Ammonia flux across the lungs in pigs with acute liver failure

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Contents

Abstract

Ammonia reduction is an important target for treatment of hepatic encephalopathy, but lack of quantitative data has limited our ability to develop novel treatments. The aims for the present study were to determine the role of the lungs in interorgan metabolism in pigs with acute liver failure (ALF) and to study the impact of sample handling on the determination of ammonia in an experimental model of ALF.

Ammonia fluxes ((A-V) * blood flow) were measured across the lungs in pigs with ALF. Blood samples were analyzed fresh and after being frozen on -70 C° for a period of more than 4 weeks.

Pigs with ALF developed hyperammonemia. Ammonia flux across the lungs was not significantly different from zero. We found an excellent correlation between fresh and frozen samples for detection of ammonia.

Although the lungs are metabolic active, ammonia flux remains zero during the experimental period. Thus in this model the lungs neither produce or take up ammonia. EDTA plasma can be stored for 4 weeks in -70°C for determination of plasma ammonia with an excellent correlation to ammonia analysed freshly.

Introduction

Acute liver failure (ALF) results in disturbed body nitrogen homeostasis due to impaired hepatic urea synthesis capacity [1,2]. This leads to an alteration in interorgan ammonia trafficking and hyperammonemia, which contributes to the risk of development of cerebral edema [3]. The urea-cycle is an important maintainer of nitrogen homeostasis, which takes place in the periportal hepatocytes. Ammonia is a substance in many biochemical processes of the body, but it is cytotoxic when the plasma-levels are increased [4,5]. Ammonia joins the urea cycle in the form of free NH_4^+ or as glutamate and aspartate (figure 1). Urea is the only new substance produced in this cycle. In acute liver failure (ALF) patients develop a hepatocellular dysfunction with reduced urea cycle capacity[2]. Furthermore, hyperammonemia is also caused by a significant portacaval shunting, which allows ammonia ions to escape clearance by the liver[1]. Elevated level of ammonia is toxic[6] and closely linked to the development of hepatic encephalopathy and brain edema.

In this situation the most important temporary alternative pathway for ammonia detoxification is the formation of glutamine from ammonia and glutamate catalysed by the enzyme glutamine synthase (GS)[7]. GS is found in many organs in the body and the activity of the enzyme is enhanced by glucocorticoid hormones, stress, and lack of glutamine[8]. Glutamine taken up by other organs is split by the intra-mitochondrial phosphate-dependent enzyme glutaminase (PAG) again into glutamate and ammonia [9]. PAG is enhanced by increased levels of glutamine[10] and are shown to be activated by glucagons and angiotensin II[11]. Accordingly, ammonia metabolism is closely related to glutamine and glutamate metabolism.

Previous studies in animal models and patients with liver disease have pointed to an important role for the gut and kidneys in the production of ammonia and the muscles in the removal of ammonia[1]. The lungs contain both PAG and glutamine synthase (GS)[12], which

suggest that they could play an important role in interorgan ammonia metabolism. Accordingly, we hypothesized that the lungs might play a significant impact on interorgan ammonia metabolism in ALF.

Furthermore, correct blood sampling procedure for ammonia determination is crucial to in order to minimize systematically errors due to wrong sample handling. Four concerns are of importance: 1) Smoking is a source of ammonia-contamination, both from the patient and of the technologist. 2) Laboratory atmosphere. The analysis should be done in a special laboratory to avoid contamination 3) Poor vein puncture technique. EDTA and heparin are acceptable anticoagulants. Air in contact with the specimen can cause ammonia levels to increase by 100-200 µg/L. Full glasses for sampling would remove this contamination factor. 4) Metabolism of nitrogenous constituents in the sample is a source of ammonia contamination. Therefore the sample must be put on ice and centrifuged immediately and the analysis performed at once[13].

In our study it takes a lot of planning to perform ammonia-analysis because of the fact that the samples should be run immediately to avoid ammonia contamination. The Department of Clinical Chemistry at the University Hospital Northern Norway routinely receive samples for ammonia-analysis, which have been transported over large distances across the county. If the specimen could be frozen for a period before analysis, with minimal impact on the results it would be a significant logistic improvement. Thus in the present study we wanted to determine the most appropriate sample handling procedure (freshly processed or after a freezing period > 4 weeks at − 70°C).

The present study was therefore designed to determine the role of the lungs in interorgan ammonia metabolism in pigs with ALF and to determine the impact of sample handling on ammonia measurements in an experimental model of ALF.

Methods

Study outline

A total of 9 female Norwegian Landrace pigs weighing 28-36 kg were used in this study. They were randomised to either hepatic devascularisation (n = 6) or sham operation (n =3).

Baseline measurements were performed right before the laparatomy. T=0 hrs was defined as 30 minutes after the induction of ALF or sham operation. All animals were studied for the whole experimental period, which lasted for eight hours after Alf induction. The pigs were sacrificed with an overdose of pentobarbital and potassium chloride at t=8hrs.

Animal preparation

The Norwegian Experimental Animal Board approved the study. The pigs were kept in the animal department for at least 2 days before the experiment. The conditions in the animal room were strictly controlled at a temperature of $21 \pm 1°C$, relative humidity of $55 \pm 10\%$ and a 12:12 h light /dark cycle. The animals were fed with Combi Fri chow (Felleskjøpet, Trondheim, Norway). All animals were fasted overnight with free access to water. The pigs were given ketamine 20 mg kg^{-1}(Ketalar®, Pfizer inc, New York, USA) and atropine 1 mg (Nycomed Pharma, Oslo, Norway) intramuscularly as pre-medication.

Anesthesia was induced with an intravenous bolus of 10 mg kg^{-1} pentobarbital (Pentobarbital®, Nycomed Pharma, Oslo, Norway) and 10 μg kg^{-1} fentanyl (Leptanal®, Janssen Pharmaceutica, Beerse, Belgium). Anaesthesia was maintained with a central venous infusion of 4 mg $kg^{-1}h^{-1}$ pentobarbital, 0.02 fentanyl mg $kg^{-1}h^{-1}$ and 0.3 mg $kg^{-1}h^{-1}$ midazolam (Dormicum®, Roche, Basel, Switzerland). All animals received 500 mL 0,9 % NaCl containing 625 mg of glucose as a preoperative volume load in order to prevent any

preoperative dehydration. Anaesthesia was stopped after the liver was devascularised. If there were clinical signs of light sedation, small doses of fentanyl and midazolam were given as a bolus, but the animals rarely required more than a total of two doses after t = 0hrs. Sham-operated animals received continuous anaesthesia during the experimental period, and received equal amount of intravenous fluids.

Tidal volume was adjusted by means of repeated arterial blood gas analyses to achieve a PCO_2 within the range 4.5 − 5.0 kPa during surgery. After t = 0 hrs no adjustments of ventilation were performed. Core body temperature were maintained at 38.5 ± 1°C with a heating pad and blankets.

During the experiment, 0,9 % NaCl were infused at a rate 3 ml $kg^{-1} h^{-1}$. 50 % glucose was infused at a rate of 0.3024 ml $kg^{-1} h^{-1}$ to shams and 0,6048 ml $kg^{-1} h^{-1}$ in the ALF group from t=0 hrs. 20 % human albumin (Albumin 200 mg/mL, Octapharma®, Hurdal, Norway) was continuously infused from t = 0hrs in both groups at a rate of 0.66 ml $kg^{-1} h^{-1}$.

Positioning of catheters

A 5 French Swan-Ganz catheter (Baxter Health-care Corp, Irvine, Calif., USA) was floated into the pulmonary artery via the right external jugular vein. Arterial and mixed venous blood samples were drawn from catheters in the common carotid artery and from the Swan-Ganz catheter. A central venous catheter (Secalon T, Ohmeda, Swindon, UK) was introduced into the left external jugular vein for administration of anaesthesia and fluids. The bladder was drained via a cystotomy and diuresis was carefully measured every other hour before urine samples were drawn. All wounds were sutured with polyglycolic acid 2-0 after completion of surgery. Normal saline was used to flush all catheters.

Measurements

Calibrated transducers (Transpac 3, Abbott Critical Care Systems, Chicago, Il. USA) were used for continuous pressure measurements. Cardiac output was measured in triplicate and the results expressed as the mean value (thermodilution method, 5 ml of normal saline as injection material at < 5 °C). A single investigator made all measures over a period of < 4 s at end-expiration. (Com-1, American Edwards Laboratories, Santa Ana, Calif., USA). Core Haemodynamic measurements were followed by the withdrawal of mixed venous and arterial blood samples, which were immediately analysed at 37 °C for partial pressure of oxygen and carbon dioxide, pH, base excess and oxygen saturation (Rapidlab 865, Chiron Diagnostics Ltd, Essex, England). Body temperature was measured via the pulmonary artery. Blood measurements were performed every other hour for 8 hours.

Devascularisation of the liver

A midline incision was made from the xiphoid to the pubis. The portal vein was dissected free from surrounding tissue and lymph nodes from the porta hepatis to the confluence of the splenic vein. The inferior caval vein, immediately cranial to the renal vein, was then cleared and clamped with an exclusion clamp, and a 1.5 cm long longitudinal incision was made. The portal vein was then clamped, transposed and anastomosed end-to-side to the inferior caval vein, using a continuous over-and-over polypropylene 5-0 sutures. The total period of portal veil occlusion was between 11 and 15 min. During this time, and for a further 10 min, 1000 ml of 0,9% NaCl was infused to every animal in order to maintain the arterial pressure. Dissection of the structures in the hepatoduodenal ligament was performed carefully to ensure that also the arterial blood supply to the liver was completely interrupted. The common bile duct was preserved, but two polyglycolic 2 ligatures tied the hepatic artery. Finally, the abdominal wound was closed in two layers with polyglycolic acid 2-0. Sham-

operated controls received equal amounts of intravenous fluids and underwent the same surgical intervention, except for the devascularisation procedure.

Blood sampling

Arterial and mixed venous blood samples were drawn from the carotid artery and the pulmonary artery, respectively. EDTA-plasma was drawn into vacutainers and kept on ice water (4°C) until further processing at the Department of Clinical Chemistry. Samples were immediately centrifuged for 10 minutes at 4°C. 1 ml plasma was pipetted into plastic-containers and put on ice water. Hemolysis was checked visually and when in doubt by a serum-index. Two aliquots EDTA plasma were pipetted of which one was frozen and the other immediately analysed for ammonia. The frozen samples were kept on airtight tubes, which were completely filled to avoid any chemical reaction with air inside.

Samples were run on a Hitachi Modular PP (Roche diagnostics, Germany). The test principle of the Hitachi Modular system is an enzymatic kinetic assay. In the reaction catalyzed by glutamate dehydrogenase, ammonia reacts with alfa-ketogrlutarate and NADPH to form glutamate and NADP+. The amount of NADPH oxidised (disappearance rate) during the reaction is equivalent to the amount of ammonia in the specimen and can be measured photometrically by the resulting decrease in absorbance.

The other aliquot was immediately put into -70°C and kept frozen for a period of more than 4 weeks. Samples were then thawed on ice-water (4°C), carefully mixed at least 20 times before they were analysed as described above.

Only blood samples from the first two pigs (one sham and one ALF operated pig) were used to test the correlation between freshly analysed versus frozen blood samples. Blood samples from the consecutive seven pigs were frozen in -70°C for > 4 weeks.

Calculations and statistics

The ammonia-flux across the lungs was calculated as the arterial-venous concentration difference times the plasma flow. The lungs are different from other organs because they receive venous blood and deliver arterial oxygenated blood. This is why the concentration difference is calculated by arterial-venous concentration. Positive values will thus reflect ammonia release, while negative values reflects ammonia uptake.

Statistical analysis was performed using the Statistical Package for the Social Sciences, version 11.0 for Windows (SPSS, Chicago, Ill., USA). Data are expressed as mean ±SEM. Two-way ANOVA was applied to test for differences within and between groups over time. An overall significance in analyses of variance for repeated measurements (F-test $P \leq 0.05$) may be attributable to either the effect of group (P_G) or the interaction for group and time (P_{GT}). Overall significance for the effect of group means that the groups were different when all the repeated measurements were taken together (independent of time), while a significant interaction denotes a different time course in the two groups. $P_T \leq 0.05$ denotes a significant change during the experimental period, but without any difference between the groups. Huynh-Feldt epsilon factor adjusted probability levels were used when Mauchly's test of sphericity was significant. Probability values $P \leq 0.05$ were considered significant for all tests applied.

Results

Core body temperature did not alter significantly within or between the groups during the study period (38.5 ± 1°C). Cardiac output increased significantly in the ALF group compared to sham operated controls ($P<0.01$) (figure 2).

Fresh vs. frozen samples for analysis of ammonia

There was an excellent correlation between arterial ammonia levels in freshly processed and frozen samples ($R^2= 0,9998$) (figure 3).

Ammonia flux across the lungs

Arterial ammonia levels in sham operated animals remained unchanged from baseline levels, while ammonia increased significantly in pigs with ALF ($P_{GT} < 0.001$) (figure 4). Flux was not different from zero in any group at any time point (figure 5). Furthermore, there was no difference between the groups at any time point.

Discussion

Treatment of hepatic encephalopathy aims to lower arterial ammonia levels in the body. However, incomplete understanding and lack of quantitative data regarding interorgan metabolism of ammonia has limited our ability to develop effective treatment concepts. Studies in patients with cirrhosis have identified important roles for the kidneys and muscles in addition to the gut to be involved in ammonia homeostasis.

Normally ammonia is produced in the gut and kidneys. The small intestine and colon contributes quantitatively with equal amounts[14]. The PAG activity in the intestines is high while the GS activity is low[7, 15], which makes it to a major glutamine consuming organ[16]. The ammonia produced in the small intestine is from amino acid breakdown[17], especially glutamine[7, 15], while the ammonia production in colon is caused by bacteria breakdown of amino acids and urea[17]. The release of ammonia from the portal drain viscera does not seem to alter during experimental acute liver failure[18]. Accordingly, release of ammonia from the splanchnic area to the general circulation is therefore mainly dependent on the degree of portacaval shunting and not so much dependent on existing amino acid breakdown.[1, 19].

The kidneys contain both GS and PAG[20]. Normally about 70 % of the ammonia produced in the kidneys is released into the veins of the kidneys. The other 30 % are excreted through urine[1]. The kidney is therefore an organ, which contribute with ammonia to the general circulation.

During liver failure the kidneys contribute to maintain the hyperammonemic state because they release ammonia into the renal vein. However, some studies show that the kidneys can adapt to hyperammonemia, by reducing the release of ammonia to the systemic circulation and at the same time increase the excretion of ammonia to urine[1].

The skeletal-muscles are our most important glutamine synthesising organ[21]. A study of simulated upper GI bleeding show that muscles have a positive uptake of ammonia and that

they quantitatively are more important than the liver itself[22]. Due to these findings and because the muscles quantitatively is the most important location for GS, one might assume that the muscles play an important role during liver failure.

As interorgan ammonia trafficking is difficult to study in human ALF, we decided to use a well-characterised porcine model of ALF to determine ammonia flux across the lungs in pigs with acute liver failure (ALF)[23, 24]. In this model animals develop hyperbilirubinemia, hyperammonemia, hyperdynamic circulation, intracranial hypertension, renal dysfunction and an abnormal hemostasis[23]. This model was thought to be suitable for our study, as our main focus was to evaluate interorgan ammonia metabolism in animal model of ALF that could be repeatedly sampled and that depicted the characteristic end-organ dysfunction typically seen in human ALF[25].

Hyperammonia developed shortly after ALF induction to arterial levels previously reported in experimental and human ALF[18] Arterial ammonia-levels remained within the normal range for sham operated pigs[23].

The present data, however, did not reveal positive or negative fluxes across the lungs. This could mean that the lung do not contribute to removing ammonia during ALF. However, there are many factors that should be considered before this conclusion can be made. It has been shown that the kidneys, the gut and the skeletal muscles most likely contribute to the ammonia-metabolism during ALF. These organs contain PAG and /or GS (described above) and indicate that other organs containing these enzymes could be involved in ammonia metabolism during ALF. It is a fact that the lung-tissue contains both GS and PAG and therefore has potential to be involved in ammonia-metabolism. Stress is proven to increase GS[8]. It might be correct to assume the pigs are stressed because of the surgery and therefore the GS in lungs increase. This could cause a positive ammonia flux across the lungs

Because of large variations in our results it is difficult to say whether stress plays a role at all. Are some of the pigs more stressed than others? Some of the pigs needed more anaesthesia than others (both ALF pigs and shams). This could be a stressing factor for the pigs and thereby increase GS. Because of large variability in the results and small number of pigs this has not been compared in this study. It could be of interest to study the ammonia-flux across the lungs in stressed animals with high cortisol-levels to get a better understanding of this.

Lack of glutamine also enhance activity of the enzyme GS. This can be of importance for handling of ammonia in the lungs. If there is excess of glutamine in the lungs, enzymatic activity of GS will decrease and ammonia will not be removed. Glutamine levels in the blood and in lungs should have been measured and compared to the ammonia-levels at different stages during the experiment. This way we could have got an impression of whether glutamine-levels influence on the lungs during ALF.

For the lung to be able to remove ammonia it is necessary to have access to glutamate. Studies have shown that glutamate levels decrease after ALF induction[18]. This causes a substrate deficiency for the reaction processed by GS. The mechanism of this glutamate depletion is not known.

PAG activity will increase when glutamine levels rise. This causes release of ammonia and glutamate. Because of high glutamine levels during ALF, PAG activity is probably increased also in the lung. This will supply glutamate to the glutamine production but it will also replace the ammonia removed by the same reaction.

To summarise we see that PAG and GS both in theory influence the ammonia-flux across the lung during ALF. In the present experiments we did not specifically measure the activity of those enzymes. However, this should be done and hopefully given us a better picture of the role of the lungs in pigs with ALF.

In the present study the animals developed typical pattern of hyperdynamic circulation as previously reported by our group[24]. The hyperdynamic circulation makes it difficult to calculate the flux across the lungs. Because of small V-A differences and enhanced CO the findings in this experiment are uncertain. If these measurements can be done more accurate(by perivascular flow probes) this might give a better picture of the blood flow (CO) to the lungs during ALF.

We found an excellent correlation between frozen and fresh ammonia samples. Accordingly, we came to the conclusion that there should be no concerns regarding whether samples for ammonia determination might be freezed or not. However, one must be aware of systematic errors with the procedure., which are most likely to be related to the freezing and thawing procedures.

In conclusion, ammonia flux measurements across the lungs are hampered by methodological difficulties. However, the lung is not consuming or producing ammonia in the present porcine model.

Acknowledgements

Figure 1

Urea cycle. (Wikipedia, the free encyclopedia. http://en.wikipedia.org/wiki/Urea_cycle)

Colour-codes: **Enzymes**, **coenzymes**, **substrate name**, **inorganic molecule**, **The nitrogen from Aspartate and ureas origins from these substrates**, **NH_4^+ and the nitrogen from urea origins from this** , **HCO_3^- and ureas carbon origins from this.**
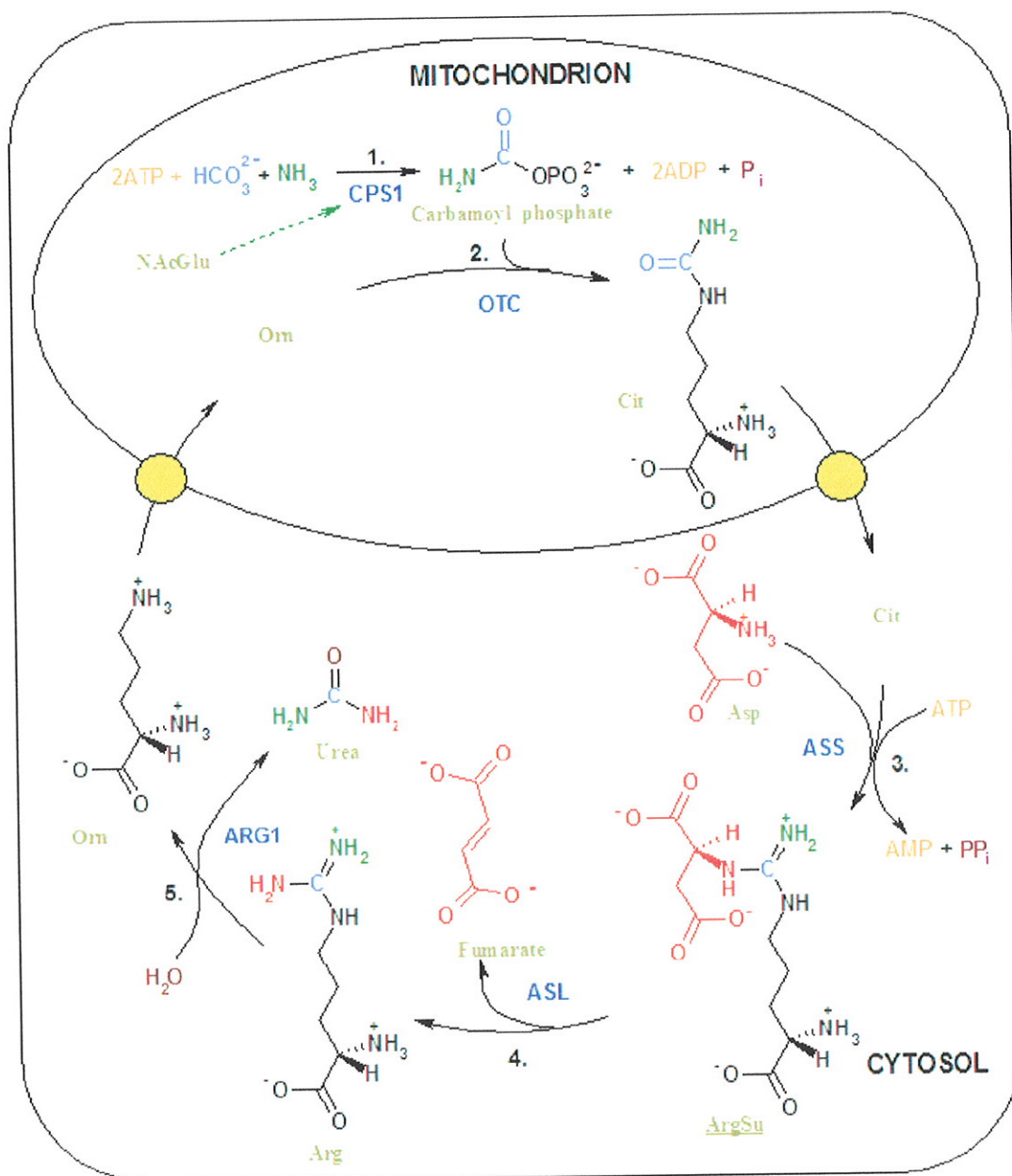
Figure 2

Cardiac output in pigs with acute liver failure and in sham operated controls.
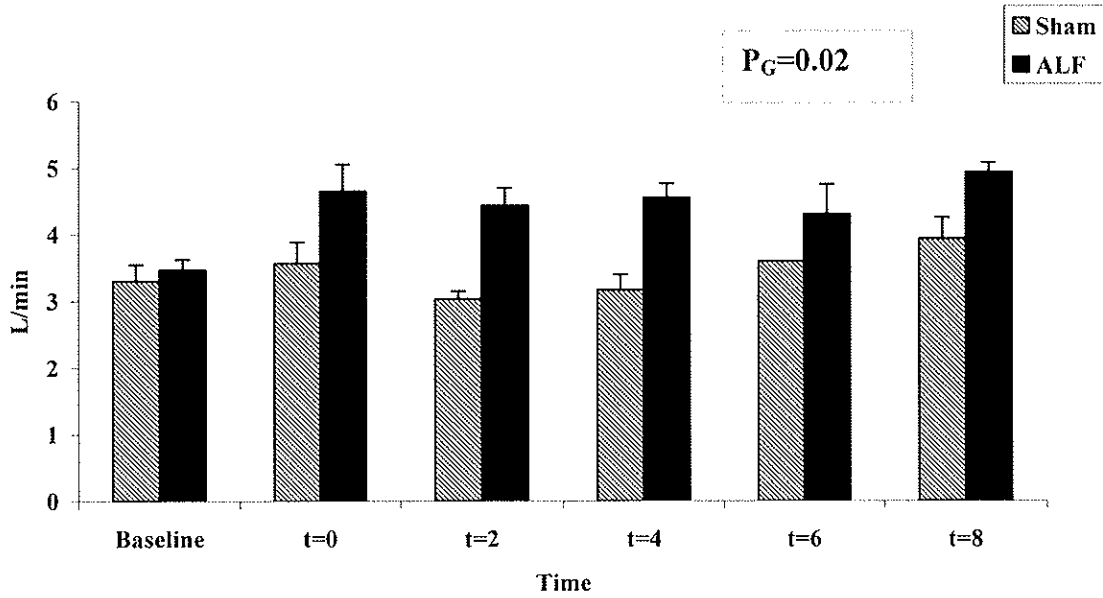

Figure 3

Correlation between fresh and frozen samples for ammonia analysis.

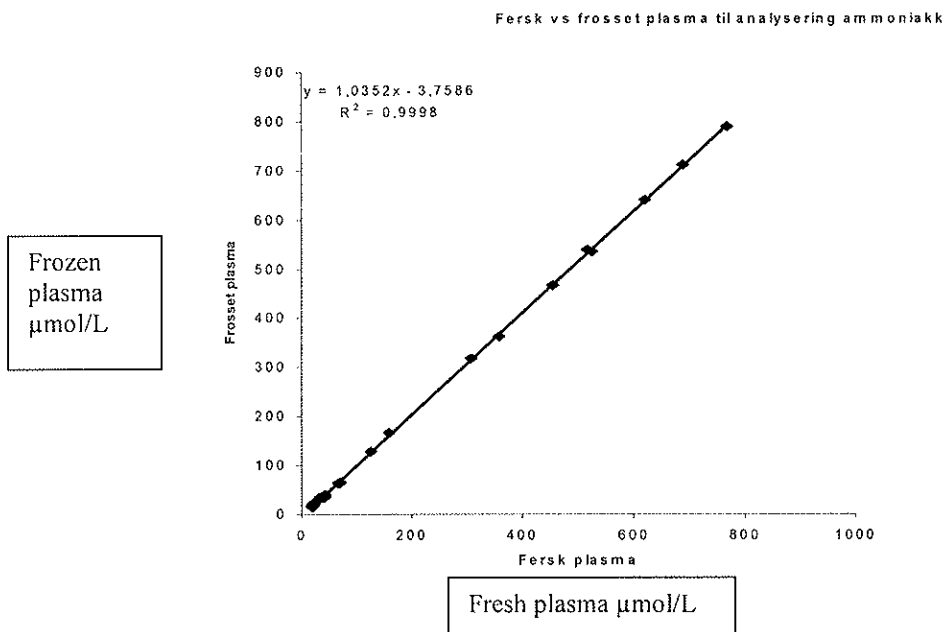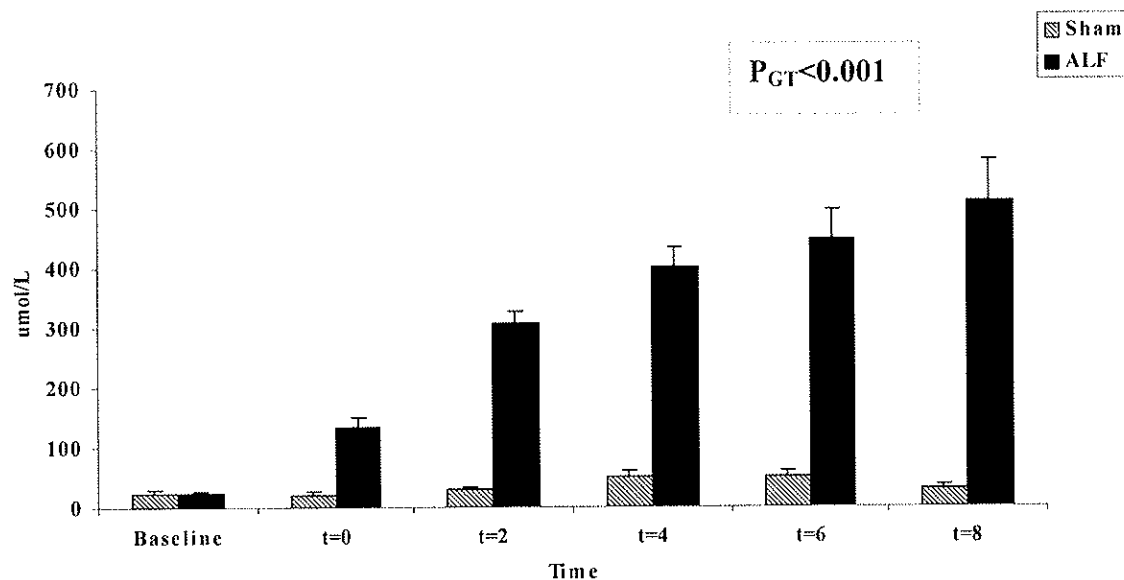(Made by the Dept of Clinical Chemistry, University hospital Northern Norway.)
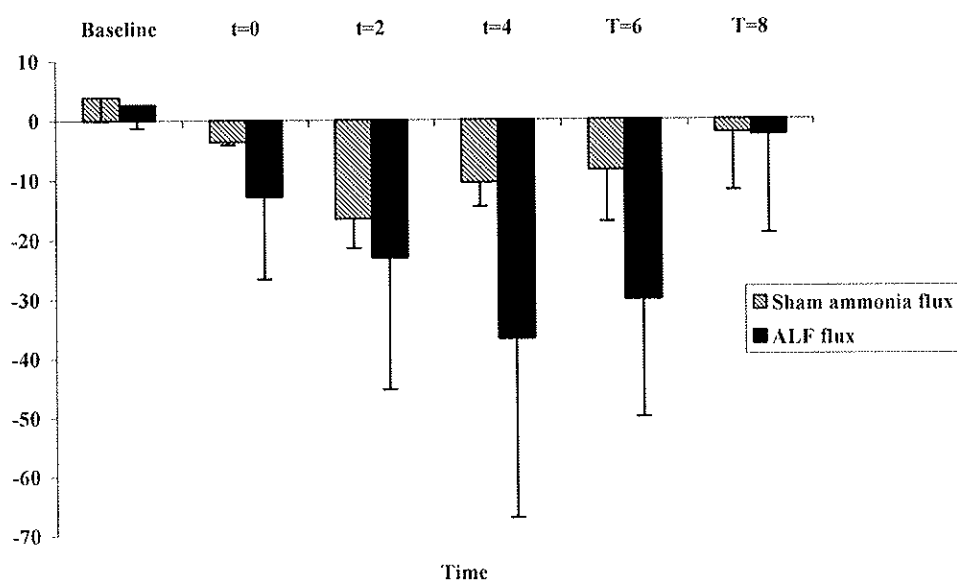
19

Figure 4

Arterial ammonia levels in pigs with acute liver failure and in sham operated controls.

Figure 5

Ammonia flux across the lungs in pigs with acute liver failure and in sham operated controls.

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