

Xenogeneic, Extracorporeal Liver Perfusion in Primates Improves the Ratio of Branched-Chain Amino Acids to Aromatic Amino Acids (Fischer's Ratio)

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Key Words

Extracorporeal liver perfusion · Amino acids · Hepatic failure · Hepatic encephalopathy · Primate · Dialysis · Xenogeneic liver perfusion

Abstract

In fulminant hepatic failure (FHF), the development of hepatic encephalopathy is associated with grossly abnormal concentrations of plasma amino acids (PAA). Normalization of the ratio of branched-chain amino acids to aromatic amino acids (Fischer's ratio) correlates with clinical improvement. This study evaluated changes in PAA metabolism during 4 h of isolated, normothermic extracorporeal liver perfusion using a newly designed system containing human blood and a rhesus monkey liver. Bile and urea production were within the physiological range. Re-

lease of the transaminases AST, ALT and LDH were minimal. The ratio of branched (valine, leucine, isoleucine) to aromatic (tyrosine, phenylalanine) amino acids increased significantly. These results indicate that a xenogeneic extracorporeal liver perfusion system is capable of significantly increasing Fischer's ratio and may play a role in treating and bridging patients in FHF in the future.

Introduction

Fulminant hepatic failure (FHF) is regularly associated with encephalopathy [1]. If supportive measures fail, then the stage of encephalopathy progresses and brain edema develops [2]. For many patients, liver transplantation is the therapy of choice that yields the best prognosis for survival and quality of life

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[3–5]. Transplantation must be carried out before irreversible neurologic complications secondary to brain edema occur. Apart from liver transplantation, therapeutic measures such as continuous hemofiltration and plasma exchange have not been very successful in preventing FHF patients from developing brain edema to date [6].

During FHF, grossly abnormal concentrations of a wide variety of plasma amino acids (PAA) can be measured. PAA play a decisive role in the pathophysiology of hepatic encephalopathy (HE) and brain edema [7–9]. Increasing the ratio of branched-chain amino acids (BCAA) to aromatic amino acids (AAA) correlates well with an improvement in HE [10]. Under clinical conditions the source of PAA is difficult to determine. The source can be either the diseased liver or the body muscle mass.

During the late 60s and early 70s, extracorporeal liver perfusion with livers from different species was investigated as a method to treat patients in FHF, expecting the affected liver would regenerate [11, 12]. Recently, the same technical approach of liver perfusion has been applied in FHF patients waiting for liver transplantation [13]. So far, no evidence has been presented that a normothermic extracorporeal liver perfusion (NELP) system is capable of improving the BCAA to AAA ratio.

This study examines the role of an improved NELP system utilized under defined conditions on changes in PAA levels and the BCAA/AAA ratio.

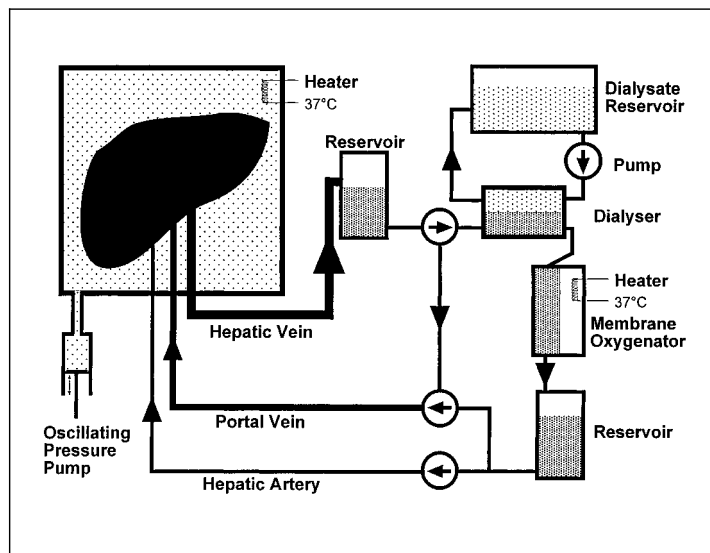
Methods

Six rhesus monkeys (*Macaca mulatta*) of either sex weighing 10.3 ± 0.6 kg were used as multiple organ donors. All animals received a standard commercial diet and were allowed free access to food and water prior to hepatectomy.

Organ Harvesting. After intramuscular premedication with ketamine hydrochloride, anesthesia was induced with etomidate and azaperone followed by endotracheal intubation. Maintenance of anesthesia was carried out with halothane, fentanyl and a mixture of oxygen and nitrous oxide on a mechanical ventilator. A midline abdominal incision was made from the xiphoid process to pubis. The liver was dissected and the infra- and suprahepatic vena cava, portal vein, common bile duct, and hepatic artery were exposed. The common bile duct was ligated and divided as distally as possible. The gallbladder was excised and the cystic duct ligated. Branches of the celiac and hepatic arteries to the stomach and duodenum were ligated and divided along with the coronary artery and phrenic veins and all connective tissue surrounding the liver. A ligature was placed around the descending thoracic aorta. The kidneys and ureters were manipulated before organ perfusion. A thoracotomy was carried out, and the heart and lungs were prepared for organ removal. For organ perfusion one catheter was placed in the abdominal aorta and a second catheter in the portal vein. The portal vein and the abdominal aorta were perfused with 400 ml University of Wisconsin (UW) solution, respectively. For storage, the liver was wrapped in a double plastic bag and stored at 0°C in UW solution. The storage period varied from 60 to 80 min.

Liver Perfusion. At the end of the storage period, livers were prepared for perfusion by inserting spring perfusion cannulae into the portal vein, vena cava and hepatic artery. Then the liver was placed in a plastic bag and immersed in water (37°C) contained in a specially designed perfusion chamber. The liver was subjected to intermittent oscillations produced by an external pump. The pressure difference was in the range of 0 to 25 cm H₂O. The liver was perfused via cannulae in the portal vein and hepatic artery. The suprahepatic vena cava was ligated while blood outflow was allowed through the infrahepatic vena cava. According to physiological conditions, the mean arterial pressure was adjusted to 100 mm Hg and the portal venous pressure to 15 cm H₂O. The flow rates measured under these conditions were about 150 ml/min through the hepatic artery and 250 ml/min through the portal vein. Our perfusion system allows for easy control and adjustment of flow rates and perfusion pressures. The Dideco D701 pediatric hollow fiber oxygenator was supplied with a mixture of 95% oxygen and 5% carbon dioxide. Bile production was measured continuously. Since the livers were stored in UW solution, we flushed them with warm and equilibrated perfusate according to our clinical protocol. The first 200 ml of perfusate were discarded before perfusion was started.

Fig. 1. The liver was placed in a sterile plastic bag, which was then immersed at 37°C in water and was subjected to intermittent oscillations produced by an external pump. The liver was perfused via cannulae in the portal vein and hepatic artery. The suprahepatic vena cava was ligated while blood outflow was allowed through the infrahepatic vena cava. The Dideco D701 pediatric hollow-fiber oxygenator was supplied with a mixture of 95% oxygen and 5% carbon dioxide. The simultaneous dialysis circuit consisted of two Stöckert roller pumps, a Gambro ALWALL GFS 12 fiber dialyzer and a reservoir containing 10 liters of dialysate.



Perfusate. The perfusate consisted of 800 ml human blood and 400 ml of a balanced electrolyte solution to give a total volume of 1.2 liters. The blood was donated by healthy blood donors 90 min before each perfusion. The monkeys were tested not to have any preformed antibodies for blood groups O and B. Only blood from donors of blood groups O and B was used as perfusate.

Dialysis. The dialysis circuit consisted of a Gambro ALWALL GFS12 fiber dialyzer (Gambro, Lund, Sweden), two Stöckert roller pumps (Stöckert-Shiley, Munich, Germany) and a reservoir for the dialysate. The perfusate was pumped at a steady rate of about 150 ml/min through the dialyzer. The dialysate was pumped at a rate of 1,000 ml/min from the reservoir and back in a recirculating fashion. Both sides of the dialyzer were controlled and if necessary adjusted to keep the volumes of perfusate/dialysate constant. The perfusate which had passed the dialyzer is pumped back into the main perfusion circuit to be oxygenated. The perfusion-dialysis apparatus including all tubes and the fiber oxygenator were primed with an albumin-containing electrolyte solution to avoid hemolysis. The priming solution was discarded after 10 min of perfusion. Heparin was continuously injected at a rate of 2,500 U/h to protect the perfusate in the circuit from clotting. Figure 1 shows a diagram of the perfusion circuit.

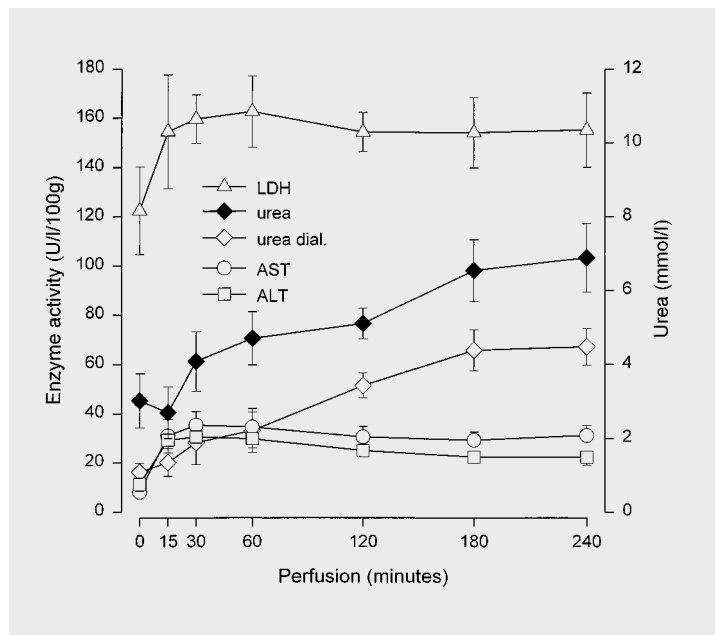
Dialysate. Ten liters of dialysate were prepared before each experiment. The dialysate consisted of commercially available acid and basic bicarbonate

concentrate and distilled water. The dilution ratios were 1:1.78 and 1:42.22, respectively. The prime buffer of the dialysate was sodium bicarbonate. The electrolyte concentrations of the dialysate (in mmol/l) were Na⁺ 141, K⁺ 2.9, Ca²⁺ 0.8, Mg²⁺ 0.38, Cl⁻ 113, CH₃COO⁻ 2.5 and HCO₃⁻ 37. The osmolality of the dialysate was 296 mosm/kg.

Amino Acids. The samples were deproteinated by adding 50 mg sulfosalicylic acid (Merck) per 1-ml sample, centrifugated at 10,500 g for 5 min, ultrafiltered through a membrane with a pore size of 0.2 μm (Minisart NML, Sartorius), adjusted to a pH of 2.2, and finally diluted 1:2 with diluent containing norleucine as an internal standard prior to injection. Analyses were performed on a Pharmacia/LKB Alpha Plus amino acid analyzer containing ultrapack cation-exchange resin column (Pharmacia, Uppsala, Sweden) and equipped with a Shimadzu integrator. The post-column ninhydrin reaction was carried out at 135°C. The system used changes in the eluent lithium citrate buffer from pH 2.8/0.2 mol/l to pH 3.55/1.65 mol/l and a regeneration solution of 0.3 mol/l lithium hydroxide. Changes in column temperature were between 32 and 75°C.

Analyses. Six livers were perfused. Perfusate and dialysate samples were taken before, during (at 15 and 30 min) and after perfusion (every 30 min). Perfusion was carried out over a period of 4 h. Enzyme activities are given in units per liter per 100 g liver weight. Electrolyte concentrations are given in millimoles per liter. Amino acid levels are expressed as means ± SEM (in

Fig. 2. The activities of AST, ALT, and LDH (U/l/100 g) increased after reperfusion until 15 min. This was attributed to hepatocellular damage caused by preservation injury. During the following 225 min, the enzyme activity remained fairly constant. Based on the significant increase in urea (mmol/l) in both perfusate (1.2 liters of circulating volume) and dialysate (10 liters of circulating volume) and a normal production of bile, the system apparently provided physiological conditions for the perfused livers.



μmol of amino acid per liter of sample). Comparison between the BCAA/AAA ratios was done by Student's t test. A p value <0.05 was considered statistically significant.

Results

All livers regained a pink and homogenous appearance within 10 min of starting perfusion. There was no macroscopic evidence of inadequate perfusion. As observed from on-line monitoring, the vascular resistance was highest at the start of perfusion, but settled then down to a steady state after about 20 min. No technical failures or interruptions occurred during the perfusion of these 6 livers.

Electrolytes, Enzymes, Urea and Bile Production

The concentration of potassium, sodium and calcium in the perfusate varied within physiological limits. The dialysate neutralized

excess electrolyte released after reperfusion. As it has been shown recently [14], the dialysis unit assists in maintaining physiological concentrations of pH and electrolytes throughout the perfusion period.

During the whole perfusion process, no enzyme activity could be detected in the dialysate. Figure 2 shows the changes in enzyme activity in the perfusate and urea concentrations in perfusate and dialysate during the 4 h of liver perfusion. The activity of AST in the perfusate before reperfusion was 8 U/l/100 g. Following 15 min of perfusion it increased to 25 U/l/100 g. After 240 min of perfusion the activity measured was 27 U/l/100 g. ALT increased from 11 U/l/100 g before reperfusion to 21 U/l/100 g 15 min thereafter. The activity of ALT for the remaining 225 min of perfusion was 17–23 U/l/100 g and without a clear trend in either direction. LDH activity increased from 123 U/l/100 g before reperfusion to 155 U/l/100 g after 240 min. Similar to

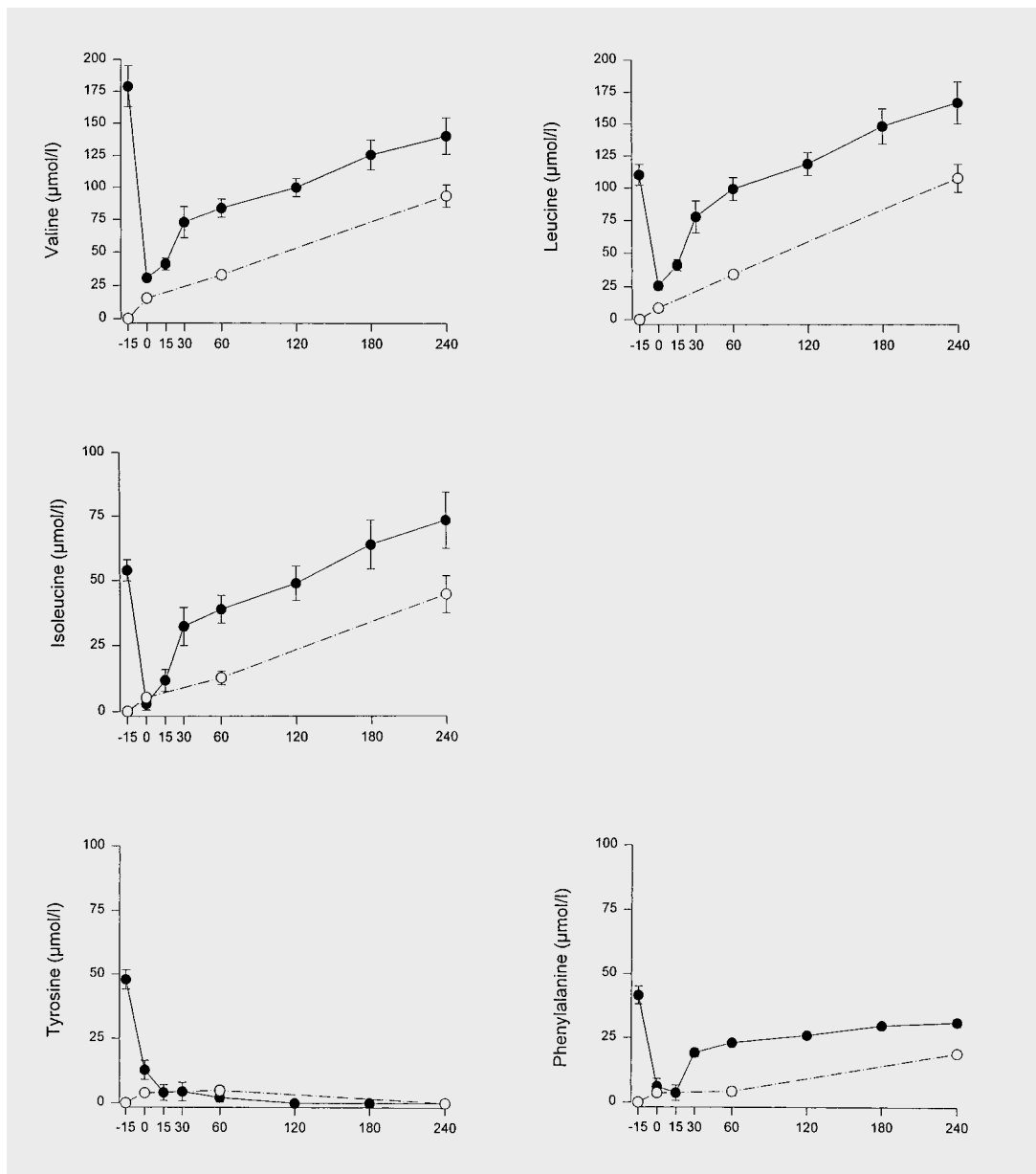


Fig. 3. The BCAA (valine, leucine, and isoleucine) and the AAA concentrations (tyrosine and phenylalanine) are given during 4 h of liver perfusion. PAA concentrations ($\mu\text{mol/l}$) of the perfusate (closed symbols) and dialysate (open symbols) are given. The point -15 corresponds to initial levels in the perfusate and dialysate.

Time point 0 min reflects the concentrations of PAA after an equilibration period of 15 min between the perfusate (1.2 liters) and the dialysate (10 liters) just prior to liver reperfusion. Positive time points correspond to the period of liver perfusion. Means \pm SEM.

AST and ALT, the largest increase in LDH release was measured immediately after reperfusion.

During the equilibration period, the concentration of urea in the perfusate was 2.8 mmol/l. The concentration of urea at the end of perfusion was 6.9 mmol/l in the perfusate. The amount of urea in the dialysate paralleled the increase in the perfusate. After equilibrium, but before reperfusion, the urea concentration was 1.1 mmol/l. It increased steadily reaching 4.5 mmol/l at 240 min. Bile production was noted about 20 min after reperfusion. The average amount of bile produced per 100 g liver weight was 2.6 ml during the 1st h of perfusion, 3.5 ml during the 2nd h, 3.3 ml during the 3rd h and 2.5 ml during the 4th h of perfusion.

A more detailed description of perfusion parameters has been given recently [15].

Amino Acids

The concentrations of amino acids in the perfusate and dialysate are given in figures 3 and 4. Time point -15 min corresponds to the PAA concentration of the perfusate just before starting dialysis. At time point 0 min the perfusate had been dialysed for 15 min, but reperfusion of the liver had not begun.

The ratios of BCAA/AAA [(valine+leucine+isoleucine)/(tyrosine+phenylalanine)] in the perfusate are given in figure 5. At -15 (before dialysis), 0, 60 and 240 min, the ratios were 3.1, 3.8, 8.8, and 12.1, respectively, in the perfusate. Calculation of the ratios for the dialysate gave very similar results. At 0 min, the ratio was 3.6, at 60 min the ratio was 8.7 and at 240 min it was 12.9. The increased BCAA/AAA ratios between 0 (before liver perfusion) to 60 and 240 min after liver reperfusion were statistically significant in both the perfusate ($p < 0.01$ and $p < 0.001$) and dialysate ($p < 0.01$ and $p < 0.001$).

Discussion

Approximately 2,000 cases of FHF are diagnosed annually in the United States, with mortality approaching 80% [16]. Despite improvements in conservative medical treatments, liver transplantation remains the therapy of choice for a large number of patients [17].

In patients with FHF, PAA levels deviate considerably [18]. The BCAA valine, leucine and isoleucine are low – in contrast to most other PAA [19]. There is strong clinical and experimental evidence that elevated PAA levels are a prerequisite for the development of HE and brain edema [9, 10]. Normalization of PAA has been previously attempted by hemodialysis and plasma exchange [20]. In a group of 26 patients with FHF and signs of grade IV coma, levels of BCAA were lowest in those patients that subsequently died [18]. The adjustment of the molar BCAA/AAA ratio from 1 (e.g. in hepatic coma) towards 3 (normal subjects) has a beneficial effect upon encephalopathy [19]. The BCAA/AAA ratio is increased in patients receiving supplemental BCAA [21]. The role of oral BCAA supplements in the treatment of chronic HE is not yet established, and conflicting opinions have been advanced [22–26]. Two meta-analyses of randomized clinical trials support the view that BCAA are improving hepatic encephalopathy even when given orally [27, 28]. Although BCAA and AAA play important roles in the development of brain edema, experimental evidence is lacking that an extracorporeally perfused liver can improve this ratio. Liver function in an NELP system is determined by appropriate physiological perfusion conditions.

We have developed a perfusion system in which the liver is immersed in a closed perfusion chamber that allows for intermittent pressure oscillations. This proved to be indis-

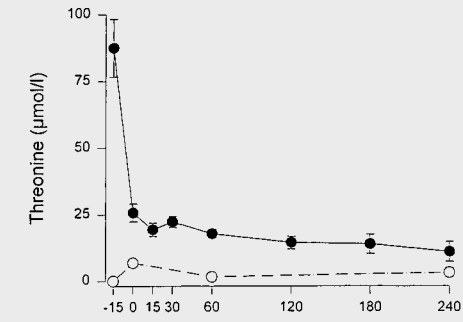
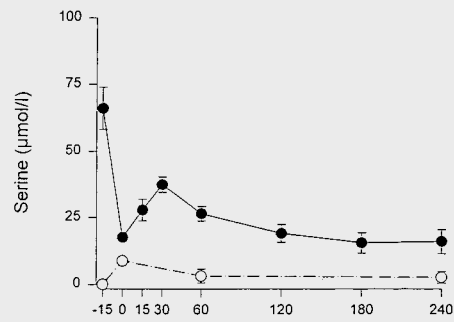
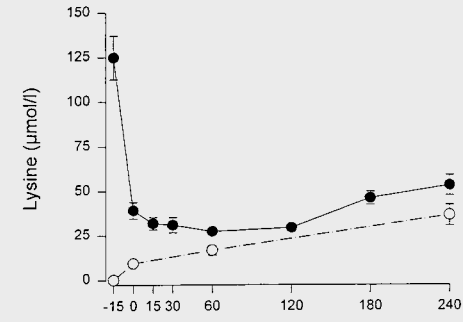
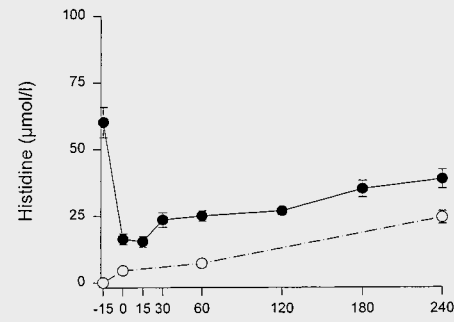
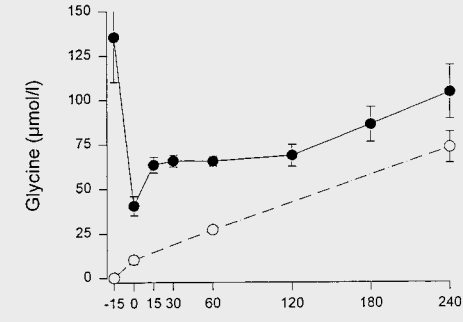
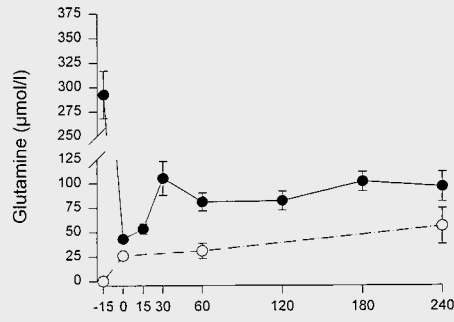
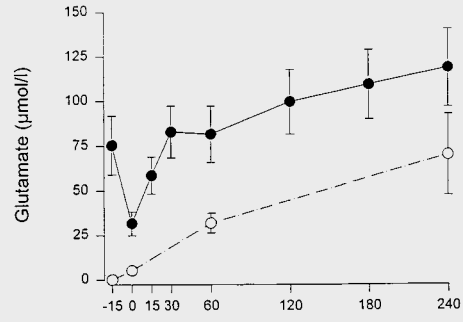
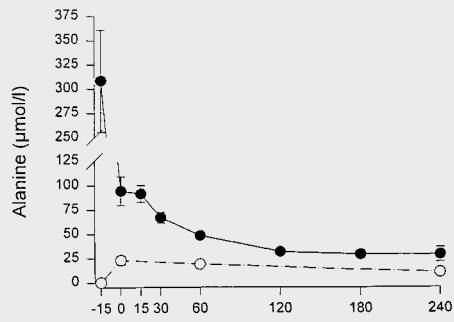
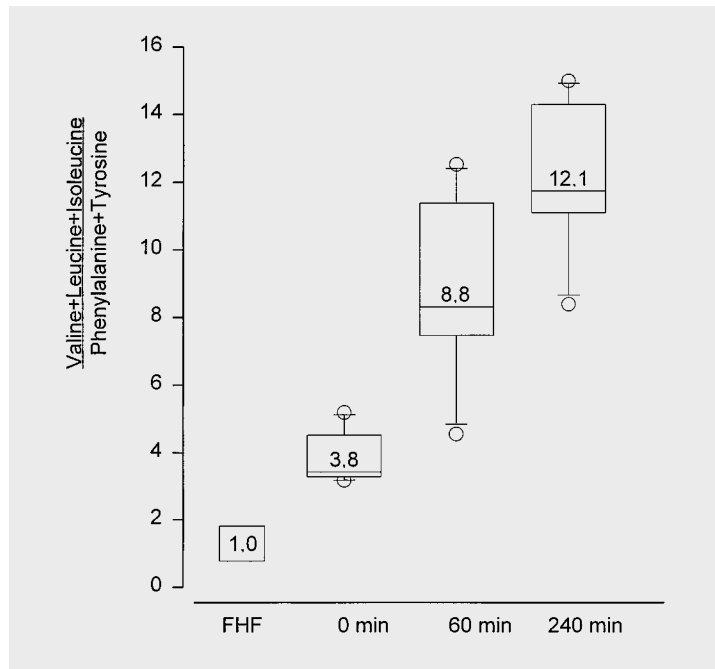


Fig. 5. The ratios of valine+leucine + isoleucine / tyrosine + phenylalanine concentrations were calculated on the basis of their perfusate concentration ($\mu\text{mol/l}$) at 0, 60, and 240 min. In FHF, the ratio seen clinically is close to 1. The ratio increased from 3.8 before liver perfusion (i.e. 0 min) to 8.8 after 60 min and further increased to 12.1 after 240 min of perfusion. Both increases were statistically significant.



pensable for homogeneous peripheral perfusion particularly in the portal venous system [29]. Recently, we have shown that NELP can be further improved by simultaneous dialysis of the perfusate [14].

On the basis of physiological amounts of bile produced (11.9 ml/100 g/4 h), very low activities of AST, ALT, and LDH (fig. 2), and the lack of gross or histologic evidence of poorly perfused areas, it is our opinion that this system provides excellent physiologic perfusion conditions.

Since PAA metabolism depends on the functional integrity of the liver, the results of a

Fig. 4. The concentrations of alanine, glutamate, glutamine, glycine, histidine, lysine, serine, and threonine in the perfusate and dialysate are shown. Perfusion conditions and parameters were identical to those in figure 3.

carefully perfused extracorporeal liver should be similar to those found under clinical conditions. We therefore analyzed changes in PAA concentrations within the perfusate. The design of our perfusion system consists of two closed circuits, one of 1.2 liters for the perfusate and one of 10 liters for the dialysate. Due to the circuit design of our perfusion, all concentrations of PAA within the perfusate decrease from the moment the dialyzer was connected to the perfusion system. This is the result of a dilution phenomenon since PAA cross the membrane of the dialyzer. Therefore, concentrations of PAA in the dialysate were included in the calculation for uptake or production of amino acids.

Once equilibration of the perfusion and dialysis circuit was established, reperfusion of the liver was started at time point 0 min. Following reperfusion, changes in PAA concentrations in the perfusate or dialysate could

only be attributed to their release or utilization by the liver.

The concentration of all BCAA – valine, leucine and isoleucine – increased significantly in the perfusate as well as in the dialysate during the 4-hour perfusion. The AAA phenylalanine increased slightly to plasma concentrations noted before perfusion while the other AAA tyrosine decreased during perfusion. As a result, the BCAA/AAA ratio increased from 3.8 to 8.8 ($p < 0.01$) after 60 min and to 12.1 after 240 min of perfusion ($p < 0.001$). The increase from 3.8 (normal ratio) towards artificially high ratios of 8.8 and 12.1 should provide a HE patient with some benefit since the patient's pathologically low ratio of around 1 would increase substantially. This view is further supported by a similarly increased ratio in the 10 liters of dialysate.

Although perfusate concentrations of threonine, alanine and tyrosine were initially low, these dropped further during perfusion. Serine was released after reperfusion and then taken up by the liver, which may be due to a gluconeogenic effect because the rate of glucose synthesis from alanine and serine is far higher than that observed for all other PAA during liver perfusion [30]. While this might not play an important role in the elevated PAA levels during FHF, threonine, serine, alanine and tyrosine are likely to be metabolized in larger quantities if the extracorporeal

liver perfusion is performed as an isolated perfusion over a longer period of time, i.e. for preservation or pharmacological studies. We would suggest to substitute for their loss either by addition to the perfusate or to the dialysate. This suggestion is further based on an observation during normothermic liver perfusion of ischemia-damaged livers in which addition of amino acids was of particular importance for liver resuscitation [31].

The concentration of glycine, histidine, lysine, glutamine, and glutamate increased in the perfusate as well as in the dialysate. We cannot conclude with certainty that the extracorporeally perfused liver would release these PAA when perfused with high concentrations of PAA, e.g. in patients with FHF, but it seems rather unlikely that it will metabolize large quantities of these amino acids [32].

More than 600 enzymatic systems are known currently causing many metabolic changes in case of liver failure [33]. More likely, HE is of a multifactorial origin. In this context, the importance of the amino acid pattern in the pathogenesis of HE becomes more and more evident [26, 34].

For the first time we could show that an improved NELP system significantly raises the BCAA/AAA ratio. This system therefore may improve the encephalopathic state of FHF patients waiting for liver transplantation.

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