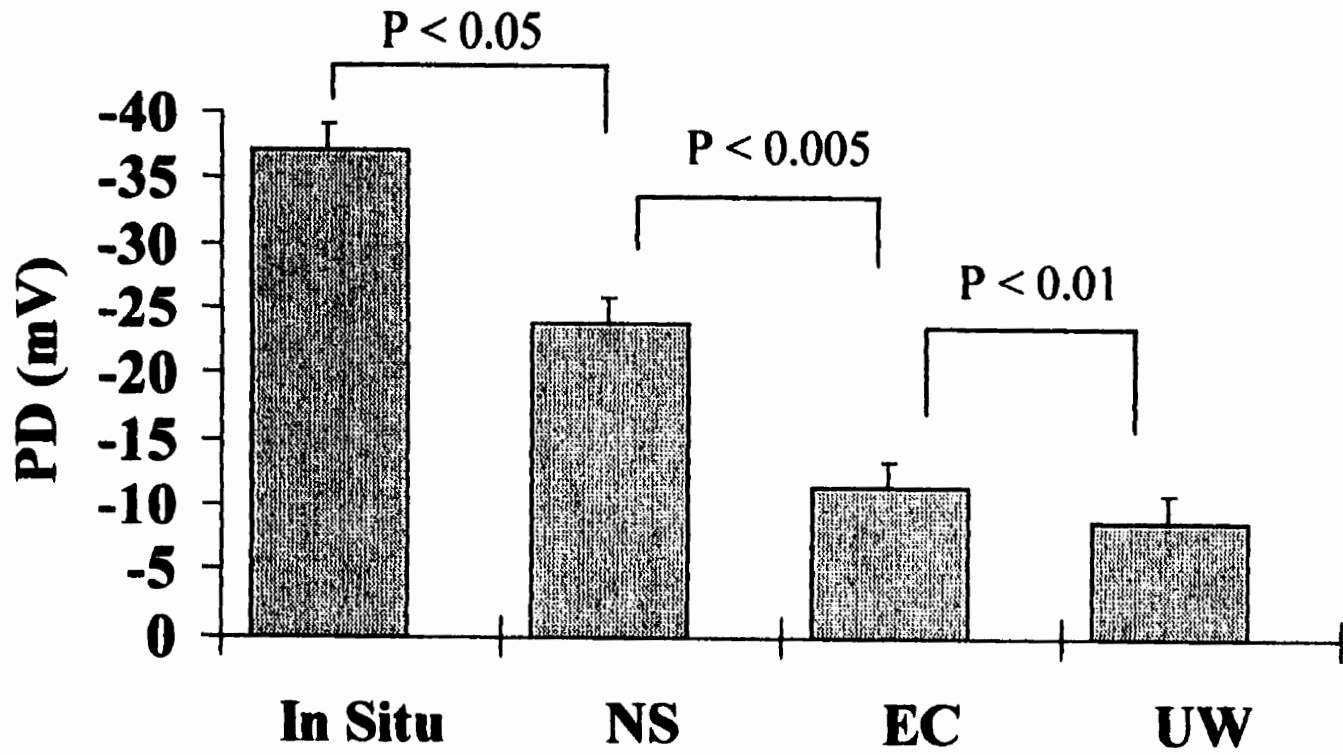
Figure 9: Membrane Potentials over Time
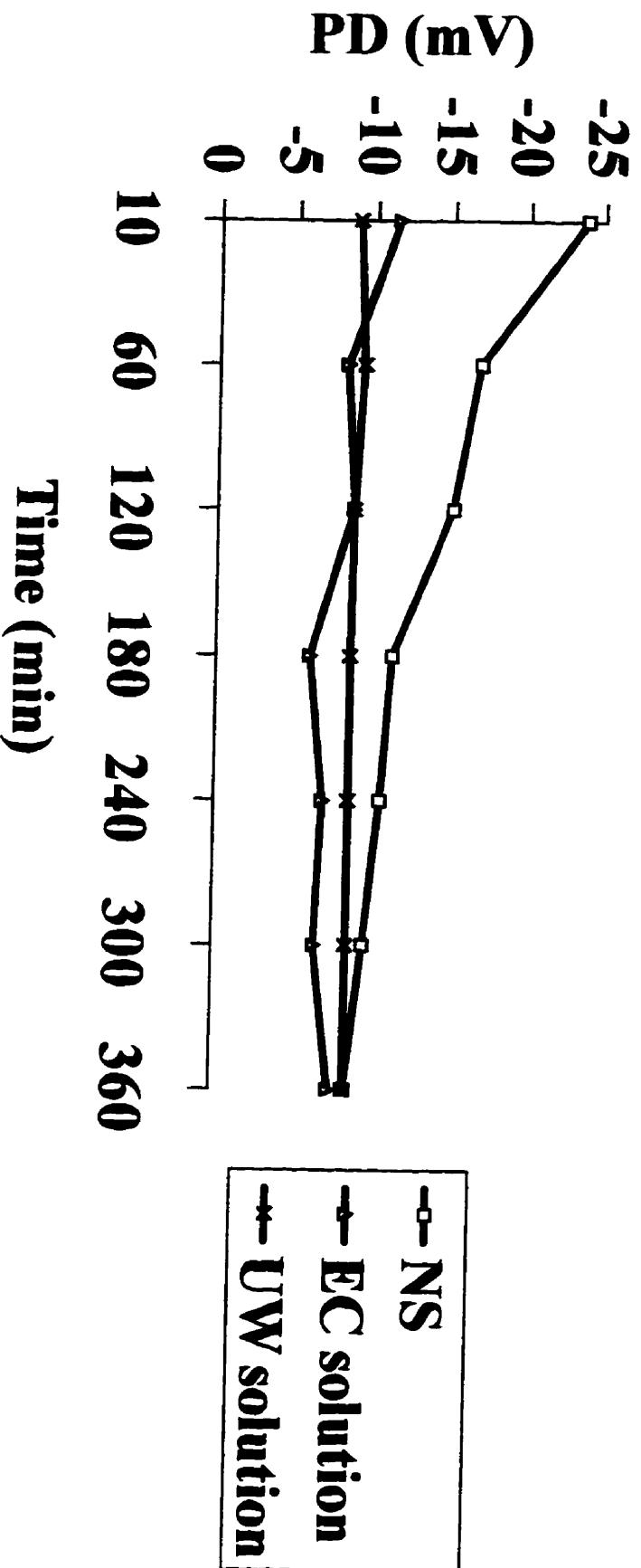
Figure 10: Titration of Modified Saline with Ethanol

Legend:
- NS
- UW solution
- MNS (0.03% ethanol)
- MNS (0.06% ethanol)
- MNS (0.10% ethanol)

Y-axis: Membrane Potential (mV)

X-axis: Time (minutes)

Figure 11: AST Levels at 6hrs in Perfusate Bath

* P < 0.05 vs Warm Perfusion

Liver transplant studies

NS versus MNS: Donor and recipient body weights were similar in the NS (221.2±13.1 grams and 220.8±16.5 grams respectively) and MNS (205.2±26.0 grams and 204.2±25.2 grams respectively) groups (table 4). Donor surgery in both groups was consistently under 30 minutes per animal. Portal clamping times in recipients for the NS and MNS groups were 13.1±1.9 minutes and 13.1±1.2 minutes respectively. The inferior vena cava clamping time in NS and MNS groups were 19.0±3.7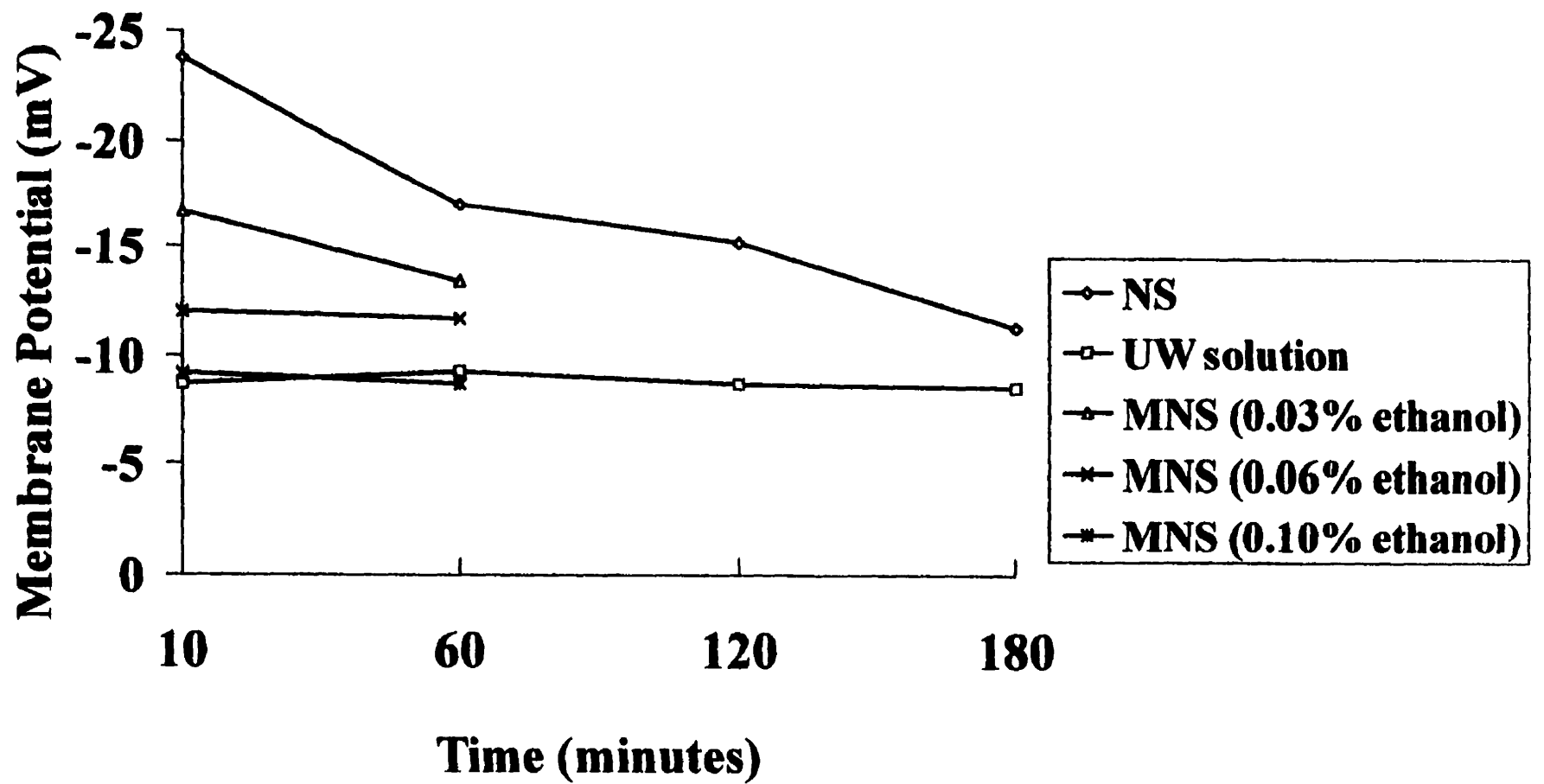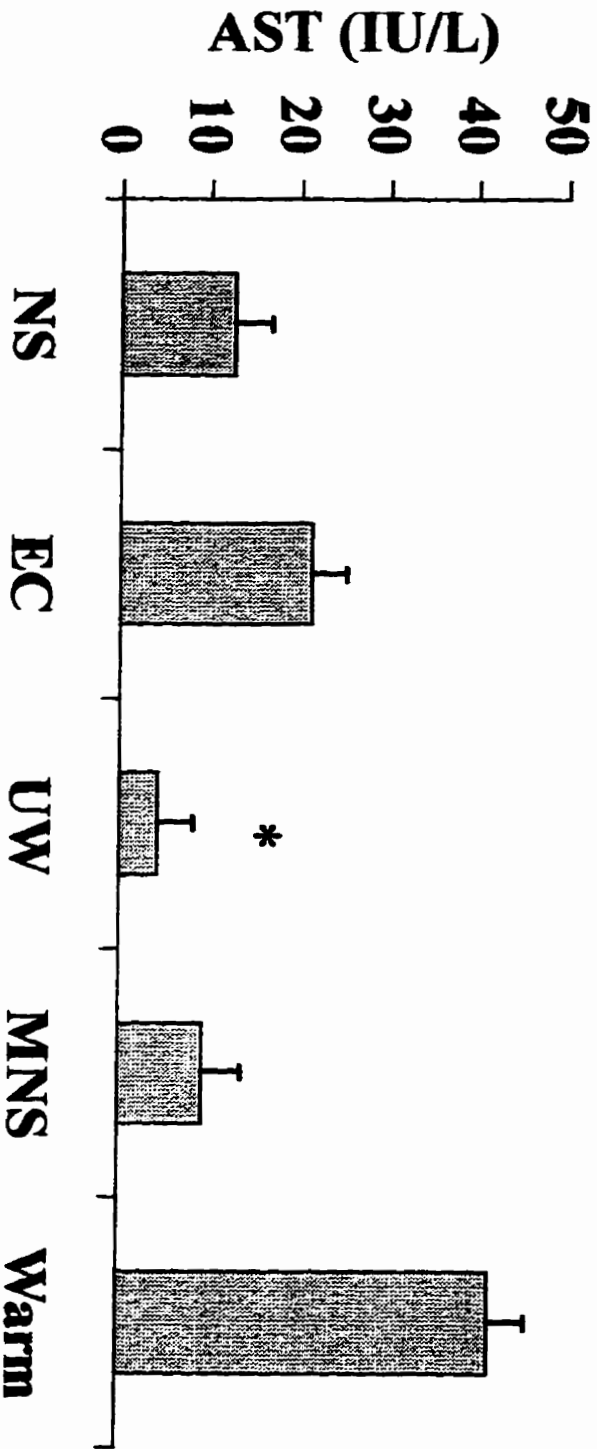 minutes and 17.3±1.4 minutes. The mean preservation times for NS and MNS were 355.0±16.1 minutes and 360.3±14 minutes (Table 5). In summary, there were no significant differences in surgical or preservation times between the two groups.

Survival: Survival in the NS group at 10 days was 1 out of 6 rats (17%). Four of the non-survivors in the NS group died within 12 hours of surgery. One rat lived for two days. Survival in the MNS group was 4 out of 6 rats (67%) (Table 6). The two non-survivors both died within 12 hours of surgery. The survival rate in the MNS group was significantly improved when compared to the NS group (P < 0.05). Autopsies were performed on all rats and the results were negative for signs of internal bleeding or complications arising from anastomoses. Histology was not performed as the majority of the livers in the non-survivors had under gone rapid and extensive autolysis.

Enzymes: The mean AST levels in surviving rats in the MNS group on day 8 were 41.2±26 I.U. The AST level on day 8 in the NS survivor was 56.9 I.U. However, there were too few survivors to analyze and compare the AST values in the NS group.

UW versus MNS: Donor and recipient body weights were similar in the UW (216.5±22 grams and 223.8±18.1 grams respectively) and MNS (229.5±9.9 grams and 244.8±12.6 grams respectively) groups (table 7). Donor surgery in both groups was consistently under 30 minutes per animal. Portal clamping times in recipients for UW and MNS were 11.3±1.6 and 11.7±1 minutes respectively. The inferior vena cava clamping time for the UW and MNS groups were 15.2±2.1 and 15.3±2.1 minutes. The mean preservation time for UW and MNS were 2193.3±21.4 and 2190±26.8 minutes respectively (table 8). These were no significant differences between these groups surgical or preservation times.

Survival: There were no 10 day survivors in either the MNS or UW groups. At

implantation, the MNS livers were pale, and markedly swollen. The blood vessels were intact at the anastomosis sites. Although the livers in the MNS group were markedly swollen at the time of implantation, there were no surgical complications resulting from the edematous livers. Five rats in the UW group died within 2 hours of surgery. One rat survived for 2 days postoperatively (tables 9 and 10). Autopsies on all animals were negative for internal bleeding and complications arising from anastomoses.

Table 4: Comparison of animals in the liver transplant groups

	Donor weights (grams)	Recipient Weights (grams)
NS group	221±13	220±16
MNS group	205±26	204±25

Table 5: Comparison of surgical and preservation times in the liver transplant groups

	Portal clamp time (minutes)	Inferior Vena Cava clamp time (minutes)	Preservation Time (minutes)
NS group	13.3±1.9	19±3.7	355±16.1
MNS group	13.3±1.2	17.3±1.4	360.3±14

Table 6: Survival (10 days) post liver transplant with 6 hour preservation time

	Number of Survivors (N=6/group)
NS group	1
MNS group	4*

* $P < 0.05$

Table 7: Comparison of animals in the liver transplant groups

	Donor weights (grams)	Recipient Weights (grams)
MNS group	229±9	244±12
UW group	216±22	223±18

Table 8: Comparison of surgical and preservation times in the liver transplant groups

	Portal clamp time (minutes)	Inferior Vena Cava clamp time (minutes)	Preservation Time (minutes)
MNS group	11.7±1	15.3±2.1	2190±26
UW group	11.3±1.6	15.2±2.1	2193±21

Table 9: Survival (2 days) post liver transplant with 36 hour preservation time

	Number of Survivors (N=6/group)
MNS group	0
UW group	1

Table 10: Survival (10 days) post liver transplant with 36 hour preservation time

	Number of Survivors (N=6/group)
MNS group	0
UW group	0

Intracellular Calcium studies: Baseline measurements were taken prior to suspending the cells in either NS, UW or MNS solution (N=3-4/group). Subsequent intracellular calcium levels were expressed as a percentage of baseline. The one minute results in NS, UW and MNS were 99±21%, 88±4% and 90±4% respectively. The five minute calcium levels were 82±16%, 61.5±15% and 70±11% respectively. By 15 minutes, the intracellular calcium levels had dropped to 64±15%, 37±24% and 56±13% (figure 12). The UW group resulted in the lowest intracellular calcium level when compared to NS and MNS. However, both the UW and MNS results were significantly lower than the NS values at each time indicated (p < 0.005).

Figure 12: Changes in Intracellular Calcium over Time
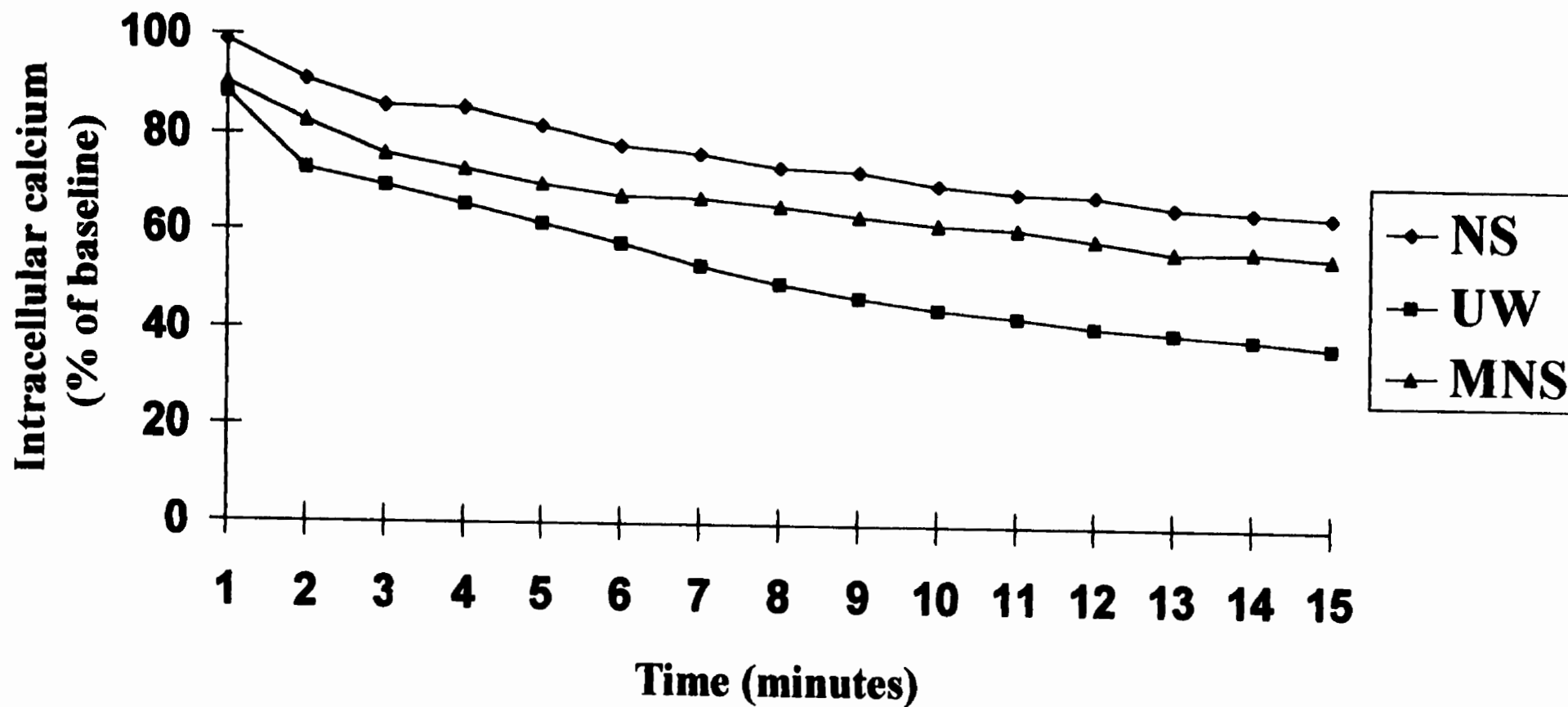
Discussion

The results of this study provide new insights into transplant-related physiology and pathophysiology. Specifically, the data indicate that the membrane potential of the liver significantly depolarizes following its excision and placement in organ preservation baths. The data also indicate that the extent of the depolarization process is perfusate/bath-dependent with relatively limited depolarization being associated with normal saline, moderate depolarization with Euro-Collins solution and most marked depolarization with Wisconsin solution. Of potential clinical interest was the finding that by modifying normal saline such that changes in hepatic membrane potentials resembled those obtained with Wisconsin solution, there was improved survival and a trend towards reduced biochemical injury following transplantation. Finally, the results indicate that differences in bath solution-induced membrane potentials are associated with changes in intracellular calcium homeostasis.

Although hepatocyte membrane potentials have variously been reported to remain unchanged, hyperpolarize, or depolarize following partial hepatectomy, the results of the present study are the first to describe changes in hepatocyte membrane potential following complete excision of the liver[38,50,58,59,60]. That the liver depolarizes with excision is in keeping with reports describing depolarization following partial hepatectomy as the liver in both conditions is in a replicative state[38].

Examination of hepatocyte membrane potentials following orthotopic liver transplantation would ideally document return of the membrane potential to it's insitu baseline. However, in the immediate postoperative state, the animals are unstable and require closure of their abdominal wall, to help minimize the surgically induced massive fluid and heat abnormalities. At this stage, the animals do not tolerate any further interventions. Attempts to re-enter the abdomen in these transplanted rats at later dates, in the hope of measuring membrane potentials, were met by dense adhesions of the small bowel and abdominal wall to the transplanted liver. These adhesions were atypical of re-operative liver surgery, as they were denser than fibrinous adhesions usually found in the post-operative period following other abdominal surgery, likely due to the extensive manipulations required during the transplant

procedure. Therefore, even if an appropriate site on the transplanted liver would have been accessible, the dissection itself, may have caused sufficient variations in the membrane potential that the results would not be reliable. Furthermore, the results were unlikely to be reproducible as each rat was different, and there could be no acceptable controls for this situation.

The mechanism whereby the liver depolarizes following resection remains to be determined. The finding that the extent of the PD changes were perfusate/bath solution-dependent suggests that the solutions per se rather than extrinsic, neuronal innervation are more relevant. In future experiments, we hope to demonstrate that immediately following transplantation hepatocyte membrane potentials return to pre-transplant values further suggesting that hepatic perfusion rather than neuronal innervation plays a more important role in regulating PD changes in this setting. These future studies are provisional on a new method of overcoming the post-operative problems with adhesions.

The principle purpose of the present study was to determine whether the membrane potential changes observed were of pathophysiologic importance or merely secondary to changes in hepatocyte viability and/or function. The finding that Wisconsin solutions were associated with the most marked changes in hepatic PD (depolarization of approximately 15 mV) argues in favor of a pathophysiologic role, particularly in light of the fact that depolarization had previously been considered evidence of hepatocyte injury. Even more compelling was the fact that by modifying normal saline solutions such that membrane potential changes resembled those associated with Wisconsin solution, improved survival resulted and a trend towards less biochemical evidence of hepatic injury post transplantation was seen. Unfortunately, our inability to identify an agent which consistently stabilizes hepatocyte membrane potentials at baseline values precluded any attempt to supplement Wisconsin solution with this agent, and thereby determine whether the outcome would have been compromised.

The improved survival in the modified normal saline transplant group over the normal saline group further strengthens the membrane potential data, suggesting that hepatic PD's are of pathophysiologic importance. Although the sample size is small, given the complex nature of

the surgery, it is in keeping with published studies for rat liver transplant research and in fact larger than some reported studies[8,9,54,61,62,63]. To eliminate the potential for causes of death in these animals other than preservation injuries, Lewis rats were used to avoid rejection and standard autopsy techniques were utilized as documented in other preservation research studies[7,8,61]. Furthermore, the survival period of 10 days has been shown to be an adequate period of time for determining preservation related deaths[62,63].

The selection of modified normal saline for comparison to University of Wisconsin solution was a practical choice. To compare UW to a modied version of UW, was unlikely to have resulted in any differences in survival, as UW by itself is a very effective preservation solution. The decision to use 36 hours for comparing modified normal saline to UW was a difficult one. Below twenty-four hours of preservation, UW results in 100% survival in animal models. The half life for 50% survival with UW solution is not clearly defined. In fact, centers have documented this time to lie between twenty-four and forty hours. If a shorter preservation time was used, the superior ability of UW as a preservation agent might have masked any beneficial effects that the modified saline would have demonstrated.

In retrospect, the failure of modified normal saline solutions to compare favorably with the results of Wisconsin solutions following 36 hours of liver preservation time should have been predicted. Regardless of PD values, the hypo-osmolarity of the saline solutions should have been predicted to result in swollen and compromised livers. Whether the addition of an osmotically active substance to the modified normal saline solution would have circumvented this problem, deserves further study.

Calcium exists in many concentrations, forms (bound and unbound) and locations (microsomal, mitochondrial) throughout the cell. Since proteolytic enzyme activity was not directly measured, it was impossible to determine whether the decreased total intracellular calcium concentrations documented in this study with Wisconsin and modified normal saline solutions, were sufficient to result in calcium-related changes in proteolytic enzyme activity. Nonetheless, the fact that depolarized hepatocytes either limit the influx of extracellular calcium or enhance the efflux of intracellular calcium supports the possibility that calcium-mediated proteolytic activity is attenuated by Wisconsin and other membrane depolarizing

solutions.

Unfortunately, it is impossible to measure intracellular calcium levels in whole excised livers or in transplanted liver. As a result, the calcium studies were performed in isolated cultured hepatocytes. The results of these studies are suggestive but not indicative of the effects that UW and modified saline might have in whole excised livers during cold preservation. This data still is supporting the role of intracellular calcium levels in predicting clinically useful preservation solutions as the higher calcium levels occurred in the poor preservation solutions while the low calcium levels were in the more effective solutions (UW and MNS).

Future studies on preservation solutions should focus on pharmacologic interventions to prevent preservation injuries. For example, the effects of calcium channel blockers on hepatocyte membrane potentials has not been determined. Furthermore, the role of pharmacologically altering the donor liver prior to harvesting is another option in the hope of minimizing re-perfusion injuries.

In conclusion, the results of this study further our understanding of the physiologic and pathophysiologic changes that occur in association with liver transplantation. They also point to the development of new, perhaps less expensive preservation solutions that are based on their effects on hepatocyte membrane potentials and intracellular calcium levels.

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