A Gravimetric Technique for Measurement

of Brain Water in Pigs with Acute Liver Failure

5th year project

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Abstract

Background: Hepatic encephalopathy (HE) is a serious complication to acute liver failure (ALF) where increased intracranial pressure (ICP) leads to brainstem herniation and consequently death. The present study attempts to look at the amount of cerebral oedema in different brain regions and the effect of albumin dialysis, in the form of Molecular Adsorbent Recirculating System (MARS), in a porcine model of acute liver failure.

Methods: 21 female Norwegian Landrace pigs were used for the study. Brain samples from the frontal cortex, frontal white matter, and cerebellum were obtained six hours after induction of ALF. ALF was induced by hepatic devascularisation and followed with (ALF-MARS) or without (ALF) a 4 hour MARS treatment, which started 2 hours after ALF induction. A sham operated control group was included. A gravimetric technique was used to determine % brain water in specific regions of the brain. A linear density gradient was obtained in a graduated cylinder by mixing two organic solutions with known specific gravity Kerosene=0.9750; Bromobenzene=1.0650. Small samples of tissue were placed into the cylinder and equilibration depth was measured. Using K_2SO_4 with known specific gravity as standards the specific gravity of the tissue could be calculated.

Results: Brain water content increased in ALF compared to sham in frontal cortex 82.0% vs 78.6%, p<0.05, and white matter 72.9% to 69.5%, p<0.05, whereas no significant change was found in the cerebellum 78.1% to 76.2%, p=0.0691. Brain water content decreased in ALF-MARS vs ALF 65.9% vs 72.9%, p<0.05 in the white matter, while there was no significant difference in the frontal cortex.

Conclusion: Pigs with ALF developed an increase in brain water in both the frontal cortex and frontal white matter, but remained unchanged in the cerebellum. MARS treatment significantly decreased the degree of brain oedema in the white matter while having no effect on the brain oedema in the frontal cortex.
1. BACKGROUND

1.1 Acute Liver Failure

1.1.1 Definition

Acute Liver Failure (ALF) is defined as the development of hepatic encephalopathy (HE) in the presence of acute liver injury of less than 8 weeks duration (1).

1.1.2 Frequency and causes

ALF is a rare condition. It is estimated that 5 of 6000 hospital admissions are due to ALF (2). Viral hepatitis and drug-induced liver injury account for most cases of ALF (Table 1), respectively approximately 70% and 20% (2). However regional variations in both frequency and etiology are wide.

<table>
<thead>
<tr>
<th>Cause</th>
<th>Agent responsible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral hepatitis</td>
<td>HAV (0.2-0.3%), HBV (1-2%), HCV (1%), HDV (super-infection, 20%), HEV (10-20%), HCV+HAV (30-40%), HSV (type 1,2), CMV, EBV, VZV, Adenovirus, Parainfluenzavirus, Coxackievirus.</td>
</tr>
<tr>
<td>Bacteria and parasites</td>
<td>Leptospira, Listeria, Malaria, M.tuberculosis, Rickettsia</td>
</tr>
<tr>
<td>Drug-related liver injury</td>
<td>Paracetamol, halothane, allopurinol, amiodarone, carbamazepine, interferon, isoniazid, MAO-inhibitors, methotrexate, methyldopa, rifampicin, sulphasalaxine, tetryxyline, valproic acid, &quot;ecstasy&quot; Idiosyncratic reactions</td>
</tr>
<tr>
<td>Alcohol</td>
<td></td>
</tr>
<tr>
<td>Toxins</td>
<td>Carbon tetrachloride, Amanita phalloides, phosphorus</td>
</tr>
<tr>
<td>Vascular events</td>
<td>Ischemia</td>
</tr>
<tr>
<td>Miscellaneously</td>
<td>Veno-occlusive disease</td>
</tr>
<tr>
<td></td>
<td>Budd-Chiari syndrome, heatstroke, veno-occlusive disease, malignant infiltration, heart failure</td>
</tr>
<tr>
<td></td>
<td>Wilson's disease, Acute fatty liver of pregnancy, HELLP syndrome, Reye's syndrome, autoimmune hepatitis, septic cholangitis</td>
</tr>
</tbody>
</table>

Table 1: Principal causes of acute liver failure (1,2)
1.1.3 Clinical findings and complications

ALF is a devastating condition. ALF results in rapidly progressing multiorgan dysfunction with a dramatic impact on the brain. The course of disease can advance within a matter of days or, in a subacute form, take several weeks. (1,2)

General symptoms. Fatigability, loss of appetite, nausea, weakness, lassitude, meteorism, apathy and disruption of the circadian rhythm.

Hepatic encephalopathy (HE). The current definition of ALF is dependent on the development of HE. Associated with development of cerebral oedema, intracranial hypertention (IH) and subsequently herniation is it a leading cause of death. HE is classified as Grades I-IV, describing the progression from normal mental status to coma (table 2).

<table>
<thead>
<tr>
<th>Grade</th>
<th>Spontaneous survival</th>
<th>Clinical findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>70 %</td>
<td>Subtle changes in affect, dysarthria, mild confusion.</td>
</tr>
<tr>
<td>II</td>
<td>60 %</td>
<td>Inappropriate disinhibited behaviour (agitation, aggression and drowsiness), muscle tremor, asterixis.</td>
</tr>
<tr>
<td>III</td>
<td>40 %</td>
<td>Somnolent, stupor with marked confusion, incoherent speech, hyperreflexia, Babinski’s reflex, clonus, spasticity, nystagmus. EEG: slowing down of basic activity, mainly biphase and triphase potentials are seen.</td>
</tr>
<tr>
<td>IV</td>
<td>20%</td>
<td>Coma, areflexia, absence of corneal reflex, loss of tonicity. EEG: isoelectric line.</td>
</tr>
</tbody>
</table>

Table 2: The grades of hepatic encephalopathy in ALF (7,8)

Jaundice. Jaundice is present in most cases, but can be absent especially in hyperacute courses.

Foetor hepaticus. The sweet aromatic smell of exhaled breath (mercaptan derivatives) is a reliable sign of ALF, but it is not always present.

Liver size. The liver may be of normal size or enlarged due to hyperaemia or massive fatty infiltration. However acute atrophy can also ensue.
Coagulation disorders. Diminished synthesis of coagulation and fibrinolysis factors and inhibitors, decrease in the breakdown of activated factors, and a functional disorder of thrombocytes or thrombopenia, makes bleeding and coagulation disorders a frequent cause of death (20-25%).

Circulatory disorders. Approximately 80% of patients develop hypotension. Loss of autoregulation of vasogenic tone with reduction of systemic vascular resistance, are associated with a compensatory increased cardiac output. The exact pathogenesis of the systemic hypotension remains unclear, but NO is implicated as a main factor. The hypotension results in a considerable reduction in hepatic, cerebral and renal perfusion.

Renal failure. Renal insufficiency develops in about 50% of patient with ALF. It can be expressed in 3 forms: 1) prerenal due to hypovolaemia, 2) acute tubular necrosis, mainly secondary as a result of hypotension, or 3) hepatorenal syndrome (development of renal failure in the absence of clinical, anatomical, or pathological causes) (2).

Respiratory insufficiency. Despite hyperventilation, tissue hypoxia develops. Oxygen delivery increases as a consequence of a compensatory increased cardiac output, but the oxygen extraction ratio and oxygen consumption decreases. The basis for the microcirculatory dysfunction is poorly understood, but it is clearly excacerbated by small vessel occlusion and the development of shunts.

Infections. Patient with ALF have a greater susceptibility to infections. In 10% of cases this is the reason for their death. The respiratory tract and the urinary passages are most frequently affected.

Others. Acid-base disorders, hypoglycemia, pancreatitis
1.1.4 Prognosis
Mortality rates are high, ranging from 40 to 80%. This rate varies widely owing to a number of reasons. For example there is a better prognosis for poisoning from paracetamol since successful therapy procedures have been established. Young patients (10-40 years of age) generally have a better prognosis. A poor outcome can be expected in coma stages III and IV due to development of complications, especially with younger (<10) and older (>40) patients. ALF due to viral hepatitis and various drugs likewise have a less favourable prognosis.

1.2 Cerebral oedema

1.2.1 Different types of cerebral oedema
Principally there are three types of cerebral oedema:

1) Cytotoxic: The oedema is caused by a change of osmolarity.
   (e.g. can bee seen with hypoxia or water intoxication)

2) Vasogenic: The oedema is due to an increased permeability in cerebral vessels.
   (e.g. after a head trauma or cerebral bleeding)

3) Interstitial: Periventricular oedema caused either by increased production,
   decreased absorption or decreased transport of CSF.
   (e.g. hydrocephalus)
1.2.2 Relationship between ICP and cerebral oedema

The intracranial pressure (ICP) has been demonstrated increased in ALF (graph 1).

Graph 1: The intracranial pressure is higher at all timepoints after time zero, comparing ALF and controls.

(3)

However, it is important to differentiate the development of brain oedema from that of intracranial hypertension, whereas one represents a net increase in the water content of the brain, the other denotes a rise of pressure within the skull. Due to compensatory mechanisms in the brain (e.g., reduction of the CSF), the relationship between brain volume and ICP is not linear (graph 2). That means that the amount of brain water can not be calculated directly from the ICP, i.e. the water must be measured by specific methods.
1.3 Pathophysiology of hepatic encephalopathy and cerebral oedema

The pathophysiology of HE and cerebral oedema remains largely unknown. The most favoured view is that toxic substances elaborated by the gut are not detoxified by the liver, either because of the presence of shunts or because of inability of the injured liver to detoxify these substances. It is likely that several factors acting synergistically may give rise to the neurologic deficit. Possibly the most important toxin and certainly the most studied is ammonia. Figure 1 illustrates the strong correlation between arterial ammonia concentrations and cerebral herniation. Ammonia is clearly neurotoxic. In animals, high doses results in seizures, while lower doses cause coma (5). It is still unclear how ammonia exerts its toxic effect, but we will now present the latest theories (6,7).
1.3.1 Ammonia causes alterations in the glutamatic synapse

Recent studies suggest that a glutamateergic synaptic dysfunction is one of the main factors implicated in the pathogenesis of the hepatic encephalopathy and the cerebral oedema in ALF.

The normal glutamatic synapse (figure 2). Glutamate is the main excitatory neurotransmitter in the brain. It is synthesised in the presynaptic nerve terminal and stored there, until it is released into the synaptic cleft due to calcium dependent exocytosis. Released glutamate binds to and activates receptors both on the postsynaptic neuron (the ion-gated receptors AMPA/kainat and NMDA, and also metabotropic receptors) and the neighbouring astrocytes (AMDA/kainat and metabotropic receptors). The released glutamate is removed from the extracellular space by high affinity sodium dependent transporters located on the astrocytes, EAAT-1 and -2 (primary GLT-1 and GLAST). Glutamate uptake by the astrocytes is important since ammonia removal in the brain relies entirely upon the synthesis of glutamin by the enzym glutamin synthetase, found exclusively in the astrocytes. The resulting glutamin in the astrocytes is shuttled to the presynaptic nervterminal where it is the immediate precursor of glutamate catalysed by the enzyme glutaminase. In this way, a glutamatic-glutamin cycle is established. (7)
The glutamat synapse in ALF (figure 3). Several studies have demonstrated an alteration in the glutamatergic synaptic regulation and a disruption of the glutamat-glutamin cycle in ALF. The increase in brain ammonia will lead to an inhibition of the glutaminase in the presynaptical neurons which causes an accumulation of glutamin in the astrocytes. The astrocytes become hypertonic and cellswelling results due to osmosis. This feature has lead to the strong hypothesis about brain oedema in ALF, being fundamentally of cytotoxic nature. (7) Contrary, in chronic liver failure it is no sign of cerebral oedema, because a more slowly developing oedema results in a reduction of other osmolytes (decreased levels of myo-Inositol, cholin and taurin has been shown), i.e. osmotic compensation. (6,8)

Additionally cerebral microdialysis has shown an increased extracellular concentration of glutamat. It is suggested that ammonia both directly and indirectly is causing this increase. Increased extracellular glutamat could principally result from 1) an increased release from the presynaptic neuron, or 2) a decreased uptake in the astrocytes. The inhibition of glutaminase, due to the increased level of ammonium makes an increased release from the presynaptic neuron impossible. A significant loss of glutamat transporters is demonstrated in ALF, however this is most likely a late fenomen in ALF. Other hypotheses are now being proposed, among others a reversed transport of glutamat, a glutamat release induced by the swelling of
the astrocyte (X), and a calcium–dependent glutamat release from the astrocytes. A loss of AMPA/kainat receptors, with concomitant unchanged numbers of NMDA receptors has been demonstrated in animals with ALF, i.e. a relative increase of NMDA receptors ensues. In this way, an increased extracellular glutamat will result in an increased NMDA receptor mediated transmission. Under normal conditions, activation of neuronal nitric oxide synthase (nNOS) result from NMDA receptor activation, with production of nitric oxide (NO). In ALF this NO production is increased, i.e. a relaxation of cerebral arterioles ensues. An increased production of NO will dilate cerebral arterioles and result in an increase in CBF, compromise in cerebral vascular reactivity and subsequently a loss of cerebral autoregulation (i.e. the arterial blood pressure can directly influence the CBF). An increase in CBF will result in hyperemia in the brain, along with an even higher flow of ammonia to the brain. (7)

![Diagram of glutamate synapse in acute liver failure]

*Figure 3: a schematic illustration of the glutamat synapse in ALF. (7)*

Furthermore, the loss of arteriolar tone may unbalance the Starling forces in the brain capillaries, i.e. the hydrostatic pressure may overcome the osmotic pressure and result in an accumulation of water or osmogenic metabolites in the extracellular space. However, although an increase in blood-brain barrier permeability for certain substances has been observed in animal models of ALF, it appears late in the course of ALF (). I.e. a vasogenic oedema could also be implicated, but then as a more secondary feature. Other observations
supporting the cytotoxic ethiology of the oedema, are the appearance of the normal capillary endothelium on human brain biopsy, and that swelling of cortical astrocytes is a consistent finding. Additional indirect evidence is the absence of focal involvement on CT (as seen in cases of vasogenic oedema), normal protein levels in the CSF and the finding, in animal models, that the cortical grey matter is the main site of swelling. (4)

The resulting cerebral hyperaemia might also have other devastating effects, like increasing the level of proinflammatory cytokines in the brain. Tumour necrosis factor -alpha (TNF-α), interleukin -1β (IL-1β) and -6 (IL-6) are factors of major interest. However, it is worth mentioning that other conditions with massive cell lysis, such as necrotizing pancreatitis, rhabdomyolysis, hemolysis and burns, do not result in brain oedema (8).

Other potential toxins have been proposed, but their evidence is not nearly as strong as for ammonia. Some of these include: branched chain amino acids, alfa-ketoglutarat, phenols, mercaptans (5).

1.4 Molecular Adsorbents Recirculating System (MARS)

The Molecular Adsorbents Recirculating System (MARS) (Figure 4) is an extracorporeal blood purification system, which uses a hollow fibre dialysis module where the patient’s (in this case, animal’s) blood is dialysed across an albumin-impregnated polysulfone membrane (with a cut-off of 50 kDa), while maintaining a constant flow of 20% albumin as dialysate in the extra-capillary compartment. The premise is that toxins bound to albumin in the patient’s blood will detach and bind to the binding sites on the membrane, as albumin, when attached to polymers, have a higher affinity for albumin-bound toxins (9). These then pass on to the albumin in the dialysate (where albumin is present at a concentration (200 g/l) 5-7 times that in the plasma). The dialysate, carrying a quantity of toxins, is then cleansed by perfusing over activated charcoal and anion-exchange resin. These take up most of the albumin-bound substances. Water-soluble toxins are removed by passage through a haemodialysis/haemofiltration module, which is run in conjunction with the albumin dialysis module. The dialysate is thus regenerated, and once more capable of taking up more toxins from the blood (10,11,12). This recirculating of the MARS system, using a fixed volume (600
ml) of albumin, is considerably more cost-effective than a single-pass albumin dialysis system would be. As the present study was designed to specifically evaluate the role of albumin dialysis in liver failure, haemofiltration/haemodialysis was not performed (i.e. no removal of free water or water-soluble toxins), and the relevant ports on the circuit were clamped off. MARS was performed through a double lumen catheter in the inferior vena cava (positioned during the abdominal surgery). A blood pump was used to run the blood circuit at 150 ml/min, and the albumin dialysate was circulated by the MARS pump at 150 ml/min as well.

MARS has been used to treat patients of acute-on-chronic liver failure, hepatorenal syndom, and acute liver failure. However, the exact nature of the substances that MARS removes has never been comprehensively analysed.

Figure 4: The MARS circuit (13)
1.5 Hypothesis

(i) Accumulation of toxins (especially albumin-bound) in ALF is responsible for the occurrence of end-organ dysfunctions, and

(ii) removal of these toxins by MARS can attenuate these end-organ effects.

1.6 Aims of study

The present study attempts to look at the amount of cerebral oedema in different brain regions and the effect of albumin dialysis, in the form of Molecular Adsorbent Recirculating System (MARS), in acute liver failure (ALF).
2. METHODS

2.1 Animal preparation

The study outline is shown in figure 5.

*Animals.* 24 Norwegian Landrace pigs (25-30 kg) were randomised into three groups of 8 animals each using sealed envelope system; (1) sham operated (SHAM), (2) hepatic devascularisation (ALF), and (3) hepatic devascularisation followed by MARS therapy (MARS).

The pigs were kept in the animal department for at least 2 days before the experiments. The conditions in the animal room were strictly controlled at a temperature of 21 ± 1°C, a relative humidity of 55% ± 10%, and a 12:12-hr light/dark cycle. The pigs were premedicated with an intramuscular injection of ketamine (20 mg/kg) and atropine (1 mg). Anesthesia was induced with an intravenous bolus of 10 mg·kg⁻¹ pentobarbital and 10 mg·kg⁻¹ fentanyl and maintained with a central venous infusion of 4 mg·kg⁻¹·hr⁻¹ pentobarbital, 0.02 mg·kg⁻¹·hr⁻¹ fentanyl, and 0.3 mg·kg⁻¹·hr⁻¹ midazolam. The pigs were tracheostomized and intubated. Ventilation was maintained with an air-oxygen mixture (FIO₂ = .50) on a volume-controlled respirator. 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. However, after a few minutes on MARS treatment the animals showed clinically signs of light anaesthesia. Accordingly, the animals were kept sedated by a continuous infusion of 0.04 mg·kg⁻¹·hr⁻¹ fentanyl, and 0.6 mg·kg⁻¹·hr⁻¹ midazolam starting from t=2hrs. However, additional bolus doses were given when clinically indicated. Sham operated animals received continuous anaesthesia during the experimental period and received equal amounts of intravenous fluids. Tidal volume was adjusted by means of repeated arterial blood gas analyses to achieve a PCO₂ within the range of 4.5–5.0 kPa. After t = 0 hrs, no adjustments of ventilation were performed. Core body temperature was maintained at 38.5 ± 1°C with a heating pad and blankets. All animals received 500 mL 0.9% NaCl containing 625 mg of glucose as a preoperative load in order to prevent any preoperative dehydration. During the experiment, 0.9% NaCl was infused at a rate of 3 mL·kg⁻¹·hr⁻¹. 50% glucose and 20%
human albumin (albumin 200 mg/mL) were continuously infused from \( t = 0 \) hrs at rates of 0.6048 mL·kg\(^{-1}\)·hr\(^{-1}\) and 0.66 mL·kg\(^{-1}\)·hr\(^{-1}\), respectively. Performing an end-to-side porta-caval shunt followed by ligation of the hepatic arteries induced acute liver failure. 2500 IU heparin was given intravenously to all pigs at the start of the experiment. After \( t=0\) hrs heparin was given to keep ACT > 100 sec. In the MARS group ACT was kept >180 sec from \( t=2\) hrs. MARS was started 2 hours after the hepatic devascularisation and continued for 4 hours. The pigs were killed with an overdose pentobarbital and potassium chloride after \( t = 6 \) hrs.

![Figure 5: Time schedule](image)

2.1.1 Brain collection.

At the end of the experiment brain samples were taken from the pig for measurement of brain water in the tissue.

With the use of a circular cranial saw the brain was removed within 5 minutes. White matter was dissected from the cortex in the frontal lobe and the cerebellum, all from the left side of the brain. All dissections were maid on an ice filled petri dish and stored at 4°C until time for brain water measurement within 15-60 minutes.
2.2 Gravimetric technique for measurement of cerebral oedema

A gravimetric technique for laboratory preparation of gradient columns of specific gravity was used for measurement of brain-tissue water. By this technique, linear and repeatable density gradients were obtained from which values of tissue specific gravity could be determined. The specific gravity of solid and fresh parts of different parts of the brain; frontal cortex, white matter frontal lobe and cerebellum, were measured and converted to units of percent water per gram tissue using conversion factors derived for this purpose and applicable to studies of brain oedema. (14)

2.2.1 Specific gravity of Mixtures

Two mixtures of kerosene (K) and bromobenzene (BB) were used in the preparation of the gradient. In mixture A, the proportion of the solvents was adjusted to equal a specific gravity (sp.gr.) of 0.9750. In the more dense solution (B), the sp.gr. was adjusted to 1.0650. The sp.gr. of BB=1.49716 and of K=0.78734.

The following procedure was used for mixing 1 litre quantities of Stock A and B solutions. The volumes of kerosene and bromobenzene for the desired specific gravity of 1 litre of Stock A and B solution were computed using Equation 1:

\[
m\text{lof BB} = \frac{\text{desired Sp.gr.} - \text{Sp.gr. of K}}{\text{Sp.gr. of BB} - \text{Sp.gr. of K}} \times 1000
\]

ml of K=1000-ml of BB \hspace{1cm} (1)

In Stock A, sp.gr = 0.9750; 264.4 ml BB and 735.6 ml K.
In Stock B, sp.gr = 1.0650; 391.2 ml BB and 608.8 ml K.
Because of error in volume measurements, the specific gravity of the stock solutions was usually not equal to the desired specific gravity, and had to be corrected. To reduce the error, we mixed large quantities (1000 ml) and then adjust the specific gravity of the stock solution to the desired value and replacing with either kerosene or bromobenzene according to the following rules: If the measured specific gravity was less than the desired value, we removed $X$ ml of stock and replaced it with an equal amount of bromobenzene; $X$ is given by Equation 2:

$$X = \frac{\text{desired sp.gr} - \text{sp.grStock}}{\text{sp.grBB} - \text{sp.grStock}} \times 1000$$

(2)

If the measured specific gravity of the mixture was greater than the desired value, we removed $Y$ ml of stock and replaced it with an equal amount of kerosene; $Y$ is given by Equation 3:

$$Y = \frac{\text{desired sp.gr} - \text{sp.grStock}}{\text{sp.grK} - \text{sp.grStock}} \times 1000$$

(3)

2.2.2 Preparation of Gradient System

See figure 6. A flask B containing 100 ml of Stock B solution (sp.gr = 1.0650) was positioned 40 cm above an empty 100 ml graduated cylinder. We then placed 100 ml of a lighter mixture (Stock A, sp.gr = 0.9650) 43 cm (adjusted to 45 cm from pig 17, to try to adjust the flow rate) above solution B. The fluid kinetics of this system is such that a linear gradient column is produced if the outflow from the constantly mixed solution B to the graduated cylinder is twice the outflow from A to B. This was accomplished by using two equal lengths of polyethylene outflow tubing (PP-60, length 65 cm, outer diameter = 1.22 mm, inner diameter = 0.76 mm) from flask B to the graduate and a single equal length of tubing from flask A to flask B. The tubes were curly, so we had to warm it up for a few seconds, and stretch it out for being nearly straight under the experiment. By this technique, the specific gravity at the very bottom of the graduate cylinder is theoretically equal to that of Stock B solution (1.0650), while the specific gravity at the top flask is equal to the average of the specific gravity of the two solutions (1.020). (14)
To start the flow from the tubes we used a syringe, and without siphoning a drop of the stocks, the tubes were clamped to prevent further outflow. The downstream tips of the double connecting tubes were coiled by a thread so that they were in contact with the inner wall of the cylinder. Now, the experiment was ready to be started. The final step in making the gradient cylinder was to release all three clamps (one for each tube) at the same time and check for steady flow. During the filling process the end of the double outflow was positioned 2 mm above the fluid surface of the cylinder. A steady flow to the surface was maintained by gradually lowering the cylinder as the fluid level increased. When the cylinder volume of 100 ml was reached the tubes were clamped. The gradient was then stabilizing and calibrated with standards made up of $K_2SO_4$ of known specific gravity. (14)

We made 3 cylinders for each pig, one for each part of the brain.

Fig 6: The set-up system for the gravimetric technique for measurement of cerebral oedema (14)
2.2.3 Preparation of Specific Gravity Standards for Gradient Calibration
Reagent grade anhydrous potassium sulphate was dried at 100 °C overnight before preparation of standard solutions in distilled water. The concentrations (g/100ml) of the solutions were 6.64, 5.99, 5.34, 4.70 and 3.40 corresponding to specific gravities of 1.050, 1.045, 1.040, 1.035 and 1.025. One drop of each standard was gently placed in the column using a small syringe. The end of equilibration was recorded at the end of 1 minute. (14)

2.2.4 Preparation of samples
We used three different parts of the brain; frontal cortex, white matter in the frontal lobe and the cerebellum. Pieces of these regions were dissected from the sacrificed brain, put in the freezer for approximately 5 minutes (to make it easier cutting). Surgical instruments were used to obtain small samples (approximately 2 mm x 2 mm) from the brain. The brain was surrounded with ice while working with it. The samples were immersed in the liquid gradient and the equilibration depth was recorded at the end of 2 minutes. The equilibration depth represents the specific gravity of the sample and the percentage of water could be calculated, using Equation 4 and 5 as described below. (14)

In the sham group the water content for a specific brain area was determined by both the gradient and a conventional drying technique. In the drying technique the percentage of water in the samples was determined by measuring the wet weight, drying each sample in an oven at 100°C to constant weight, and using the equation: \(1 - \frac{\text{dryweight}}{\text{wetweight}}\) 100%. (14)
The preparation of the liquid gradients by this technique resulted in a virtually linear relationship between specific gravity and graduate division (figure 7).

Fig 7: The relationship between specific gravity and graduate division (14)

2.2.5 Measurement of Brain Water

Changes in brain water may be expressed directly in units of increased or decreased specific gravity. We preferred to characterize the oedema in more familiar terms of gram water per gram tissue (g H₂O/g tissue). Two physical parameters had to be determined before obtaining the g H₂O/g tissue from gravimetric methods; first, the specific gravity of the wet sample (sp.grₙ) was measured by the gradient tube, followed by specific gravity of the solid component (sp.grₛ), which is a constant for the animal tissue under the study. With these parameters known, the g H₂O/g tissue can be calculated by Equation 4:

\[ g \text{ H}_2\text{O/g tissue} = 1 - \left(\frac{\text{sp.gr}_w}{\text{sp.gr}_s} - 1\right) \]

(4)

The sp.grₛ of the brain tissue was determined by obtaining wet and dry weights (W, D) of all frontal cortex, white matter and cerebellum and inserting the sp.grₙ and W/D ratio into Equation 5:

\[ \text{Sp.gr}_s = \frac{1}{1 - \frac{\text{Sp.gr}_w - 1}{\text{Sp.gr}_w} \times \frac{W}{D}} \]

(5)
To help clarify the relationship between sp.g._w, sp.g._s and brain water, we rearranged the structure of equation (4) into the form of a straight line where the g H_2O/g tissue is linearly related to the reciprocal of the specific gravity of the sample. This straight line relationship is depicted graphically in figure 8.

Fig 8: Graphical description of the relationship between tissue water content, specific gravity of solid and fresh tissue. (14)

A specific gravity of wet tissue (sp.g._w) of 1.0 corresponds to 100% water. When tissue water is zero, we are left with the dry solid component and the intersection of the abscissa, or 0% H_2O, corresponds to the reciprocal of the dry solid specific gravity (sp.g._s). The form of a straight line is given by Equation 6:

\[
g \text{H}_2\text{O/g tissue} = \frac{\text{sp.g}_\text{rs}}{\text{sp.g}_\text{rs} - 1} \times \frac{1}{\text{sp.g}_\text{rw}} - \frac{1}{\text{sp.g}_\text{rs} - 1} \times 100
\]  

(6)
2.3 Source of errors

2.3.1 Possible errors in the set up system:

1. The tubing system was not properly in the bottom of the flasks.
2. The length of the tube length shrink when we’re heating it up for stretching. It’s also possible that the inner diameter shrink.
3. It was very difficult to adjust the top flask so the liquid line was horizontally.
4. Inaccurate pipetting when making the Stock A and B.
5. Inaccurate measuring of the amount of Stock A and B.

2.3.2 Possible errors in the accomplishment of the gradient cylinder:

1. The clamping of the tubes might have damaged the tubes.
2. The binding of the double tube with a tread might have damaged the tubes.
3. The binding of the double tube wasn’t good enough, so that it started dripping during the experiment a few times.
4. Disturbance of the cylinder while lowering the cylinder in the filling process.
5. The unclamping of the single and double tubes might not be exactly at the same time.
6. The dripping from the single tube into flask B was held manually. A few times the solution A didn’t drip directly into the stock B, but was held wrong so it dripped into the inner wall of flask B or on the double tubing inside flask B.
7. A few times the flask came out of position and the magnet stopped.
8. The speed on the magnet was too high a few times causing bobbles in the Stock.

2.3.3 Possible errors in the standard system

The sp.gr of the K$_2$SO$_4$ varied a lot from one day to another, and had to be adjusted almost every day. Possible explanations for this is:

1. Evaporation of water.
2. Variation of sp.gr in different temperature.
3. Inaccurate pipetting.
4. The pipette was inaccurate.
2.3.4 Possible errors in the accomplishment of the brain samples in the gradient cylinder

1. The samples were not always placed gently into the cylinder.

2.4 Statistical analysis

Results are expressed as mean ± standard error of mean. Student’s t-test was used to analyse significance of difference between means and $P < 0.05$ was taken to be statistically significant. Data was analysed using Microsoft Excel 2000.
3. RESULTS

3.1 Cerebral oedema

The % of brain water in the different part of the brain in the three randomised groups are shown in Table 3.

<table>
<thead>
<tr>
<th></th>
<th>Frontal cortex</th>
<th>White matter</th>
<th>Cerebellum</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>mean 78.575</td>
<td>69.477</td>
<td>76.298</td>
</tr>
<tr>
<td></td>
<td>(0.7746)</td>
<td>(1.0093)</td>
<td>(0.5953)</td>
</tr>
<tr>
<td>ALF</td>
<td>mean 81.957</td>
<td>71.901</td>
<td>78.133</td>
</tr>
<tr>
<td></td>
<td>(0.4814)</td>
<td>(1.0862)</td>
<td>(0.7307)</td>
</tr>
<tr>
<td>ALF-MARS</td>
<td>mean 82.049</td>
<td>65.909</td>
<td>78.896</td>
</tr>
<tr>
<td></td>
<td>(0.996)</td>
<td>(2.3282)</td>
<td>(1.7131)</td>
</tr>
</tbody>
</table>

Table 3: % water in the different parts of the brain

ALF. This table shows that brain water content increased in ALF compared to sham in frontal cortex, from 78.6% to 82.0%, p=0.0021. There are also a significantly change in the white matter, from 69.5% to 71.9%, p=0.0433. There were no significantly changes in the cerebellum, 76.3% vs. 77.1%, p=0.0691.

ALF-MARS. Brain water contents in ALF treated with MARS did not change significantly in the frontal cortex, p=0.9294. In white matter the brain water was significantly decreased, from 71.9% to 65.9%, p=0.0307.
FRONTAL CORTEX:

Figure 9: % water in frontal cortex.

WHITE MATTER (FRONTAL LOBE)

Figure 10: % water in white matter.
Figure 11: % water in cerebellum.

3.2 ICP

During this project they found that pigs with ICP developed intracranial hypertension. After 6 after ALF induction the ICP increased by 101.4 (±18.3)% (p<0.005) in the ALF group compared to the ICP at the start of the experiment. The ICP remained unchanged in the sham operated animals (16.1±7.1%). The rise in ICP was attenuated in ALF-MARS 33.4(±5.4)%. (Figure 12)
Fig. 12: ICP increases in ALF and are attenuated by MARS
4. DISCUSSION

4.1 Results

The principle findings in this study were increased brain water in frontal cortex and white matter in ALF. In the MARS-group we found increased brain water in frontal cortex, but not in the white matter. Cerebellum was not affected.

Why does MARS just show alteration in the white matter? One explanation could be morphological based; the protosplasmic astrocytes in the grey matter could be more resistant against MARS-treatment compared to the fibrillar astrocytes in the white matter. This fits with what Graham (15) states: “Because the metabolically active cellular constituents of brain tissue are located more in the grey than in the white matter, cytotoxic or cellular oedema is more prominent in grey matter, but can involve adjacent white matter.” Another possibility is that the result is caused by differences in the blood supply. Grey matter has a mean blood flow of 80 ml/100 g brain tissue/min, while white matter only have 20 ml/g brain tissue/min (16). The grey matter subsequently receives more blood borne toxins than the white matter. This higher amount of toxins will make more devastating effects in the grey matter, which will be relatively harder to treat than the slighter amount of toxins in the white matter.

Another question could be; is there a reel affection of MARS – or is it the potassium sulphate we used as standards (see above) playing around?

Our theories has been focusing greatly on the cytotoxic hypothesis. Graham says that vasogenic oedema mainly occurs in the white matter owing to the greater freedom with which water can mote through the parallel fibre bundles. Could it be that the oedema in the grey matter is mainly a cytotoxic oedema, while it is mainly a vasogenic oedema in the white matter? This gives us a possible explanation that MARS actually reduces the vasogenic oedema, but having no effect on the cytotoxic oedema.

Why isn’t the cerebellum affected at all in ALF? Is it because of the astrocytes morphology here? Is it because of a slighter blood supply to cerebellum? Or is the cerebellum actually slightly affected, but that we – because of the mixed white and grey sample didn’t succeed in proving it?
Another part of the project found that the ICP and ammonia both were reduced in the MARS-group compared to the ALF, but still elevated compared to the shams. The ammonia in the systemic circulation was however not significantly changed. As we stated in our introduction, even though the ICP is attenuated a cerebral oedema can still exist, which correlates with our findings. An attenuation of ammonia in the brain should have decreased the amount of cytotoxic oedema in the grey matter, which does not correspond with our results. However it could be that the attenuation in the ammonia caused by the MARS wasn’t enough to give significant change in the amount of oedema.

The finding of brain ammonia being reduced, while there was no reduction in the plasma ammonia, forms a hypothesis about other factors being implicated in the pathogenesis. I.e. other factors than ammonia causing some kind of alteration, which makes the ammonia uptake in the brain greater. However, again the findings of brain biopsies with normal capillary endothelium and mainly swelling of the cortical astrocytes (4,5) makes this hard to understand.

More research for a conclusion about the brain alterations in ALF and the benefit of MARS-treatment is definitely needed.

4.1.1 Statistical significance
This study was undertaken with very few animals in each group.
Although our results showed that there was no increase in the amount of brain water in the cerebellum (p = 0.0631), this could have been a significant observation if we had increased the numbers of animals in each group.
4.2 Source of errors

During the experiment we noted quite a few source of errors.

4.2.1 Potassium sulphate
The specific gravity of the potassium sulphate showed great fluctuations from one day to the other. To minimalise this we checked and corrected the specific gravity every day during the experiment. The last day of the experiment we realised that this most possible was due to temperature differences. The potassium sulphate should be kept in room temperature, not placed cold, like we did. In spite of our daily corrections, this could possible have interfered with our results. This is because the temperature of the potassium sulphate before the corrections would depend of the time we kept it in the weighing room, additionally the temperature on the weighing room where we did our correction was higher than in the room where we had the set-up system. That is the specific gravity of the potassium sulphate could have varied quite a lot during the experiment. We consider this to be the main factor that could have interfered with our results.

4.2.2 Flow rate
Theoretically specific gravity of stock AB, which represent the top of the graduated column should have been 1,020 at the end of the experiment. Measuring the stock AB after it didn’t show this value. In the first experiments the value was too high. We tried to correct this by increasing the height between stock A and B. This didn’t make any changes of consideration. Other factors that could have interfered with the flow rate, was the possible shrinking of the inner diameter of the tubes, and a possible damage of the tubes from the treads and clamping. Furthermore, the flask with stock A wasn’t completely horizontal, because of difficulties adjusting it. However, because these errors were approximately the same for all of the columns we made, we believe that they not could be of great importance for our results.
4.2.3 Gradient column

Interference with the gradient could be a significant source of error. Dripping from the tubes, problems with the magnet and the mixing process – included bobbles in the stock in flask B and in the tubes, and disturbances during the lowering of the cylinder and during the process placing the brain samples into the cylinder. We tried to decrease these sources of errors by doing the different steps in the experiment as carefully as possible, but still they could definitely make a slight difference for our results.

4.2.4 Exclusions of animals

The results from pig 13 (sham), frontal cortex of pig 16-R (ALF-MARS) and white matter in pig 2-R (ALF-MARS) were excluded because of abnormal results. Refer to the data material.
5. CONCLUSION

Pigs with ALF developed an increase in ICP and brain water in both frontal cortex and white matter, but remained unchanged in the cerebellum. MARS treatment significantly decreased the ICP and the degree of brain oedema in the white matter while having no effect on the brain oedema in the frontal cortex.
6. ACKNOWLEDGEMENT

We gratefully acknowledge Lars Marius Ytrebø and Cristopher Rose for their never ending enthusiasm and help under the experiment and writing period. We also thank Professor Arthur Revhaug for making this project possible.
7. REFERENCES


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FORKLARING TIL VEDLEGG

A gravimetric technique for measurement of cerebral edema

-in pigs with acute liver failure

Synnove Sævarsdottir Davland
Matzanne Kjærsgaard
University Hospital of Northern Norway

Acute liver failure- clinical manifestations:

- Altered mental status \( \rightarrow \) coma
- Brain edema
- Increased intracranial pressure (ICP)
- Brain stem herniation

Increased ICP in ALF

Small Increases in Brain Volume Lead to Large Increases in ICP

Different types of brain edema:

- Cytotoxic: Cell swelling due to change of osmolarity
- Vasogenic: Increased permeability
- Interstitial: Increased production, decreased transport or decreased absorption of CSF

Loss of hepatic function

\[ \uparrow \text{blood and brain ammonia} \]

hepatic encephalopathy
brain edema, ICP
Brain Herniation in Patients with Acute Liver Failure Correlates with Arterial Ammonia Concentrations

Measurement of cerebral edema


Solvents

- Kerosene (K)  Sp.G = 0.78734
- Bromobenzene (BB)  Sp.G = 1.49716
Preparation of Stocks

Stock A: Sp.G 0.9750
Stock B: Sp.G 1.0650

\[
\text{ml of BB} = \frac{\text{Desired Sp.G} - \text{Sp.G of K}}{\text{Sp.G of BB} - \text{Sp.G of K}} \times 1000
\]

\[
\text{ml of K} = 1000 - \text{ml of BB}
\]

Standards of K₂SO₄

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Sp.G</th>
</tr>
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<tbody>
<tr>
<td>6.64</td>
<td>1.050</td>
</tr>
<tr>
<td>5.99</td>
<td>1.045</td>
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<tr>
<td>5.34</td>
<td>1.040</td>
</tr>
<tr>
<td>4.70</td>
<td>1.035</td>
</tr>
<tr>
<td>3.40</td>
<td>1.025</td>
</tr>
</tbody>
</table>

The column

![The column diagram]

y = -2390x + 2744
\( R^2 = 1 \)

Specific Gravity

Samples of the brain

- Frontal cortex
- White matter (frontal lobe)
- Cerebellum

![Brain tissue and K₂SO₄ samples]
Measurement of Sp.G wet

\[ y = -2200 \times + 2344 \]
\[ y = \text{DIV} \]
\[ x = \text{Sp.G wet} \]

Calculation of Sp.Gs:

\[ Sp.G_s = \frac{1}{1 - \frac{Sp.G - 1}{Sp.G_w - 1} \times \frac{W}{D}} \]

Calculation of % water in the brain tissue:

\[ \frac{g_{m, H_2 O}}{g_{m, wet}} = \frac{Sp.G}{Sp.G_s - 1} \times \frac{1}{Sp.G - 1} \times 100 \]

Results

Frontal Cortex

White matter (Frontal lobe)

Sham

ALF

MARS
Summary of results

- **Frontal cortex**: Significant increase in brain water in both ALF and MARS compared to SHAM.
- However, no significant difference between ALF and MARS.
- **White matter**: Significant increase in brain water in ALF (not MARS) compared to SHAM.
- Significant decrease in brain water in MARS compared to ALF.
- **Cerebellum**: No significant differences.

Conclusions:

- **Frontal cortex**: Increased brain water in both ALF and MARS.
- Not similar to ICP results.

Thank you:

Christopher Røe
Arthur Rehleung
Lars Marius Giske Ytrehol
Rest of the MARS team
THE EFFECTS OF MOLECULAR ADSORBENTS RECIRCULATING SYSTEM (MARS) ON THE DEVELOPMENT OF CEREBRAL ÖDEMA IN A PORCINE MODEL OF ACUTE LIVER FAILURE


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Background: Hepatic encephalopathy (HE) is a serious complication to acute liver failure (ALF) where increased intracranial pressure (ICP) leads to brainstem herniation and death. The present study attempts to look at the amount of cerebral oedema in different brain regions and the effect of albumin dialysis, in the form of MARS, in a porcine model of acute liver failure.

Methods: Brain samples from the frontal cortex, frontal white matter, and cerebellum were obtained six hours after induction of ALF in female pigs (25-30 kg). ALF was induced by hepatic devascularisation and followed with (ALF-MARS, n=5) or without (ALF, n=8) a 4 hour MARS treatment, which started 2 hours after ALF induction. A sham operated control group was included (n=5). An intracranial pressure transducer was used to monitor the ICP. A gravimetric technique (ref.) was used to determine % brain water in specific regions of the brain.

Results: Pigs with ALF developed cerebral oedema and intracranial hypertension. At t=6, ICP was increased by 101.4(±18.3)% (p<0.005) in the ALF group compared to t=0, while ICP remained unchanged in the sham operated animals 16.1(±7.0)%. The rise in ICP was attenuated in ALF-MARS 33.4(±5.4)%. Brain water content increased in ALF compared to sham in frontal cortex 81.9(±1.4)% vs 77.7(±1.2)%), p<0.05, and white matter 69.5(±1.1)% to 72.5(±1.1)% p<0.05, whereas no significant change was found in the cerebellum 76.4(±0.6)% to 77.9(±0.7)%, p=0.0631. Brainwater content decreased in ALF-MARS vs ALF 69.0(±3.3)% vs 72.5(±1.1)%, p<0.05 in the white matter, while there was no significant difference in the frontal cortex.

Conclusion: Pigs with ALF developed an increase in ICP and brain water in both the frontal cortex and frontal white matter, but remained unchanged in the cerebellum. MARS treatment significantly decreased the ICP and the degree of brain oedema in the white matter while having no effect on the brain oedema in the frontal cortex.
The effects of MARS on the development of cerebral edema in a porcine model of acute liver failure

Marina K, King J, Brandt D, et al. (2023) Background

Acute liver failure - clinical manifestations:

- Altered mental status → comatose
- Brain edema
- Increased intracranial pressure (ICP)
- Brain stem herniation

Small Increases in Brain Volume Lead to Large Increases in ICP

ICP

Brain Volume

Cerdás and ML, Soc. In Liver Dis 2006

Aims

1) To study the amount of cerebral edema in different brain regions in pigs with acute liver failure.

2) To study the effects of MARS treatment in pigs with brain edema.

Methods

- 18 pigs randomised into 3 groups;
  1) ALF
  2) MARS
  3) SHAM (no treatment)

Methods continued:

- Different brain regions measured;
  - Grey matter frontal lobe
  - White matter frontal lobe
  - Cerebellum (medial grey and white)

- Gravimetric technique
Results

Grey matter (frontal lobe)

Sham
ALF
MARS

White matter (frontal lobe)

Sham
ALF
MARS

Cerebellum

Sham
ALF
MARS
Conclusions

**Grey matter:**  
- Increased brain water in ALF  
- No protection by MARS

**White matter:**  
- Increased brain water in ALF  
- Attenuated in MARS

**Cerebellum:**  
- No significant changes

Acknowledgements

Sytaphe S. Devland  
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Lars Marius Giske Ytrebråd  
Arthuq Revhauq
MOLECULAR ADSORBENTS RECIRCULATING SYSTEM (MARS) DECREASES BRAIN AMMONIA AND INTRACRANIAL PRESSURE IN A PORCINE MODEL OF ACUTE LIVER FAILURE

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\(^1\)Department of Digestive Surgery, University Hospital Northern Norway, Tromsø. \(^2\)Institute of Hepatology, University College London, London. \(^3\)Department of Cellular Neuroscience, Max-Delbrück Center for Molecular Medicine, Berlin

**Background:** Hepatic encephalopathy (HE) is a severe complication of acute liver failure (ALF). Death most frequently results from brainstem herniation due to increased intracranial pressure (ICP). Ammonia is strongly implicated in the pathogenesis of HE. The aim of the present study is to evaluate the effect of albumin dialysis, in the form of Molecular Adsorbents Recirculating System (MARS) on plasma and brain ammonia, and on ICP in a porcine model of ALF.

**Method:** Female pigs (25-30 kg) with ALF induced by hepatic devascularization were monitored for 6 hours, with (ALF-MARS, n=8) or without (ALF, n=8) a 4 hour MARS treatment which began 2 hours after ALF induction. Ammonia was measured in arterial blood. Cerebral microdialysis was used to measure extracellular brain ammonia. An intraparenchymal pressure transducer was used to monitor the ICP.

**Results:** At the start of MARS treatment, no significant difference in ICP, arterial and microdialysate ammonia was found between ALF and ALF-MARS groups. Comparing pre (t=2) and post (t=6) MARS treatment, the increase in microdialysate ammonia was significantly less in ALF-MARS (91.3±14.1%) compared to ALF (213.3±44.0%, p<0.05) whereas the increase in arterial ammonia did not significantly change between the two groups. The rise in ICP was attenuated in ALF-MARS (33.4±5.4%) vs ALF (53.7±1.6%, p<0.01).

**Conclusion:** MARS treatment significantly decreases brain ammonia and ICP in pigs with ALF, without decreasing blood ammonia.
MARS decreases brain ammonia and intracranial pressure in a porcine model of ALF

Background
- Hepatic encephalopathy
- Cerebral edema
- Intracranial hypertension
- Cerebral herniation

Ammonia concentration is determined by a balance between the rate of production and consumption

Intestines → Liver: Urea Excreted

Kidneys

Muscle, Brain: Glutamine Detoxification

Brain Herniation in Patients with Acute Liver Failure Correlates with Arterial Ammonia Concentrations

[Hapatol. 25: 648-653, 1999]
Aims of study

- Evaluate the effect of MARS on plasma and brain ammonia.
- Evaluate the effect of MARS on ICP.

Methods

- Arterial and venous blood samples
- Intracranial microdialysis catheter
- Intracranial pressure transducer

PLASMA AMMONIA

ARTERIAL AMMONIA

VENOUS AMMONIA
Conclusion

- MARS treatment significantly decreases brain ammonia and ICP in pigs with ALF.
- MARS has no significant effect on plasma ammonia levels.

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