Impaired gluconeogenesis in a porcine model of paracetamol induced acute liver failure

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RESULTS: No significant changes were observed in the concentrations of the amino acids studied in the animals maintained under anaesthesia only. If we look at the ALF animals, we observed a statistically significant rise of lactate ($P < 0.003$) and pyruvate ($P < 0.018$) at the end of the experiments. We also observed statistically significant rises in the concentrations of alanine ($P < 0.002$), glycine ($P < 0.005$), threonine ($P < 0.048$), tyrosine ($P < 0.000$), phenylalanine ($P < 0.000$) and isoleucine ($P < 0.01$). Valine levels decreased significantly ($P < 0.05$).

CONCLUSION: Our pig model of ALF is characterized by an altered gluconeogenetic capacity, an impaired tricarboxylic acid (TCA) cycle and a glycolytic state.

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Key words: Lactate; Pyruvate; Branch chain amino acids; Aromatic amino acids

INTRODUCTION

Acute liver failure (ALF) is a clinical syndrome defined by massive cell death in the absence of chronic liver disease, resulting in hepatic encephalopathy. Although brain oedema and brain herniation are common causes...
of death in ALF multi organ failure is also common\[^{2,3}\]. Previous studies have shown that metabolic pathways are affected in ALF and systemic changes in metabolite levels could be the pathophysiological reason behind multi organ failure in ALF\[^{4,5}\].

Orthotopic liver transplantation remains the most widely accepted treatment for ALF\[^{6,7}\]. Chronic donor shortages however, motivate the search for alternative non-surgical therapies. In that quest animal models of ALF play an important role. Most studies have explored mouse models of ALF which although useful do not adequately address the issue\[^{4,5}\]. We have recently developed in our laboratory a porcine model of paracetamol induced ALF\[^{8}\]. It is characterized histologically by severe centrilobular necrosis with coagulative necrosis. The animals invariably develop acidosis, hypoglycaemia, coagulopathy and acute renal failure.

Acidosis could be explained by lactate production and energy depletion in the liver of our animal model\[^{9}\]. Coagulopathy is due to loss of production of factors of the coagulation cascade namely Factors V and III\[^{9}\]. Acute renal failure is a characteristic of paracetamol overdose. Often patients develop acute renal failure through toxic injury and require dialysis in that setting without serious liver injury\[^{9}\].

The hypoglycaemia observed in our model is a hallmark of disrupted hepatic glucose metabolism. Studies on other animal models of ALF and cirrhosis have shown that, despite a relative glucose homeostasis, there is a decrease in gluconeogenesis and tricarboxylic acid (TCA) cycle coupled with an increase in lactate production\[^{6,10-12}\].

We hypothesized that there was a loss of gluconeogenic capacity in our large animal model with an increase in lactate production, an inhibited TCA cycle and a switch to glycolysis during injury to compensate for energy demands.

### MATERIALS AND METHODS

#### Animals

Large white pigs (median body mass 35 kg) were used for this study. Animal experiments were performed in accordance with the Home Office regulations under the Animal (Scientific Procedures) Act 1986 as per Project Licence 60/2389. All animals received humane care and study protocols complied with our institution’s guidelines.

Our experimental model was described in detail elsewhere\[^{7}\]. Briefly animals were anaesthetized with ketamine and midazolam as induction agents and maintained with isoflurane and nitrous oxide. Animals were hydrated with normal saline and glucose. All animals received similar amounts of glucose. Haemodynamic variables and intracranial pressure were continuously monitored.

Animals were pretreated with phenobarbital 20 mg orally for 5 d to induce cytochrome P450 activity. In six animals intravenous paracetamol was administered while the three animals used as controls were monitored but did not receive any paracetamol. A loading dose of paracetamol was administered by intravenous infusion (0.1875 g/kg) followed by an infusion for 12 h (1.8 mg/kg per min). Experiments lasted 28 h and at that time point any surviving animals were euthanized.

#### Sample preparation for NMR spectroscopy

Samples were prepared by adding a D2O solution of (150 μL) to plasma (600 μL) thus providing an internal field frequency lock for the spectrometer. As a reference substance 20 μL of sodium 3-(trimethylsilyl) 2, 2, 3, 3-2H4)-1 propionate (TSP) were added to the plasma. Chemical shifts were referenced internally to the singlet methyl resonance of TSP at zero ppm.

The following potentially gluconeogenic amino acids were quantified by NMR: leucine, isoleucine, valine, tyrosine, phenylalanine, histidine, methionine, alanine, threonine, glutamate and glutamine. Lactate, pyruvate and the gluconeogenic amino acids were measured to provide information on glycolysis and gluconeogenesis.

#### Proton NMR spectroscopy

\[ ^1 \text{H}-\text{NMR} \] spectra were measured from plasma samples taken from a large central vein at 5 hourly intervals until the experiments were terminated at \( t = 28 \text{ h} \). Data were acquired on a Varian INOVA 600 NMR Spectrometer operating at 600 MHz for protons. All spectra were acquired at ambient probe temperature (298 ± 0.2 °K). For each sample 128 transients (FIDs) were acquired into 32 K complex data points over a spectral width of 6 KHz. 300 pulses were applied with an acquisition time of 2.5 s to achieve better resolution followed by an additional pulse recycle time of 4 s to allow for complete T1 relaxation. Water signal suppression was achieved by applying a gated secondary irradiation field at the water resonance frequency. Spectral assignments were made by reference to literature values of chemical shifts in various media and biological fluids and coupling constants\[^{13}\]. The coefficient of variation between samples was < 6% and reproducibility for the same sample was good with a difference of < 2% on the same sample. The CMPG (Carr-Purcell-Meiboom-Gunn) sequence was applied for data acquisition, as this sequence enabled observation of a flat baseline in our spectra from plasma samples by minimising the signals acquired from macromolecules present in the plasma such as proteins and lipoproteins\[^{14}\]. NMR spectra analysis was performed using the MNova platform for NRM analysis (Mestrelab, Santiago de Compostela, Spain).

#### Statistical analysis

To compare between groups in the initial sample the Student’s \( t \)-test for parameters with non-missing values and the Mann Witney \( U \) test for parameters with missing values were used. Values were expressed as mean (range and standard error). A \( P \) value of \(< 0.05 \) was taken as statistically significant (two-tail test of significance). Numeric results are expressed as μmol/L. All analysis was done using the SPSS statistical package (Version 9.0).

#### RESULTS

On all samples studied we were able to identify the fol-
Following metabolites: lactate, pyruvate, leucine, isoleucine, valine, tyrosine, phenylalanine, histidine, arginine, glycine, alanine, threonine, glutamate and glutamine. Resonances from proline, methionine and ornithine were not suitably characterised and results from those amino acids are not available. Figure 1 shows a sample spectrum.

In control pigs there were no significant differences in the concentrations of the substrates studied at any time point sampled. Animals who received paracetamol showed statistically significant differences in the concentrations of lactate, pyruvate and the amino acids.

**Lactate and pyruvate**

Figure 2 shows the results for lactate and pyruvate. Increases in the concentration of lactate became significant at \( t = 15 \) h and at \( t = 25 \) h; compared to \( t = 0 \) an average increase of 405% was seen (\( P < 0.003 \)). Increases in the concentration of pyruvate became significant at \( t = 20 \) h and at \( t = 25 \) h; compared to \( t = 0 \) an average increase of 150% was seen (\( P < 0.018 \)).

**Amino acids**

Figure 3 shows the results for threonine, alanine and glycine. Increases in the concentration of threonine became significant at \( t = 20 \) h and at \( t = 25 \) h; compared to \( t = 0 \) an average increase of 82% was seen (\( P < 0.048 \)). Increases in the concentration of alanine became significant at \( t = 10 \) h and at \( t = 25 \) h; compared to \( t = 0 \) an average increase of 410% was seen (\( P < 0.002 \)). Finally, increases in the concentration of glycine became significant at \( t = 5 \) h and at \( t = 25 \) h; compared to \( t = 0 \) an average increase of 390% was seen (\( P < 0.005 \)).

Figure 4 shows the results for the aromatic amino acids. Tyrosine levels significantly increased at \( t = 5 \) h and at \( t = 25 \) h; there was an average increase of 1330% (\( P < 0.000 \)). Phenylalanine levels also increased significantly at \( t = 5 \) h and at \( t = 25 \) h; there was an average increase of 1420% (\( P < 0.000 \)).

Figure 5 shows the results for the branched chain amino acids. There were no statistically significant changes in the concentration of leucine between the beginning and the end of the experiments (0.17 ± 0.02 vs 0.175 ± 0.02). Isoleucine levels increased significantly at \( t = 10 \) h and at \( t = 25 \) h; an average increase of 250% was seen (\( P < 0.01 \)). Valine levels, on the contrary, significantly decreased at \( t = 20 \) h and at \( t = 25 \) h; an average decrease of 150% was seen (\( P < 0.05 \)).
Acute liver failure in humans is a deadly condition which could lead to multi-organ failure and death of the patient. Abnormalities in many metabolic activities are observed in ALF. Glucose homeostasis is impaired, with hypoglycaemia, increased concentrations of lactate and pyruvate, and increased concentrations of most gluconeogenic amino acids. The liver is a main organ for production of glucose, but its production is inhibited in ALF. The hepatic encephalopathy is caused by gut production of ammonia, a by-product of the production of alanine from glutamate. An interesting finding was that we observed no significant changes in the concentrations of glutamate, glutamine, histidine, and arginine, key metabolic components of the urea cycle. We unfortunately were unable to quantify aspartate but there is strong evidence that the urea cycle in this model remains largely unaffected by paracetamol poisoning. This is in accordance with other studies that have shown that ALF-induced hyperammonemia is caused by gut production of ammonia, a by-product of the production of alanine from glutamate.

In the model described, we have shown that although in patients with non-paracetamol induced ALF all branch chain amino acids are increased, in patients with paracetamol induced ALF valine is an exception and decreases over time. We believe our results to be in accordance with this observation.

In conclusion, in this large animal model of paracetamol induced acute liver failure we have observed an inhibition of gluconeogenesis in the liver with subsequent dysfunction of the TCA cycle. This has led to ATP and energy depletion and the liver switching to glycolysis to compensate for the new emerging metabolically centred therapies for hepatic encephalopathy.
pathways are known to exist but there are very few large animal models where the authors are able to reproduce the acute liver injury. Recently the authors have developed such a porcine model in our laboratory.

Research frontiers
Paracetamol induced acute liver injury in humans and animal models is characterised by profound hypoglycaemia in a very short time from the liver injury. The exact mechanism of hypoglycaemia remains debatable.

Innovations and breakthroughs
In this article the authors were able to provide some evidence that gluconeogenesis is impaired in a porcine model of acute liver injury as manifested by a relative increase in the plasma concentrations of the gluconeogenic amino acids. Gluconeogenic amino acids are the main substrate for glucose production after the exhaustion of glycogen stock very early in the acute liver injury.

Applications
This paper provides some insight into the metabolism in acute liver failure. It shows that gluconeogenesis a key metabolic pathway in paracetamol induced acute liver failure fails, early on in the disease. This needs to be confirmed in human studies and it could have implications in the way these patients are managed.

Peer review
The article by Dabos et al describes increased lactate, pyruvate and distinct amino acids in a pig model of paracetamol induced acute liver injury.

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