5. *IN VIVO* ANIMAL STUDIES

Overview

The following section presents two *in vivo*, clinically based animal studies that have been required in the development of the UP-CSIR BAL. The employed models are experimentally controlled versions of the severe liver injury scenarios that are likely to be encountered in the clinical application of BAL devices.

The first of these studies investigates the potential toxicity of IV injected PFC in a rat model of severe liver injury. Despite a lacking in human clinical evidence demonstrating PFC toxicity, the same has not previously been shown in a liver failure scenario. Since PFC is used in a sub-circulation of the UP-CSIR BAL and due to the (unlikely) possibility of its entrance into a patient, the necessity therefore exists to confirm its non-toxicity in a similar scenario. For this purpose a highly reproducible ¾ partial liver resection rat model is employed. No PFC toxicity or any impact on the rate of liver regeneration results. The success of this study is attributed to the employed methods and the study design.

The second study details the establishment and standardization of an ischemic surgical model of irreversible ALF in pigs. Such a model is required in the pre-clinical verification of the efficacy of a BAL. Although similar surgical models have previously been described, the statistical methods used in standardizing this one are unique. Briefly, criteria are defined based on the analysis of trends in the collected clinical data. These criteria are designed to discriminate between animals that do or do not represent a valid model of ALF. However, it is clear that induced animal models of ALF are inherently limited in the degree to which they are comparable to the human clinical scenario.

In the thoughts and recommendations that follow the impact of the chosen ischemic ALF model on the clinical testing of the UP-CSIR is examined. The many difficulties in the model are highlighted, suggesting research into alternate models, design alterations in the BAL device and the exclusion or inclusion of variables based on their newly established clinical value/s. Adding detail to this, arguments are raised regarding the choice of alternate animal models. The prognostic and clinical importance, strategies for measurement and reduction of blood ammonia in ALF and the inclusion of artificial toxin clearance devices into BAL devices are discussed.

5.1 Non-toxicity of an IV injected perfluorocarbon oxygen carrier in an animal model of liver regeneration following surgical injury

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5.1.1 Introduction

Perfluorocarbon (PFC) polymers have the properties of exceptional chemical and biological inertness. PFCs also have very high dissolving capacities for oxygen (O_2) and carbon dioxide (CO_2), making them attractive candidates as artificial O_2 carriers in erythrocyte replacement applications, including for example, plasma perfused bioartificial livers [10,88,94]. The intravascular (IV) administration of a PFC requires the development of a heat sterilizable, sub-micron droplet size emulsion that is stable in non-frozen conditions for at least two years [200,201].

In mammals the *in vivo* distribution and elimination of PFC is characterized by its half lives in the circulation and in the reticulo-endothelial system (RES). In the first 24 hours the PFC is cleared from the circulation by the mononuclear phagocyte system accumulating in the liver, spleen and bone marrow. In the second phase, lasting days to weeks, it is cleared from the RES via lipid compartments in the blood into the lungs. Thus, PFC is not metabolized; it is excreted from the respiratory system into the air. During this phase, flu-like symptoms with myalgia and light fever have been reported in clinical studies [201]. Factors affecting the clearance half lives of PFC are emulsion droplet size, molecular weight, surfactant-type, complement activation, animal species and in humans, racial differences [202-204].

In second generation PFC emulsions the toxicity problems associated with earlier attempts [205] have been overcome. Oxygent (Alliance pharmaceuticals, San Diego, USA), a PFC composed of (predominantly) perfluorooctyl bromide (PFOB) ($C_8F_{17}Br$)

emulsified in egg yolk phospholipid (lecithin) as a surfactant, has successfully progressed through both stage II and III clinical trials. In the above trials Oxygent was not found to significantly initiate either immunological or coagulative reactions in healthy volunteers. Furthermore, no subsequent perturbation of normal blood hemostatic or viscosity behaviour could be found (in fact, viscosity improved); there was no reduction in clot formation or strength and no increase in red cell hemolysis [206,207].

Lethal dose experiments in animals have shown that the value for PFOB is 41g/kg, which is remarkably non-toxic [208-212]. However, the non-toxicity of PFCs has not previously been demonstrated in a liver failure scenario. The clinical progress of acute liver failure (ALF) involves the development of a hyperdynamic circulation, a disseminated intravascular coagulopathy (DIC), renal, and eventually, multi-organ failure [213]. Since the IV administration of a toxin may produce a similar profile to the above, experiments such as these must discriminate between the two potential clinical syndromes. In this study a highly reproducible model of reversible liver failure in the form of a ¾ partial liver resection in rats was selected. This model emulates a seriously compromised liver, with failure in the beginning followed by progressive regeneration. Several previous studies have established that 100 % of such animals will recover [214-219]. Thus, the purpose of these experiments was, by using an animal model; to investigate the effects of IV administered PFOB on the recovery of a liver failure patient. They also served to preclude institutional manufacturing differences as the formulation of the UP-CSIR PFOB is similar to that of Oxygent.

5.1.2 Materials and methods

Animals

The experiments were conducted on 56 healthy female Sprague-Dawley rats of approximately 200 g each. These were housed under temperature controlled conditions in Macrolon type 3 cages with a 12 hour light-dark cycle, with sterilized wood shavings for bedding, access to standard rat food pellets and water with 10 % glucose at the University of Pretoria biomedical research center.

Experimental design (table 5.1.1)

The experimental groups were composed of 16 sub-groups in a cross-tabulated design aimed at discriminating between the effects of the surgery and the test substance (PFC). Half the animals received surgery (LI: liver injury) and the other half did not. In turn, half of each of the above received IV injections of either the test substance (PFC) in high (3 ml, 5 g/kg) or low doses (1 ml, 2 g/kg), or saline controls (3 ml). Acute and sub-acute toxicity effects were investigated by terminating experiments at either short (2-day) or longer (4-day) durations.

Table 5.1.1 The experimental sub-groups

		PFC		Saline	
Doses (day1)		LI (+)	LI (-)	LI (+)	LI (-)
Low	2 g/kg	6	6	6	6
High	5 g/kg	6	6	6	6
Durations	Terminations (3 of each dose group above)				
Short	Day 2	6	6	6	6
Long	Day 4	6	6	6	6
	Total surgery	12		12	
	TOTAL				48

Perfluorocarbon composition and dosing

Perfluorooctylbromide-lecithin emulsions were prepared according to the method of Moolman *et al*, as previously described by our group [10,88,94]. Sterile, deionized, autoclaved water was used to make up the PFOB-lecithin emulsion to 20% v/v concentration. pH was adjusted to 7.35 prior to drawing up the IV injections.

PFC doses were provided as either ‘high’ or ‘low’. The high dose (5 g/kg) was 3 ml of 20 % PFC in a 200 g rat, which was calculated to simulate exposure to the IV entry of one liter of 20 % PFC into an adult human. Hypervolumia was prevented in the animals by prior blood sampling of an equivalent volume. The low dose (2 g/kg) was chosen as 1 ml, i.e. 1/3 of the high dose. Saline dose controls (3 ml) were used as controls for the test substance. Surgical controls, i.e. surgeries without any additional treatments were also performed. All doses were introduced through the tail vein.

Liver injury (LI) model

As per the protocol first described by Higgins and Andersen in 1931 [214-219], 3/4 liver resections were carried out (on day 0) on one half (N = 24) of the animals. Briefly, while the animals were under isoflurane anesthesia, a midline incision was made, followed by complete liberation of all liver ligaments to allow the ligation of the pedicles of the median and left lateral lobes i.e. they were scissor clamped, tied off with suturing line and resected. Thereafter, the midline was sutured shut and the animals were allowed to recover. Each procedure took approximately 10 minutes. Prior to the above, 8 rats were used for perfecting the surgical procedure and their organ weights and blood indices were included as healthy controls (baselines) relative to the experimental groups.

For the anesthesia, recovery, pain and toxicity scoring protocols please refer to Appendix B.1.

Analyses

On days 0, 2 and 4, 1 ml blood samples were taken from the tail vein of all animals for blood biochemistry and haematology (tables 5.1.2,3). Upon termination of the 2 and 4 day groups, body, liver, left kidney, lungs and spleen weights were measured. The organs were first examined for macroscopic pathology and hematoxylin and eosin histology.

Statistical methods are as presented in Appendix B.1. No differences could be detected between the high-dose and low-dose PFC groups in the raw data and these were consequently included as one group in subsequent comparisons. The following sub-groups were compared for each of the measured variables to discriminate between the effects of the surgery and the PFC:

1. The surgical versus non-surgical groups at 2 and 4 days (PFC + saline).
2. The 2 versus 4 day surgical groups (PFC + saline).
3. The PFC versus saline non-surgical groups.
4. The PFC versus baseline (no interventions) non-surgical groups.
5. The saline versus baseline non-surgical groups.
6. The 2 and 4 day surgical groups versus the baselines.
7. The 2 and 4 day non-surgical groups versus the baselines.

Only significant ($p \leq 0.05$) or marginal ($p > 0.05 \leq 0.1$) differences between groups are mentioned below.

5.1.3 Results

Table 5.1.2 provides an explanation of the measured variables and their units. Table 5.1.3 provides the mean ± standard deviation of the relevant organ and body weights, blood biochemistry and haematological indicators.

Table 5.1.2 Measured variables and units

Variable	Explanation	Unit
LI	liver injury	
PFC	perfluorocarbon	
SAL	saline	
BW	mean body weight	g
+ and -	with and without	
ΔBW	change in mean body weight	g
spleen	mean spleen weight	g
spleen/BW	mean percentage spleen to body weight ratio	%
Δspleen/BW	mean change in percentage spleen to body weight ratio	%
liver	mean liver weight	g
liver/BW	mean percentage liver to body weight ratio	%
Δliver/BW	mean change in percentage liver to body weight ratio	%
Alb	mean plasma albumin concentration	g/l
ALT	plasma alanine amino transferase concentration	U/l at 37 ^{o}C
AST	plasma aspartate amino transferase concentration	U/l at 37 ^{o}C
Urea	plasma urea concentration	mmol/l
Bili-T	plasma total bilirubin concentration	µmol/l
Ammo	plasma ammonia concentration	µmol/l
RCC	blood red cell count	x 10^{12}/ l
Hkt	hematocrit	% l/l
WCC	blood white cell count	x 10^{9}/ l
Ab-Neutr	absolute neutrophil count	x 10^{9}/ l
Ab-Lymp	absolute lymphocyte count	x 10^{9}/ l
Ab-Mono	absolute monocyte count	x 10^{9}/ l
Plt-C	platelet count	x 10^{9}/ l

Table 5.1.3 Weight changes, biochechemistry and haematology

		Groups							
Variable	**Baseline (N = 24)**	**2-days +LI+PFC (N=6)**	**2-days +LI +SAL (N=3)**	**2-days -LI +PFC (N=6)**	**2-days -LI +SAL (N=3)**	**4-days +LI +PFC (N=6)**	**4-days +LI +SAL (N=3)**	**4-days -LI +PFC (N=6)**	**4-days -LI +SAL (N=3)**
BW	210.97±27.91	215.62 ±9.01	204.73 ±13.68	207.13 ±29.13	222.60 ±8.28	211.82 ±6.58	183.30 ±21.71	225.50 ±7.40	232.70 ±8.62
ΔBW		-29.45 ±16.28	-13.97 ±9.16	-3.42 ±2.56	0.40 ±3.40	-25.60 ±5.30	-18.63 ±5.08	-5.20 ±7.78	0.70 ±4.59
spleen	0.688±0.269	0.637 ±0.142	0.542 ±0.305	0.652 ±0.059	0.540 ±0.023	0.756 ±0.286	0.472 ±0.033	1.02 ±0.38	0.56 ±0.11
spleen/BW	0.326 ±0.111	0.262 ±0.068	0.246 ±0.138	0.314 ±0.050	0.243 ±0.003	0.318 ±0.118	0.235 ±0.020	0.44 ±0.15	0.24 ±0.04
Δspleen/BW		0.064 ±0.068	0.080 ±0.138	0.012 ±0.050	0.083 ±0.003	0.008 ±0.118	0.091 ±0.020	-0.114±0.146	0.085 ±0.04
liver	7.364±1.008	5.107 ±0.528	4.307 ±0.673	8.008 ±0.738	6.818 ±0.634	6.090 ±0.724	5.147 ±0.713	8.311 ±0.596	6.662 ±0.764
liver/BW	3.523 +0.457	2.104 ±0.351	1.966 ±0.269	3.842 ±0.399	3.078 ±0.394	2.566 ±0.300	2.559 ±0.352	3.605 ±0.228	2.874 +0.342
Δliver/BW		1.419 ±0.351	1.557 ±0.269	-0.319 ±0.399	0.445 ±0.394	0.957 ±0.300	0.964 ±0.352	-0.082 ±0.228	0.649 ±0.342
Alb	43.8 ±2.1	33.1 ±1.8	36.17 ±2.23	38.07 ±2.27	47.70 ±3.25	31.20 ±2.80	33.13 ±2.27	35.48 ±2.08	41.70 ±0.95
ALT	44 ±6	338 ±202	450 ±223	53 ±19	62 ± 33	66 ±12	56 ±5	39 ±8	41 ±9
AST	65 ±9	541 ±180	891 ±723	80 ±27	66 ±15	123 ±29	81 ±9	48 ±6	50.7 ±4.9
Urea	7.4 ±0.9	6.2 ±1.3	6.9 ±3.2	5.2 ±1.2	6.1 ±1.7	5.2 ±1.4	5.5 ±1.5	6.3 ±0.9	5.8 ±4.9
Bili-T	4.1 ±0.8	23.8 ±4.7	14.3 ±2.7	4.2 ±1.2	5.2 ±1.5	6. 9 ±1.9	5.6 ±1.4	5.5 ±2.9	5.6 ±2.8
Ammo	52.1 ±20.8	76.2 ±32.5	123.3 ±136.7	31.6±5.3	32.8 ±15.7	53.8 ±16.5	44.2 ±17.4	29.2 ±9.03	49.6 ±15.2
RCC	8.63 ±0.95	7.34 ±0.78	6.25 ±2.30	8.65 ±0.64	8.24 ±0.18	7.46 ±0.70	8.17 ±0.24	8.54 ±0.47	8.70 ±0.23
Hkt	43.5 ±1.4	36.2 ±0.04	30.7 ±11.0	42.5 ±1.9	40.0 ±1.7	38.2 ±3.1	41.7 ±1.5	42.0 ±1.80	42.7 ±0.06
WCC	7.90 ±1.29	10.35 ±3.75	13.58 ±6.45	5.72 ±1.17	4.94 ±1.31	5.98 ±0.92	12.99 ±1.67	6.08 ±1.78	7.53 ±1.36
Ab-Neutr	0.76 ±0.41	4.64 ±1.77	8.26 ±3.47	1.12 ±0.41	1.15 ±0.72	2.70 ±1.18	4.32 ±0.84	0.65 ±0.61	0.58 ±0.23
Ab-Lymp	6.82 ±1.35	5.20 ±2.33	3.82 ±2.76	4.35 ±0.90	3.23 ±1.37	3.34 ±1.10	7.05 ±1.27	5.14 ±1.52	6.48 ±1.36
Ab-Mono	0.24 ±0.21	0.33 ±0.20	1.27 ±0.99	0.14 ±0.08	0.50 ±0.4	0.27 ±0.20	1.53 ±0.92	0.20 ±0.12	0.34 ±0.04
Plt-C	491 ±387	783 ±145	803 ±209	686 ±130	555 ±425	425 ±207	550 ±109	366 ±158	1051 ±112

Notes:

1. In this table the high-dose and low-dose PFC groups were summed as no difference could be detected between them in the raw data.
2. All values are presented as mean ± std deviation.
3. Δ = change in value.

General observations

All animals in the control and experimental sub-groups survived for the duration of the trial. In the first two days all surgical animals demonstrated signs of trauma in the form of hunched postures, pilo-erection (ruffeled coats), red circles around their eyes and gnawing at the wood-shaving bedding material (pain). These signs decreased from 2 to 4 days. In the non-surgical sub-groups (baselines, PFC or saline) this behaviour was not present. One animal was lost from the trial due to disembowlment following the gnawing off its abdominal sutures. This loss did not impact the results as the animal was of the surgical control group.

Body weight loss was found in all groups save the non-surgical saline injected sub-group. This was greatest in the surgical groups, but did not significantly differ in the PFC or saline, 2 or 4 day sub-groups. No increases in the spleen to body weight ratios were found in the PFC versus saline sub-groups. Although kidney and lung weights were measured, no differences between the sub-groups and the baseline animals were detectable and this data was consequently excluded from Table 3. A significant increase in the liver to body weight ratios was found in all surgical versus non-surgical groups at 2 days ($p < 0.001$) and at 4 days ($p = 0.003$). In the surgical PFC or salines at 2 days and 4 days there was no difference in the liver to body weight ratios. The rate of regeneration of the livers of the surgical PFC or saline sub-groups was also not different. Thus, the PFC did not impact liver re-generation following severe injury (figure 5.1.1).

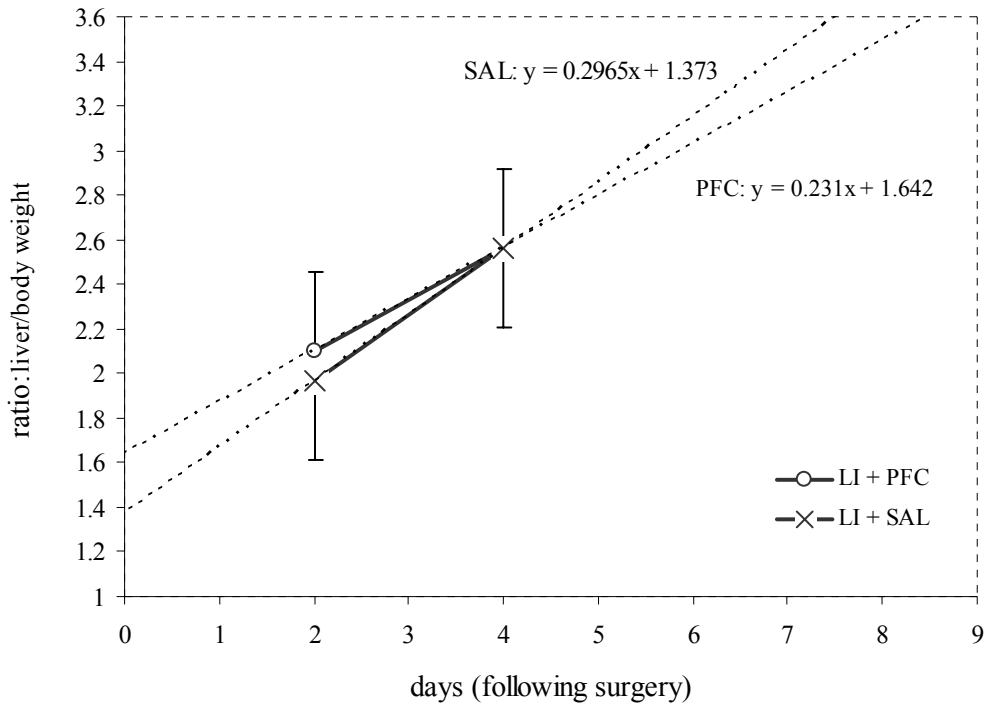

Figure 5.1.1 Liver regeneration projections, assuming linear re-growth.
The rate of regeneration after liver injury and IV dosing of PFC or saline is not significantly different. The y-intercept indicates the amount of liver initially resected. A liver/BW value of 1.5 equates to approximately a 60 % liver weight resection. The projected time to complete liver weight regeneration, i.e. to a liver/BW ratio of 3.5, is 7-8 days. This duration is in agreement with prior experience with this surgical model [214-219].

Biochemistry

Blood albumin in the surgical versus non-surgical groups (PFC + saline) was significantly decreased at 2 days ($p = 0.005$) and 4 days ($p = 0.003$). The non-surgical PFC sub-groups had significantly lower levels than both the salines and the baselines ($p = 0.001$ and $p < 0.001$ respectively). Thus, both the surgeries and the PFC decreased albumin production by the liver.

The liver enzymes ALT and AST, reflecting liver damage, were significantly increased in all surgical versus non-surgical groups, at 2 days (ALT $p = 0.001$, AST $p = 0.001$) and at 4 days (ALT $p = 0.001$, AST $p < 0.001$). At 4 days, levels were significantly higher in the surgical groups (PFC + saline) versus the baselines (ALT p

= 0.001, AST $p < 0.001$). Therefore, the surgeries rather than the PFC caused liver damage.

Bilirubin, reflecting hepatic bile removal, was significantly increased in the 2 versus 4 day surgical groups ($p = 0.001$) and also increased in the (PFC + saline) surgical versus non-surgical sub-groups at 2 ($p = 0.001$), but not at 4 days. The (PFC + saline) 4 day surgical group was significantly increased relative to the baselines ($p = 0.003$), but not the non-surgical group. Thus, surgery immediately decreased bilirubin clearance, followed by a return to normal by day 4. PFC had no effect.

Urea, reflecting hepatic (nitrogenous-waste) metabolism, was significantly lower in the non-surgical PFC and saline groups versus the baselines (PFC $p = 0.009$ and SAL $p = 0.024$ respectively). It was also significantly lower in the (PFC + saline) surgical and non-surgical 4 day sub-groups versus the baselines (+LI $p = 0.005$ and –LI $p = 0.008$ respectively). Interestingly, urea was slightly higher in the 2 versus 4 day surgical groups. It appears that neither the surgeries nor the PFC had any effect on urea production.

Ammonia, reflecting blood nitrogenous toxin levels, was increased in the 2 day versus 4 day surgical groups (but not significantly). The (PFC + saline) surgical groups at 2 days were significantly increased relative to the non-surgicals ($p = 0.002$), but only marginally increased at 4 days ($p = 0.064$). The non-surgical PFC groups (2 + 4 days) were marginally lower than the salines ($p = 0.068$) and significantly lower than the baselines ($p = 0.006$). Thus, similar to bilirubin, surgery decreased ammonia clearance with recovery to normal by day 4. Of interest, the presence of the PFC was associated with decreased ammonia levels.

Haematology

Red cell count (and hematocrit) were significantly decreased in both the (PFC + saline) 2 and 4 day surgical versus non-surgical groups (for RCC $p = 0.005$ and $p = 0.004$ respectively). The 4 day surgical group was significantly lower than the baselines ($p < 0.001$) but not so in the non-surgical group. The non-surgical saline group was marginally lower than the baselines ($p = 0.055$). RCC and Hkt were therefore decreased by the surgeries rather than the PFC.

White cell counts were increased in surgical versus non surgical groups, significantly so at 2 days ($p = 0.011$) but not at 4 days. The baselines were significantly increased relative to the non-surgical PFC groups ($p = 0.005$) but not the salines. Ab-Neutr was significantly increased in the surgical versus non-surgical groups at both 2 days ($p < 0.001$) and 4 days ($p < 0.001$). In the 2 day group this was significantly larger than in the 4 day surgical group ($p = 0.031$) and at 4 days the surgical groups had significantly larger values than the baselines ($p < 0.001$). In Plt-C the surgical 2 day groups had significantly higher counts than 4 days groups ($p = 0.004$). The surgery therefore substantially increased the WCC (especially the neutrophils) while the PFC had no apparent effect.

Macroscopic observations and histology

White droplets were macroscopically noted in the spleens and to a lesser extent in the kidneys and livers in the PFC injected 2-day animals, both surgical and non-surgical. In the 2-day surgical groups (PFC + saline) the liver remnants were blanched and tough relative to healthy livers. In the 4-day surgical groups the livers had grown back to approximately ¾ their original size and were more similar in color and texture to the (healthy) livers of the baselines, than the 2-day group. In the 4-day PFC injected groups, no white droplets could be discerned in any of the organs.

H and E histology of the livers revealed vacuolar swelling with cytoplasmic droplets and an increase in mitosis and apoptosis that correlated with the surgeries. This was more severe in the 2 versus 4 day groups. Vacuolated Kupffer cells, associated with the PFC, were especially detected in the 2-day high-dose animals in the non-surgical groups. Low-dose animals did not demonstrate this. Micro-granulomas were noted in the Kupffer cells in the 4 day PFC injected animals. In the spleens of the PFC injected animals vacuolated reticulo-endothelial cells in the blood sinuses of the red pulp were visible. Kidney sections demonstrated no specific findings. In the lungs, atelectasis presumably associated with anesthetic euthanisation, was found in the majority of the animals. Leucocyte aggregations and alveolar macrophage hypertrophy was observed in several of the PFC injected surgical and non-surgical animals in the 2 day and 4 day groups.

5.1.4 Discussion

The surgical method employed in this study was selected to model the potentially reversible acute liver failure syndrome seen in human patients. However, animal models make extension to the human clinical scenario difficult due to, amongst other reasons, species differences in response to test substances, the degree of reversibility, the disease process duration and the degree of involvement of other organ systems [220,221]. The ¾-partial liver resection in rats is an attractive model in that it is well-described, technically feasible, highly reproducible, non-toxic yet severe, but reversible within a time period sufficient to enable study. Although species differences must obviously exist, extension to the human scenario is reasonable in view of prior findings of PFOB non-toxicity in clinical studies [202,206,207].

The lack of PFOB toxicity was evident in the absence of differences between the control and experimental sub-groups for the parameters studied. Specifically, no changes in the haematological indices as markers of systemic toxicity were apparent in the PFC versus saline injected groups. Similarly, the biochemical indices including bilirubin clearance, urea production and liver enzymes levels were also not impacted by the PFC. Bearing in mind the findings it is therefore safe to assume that this study was successful in meeting its aims. That is, PFC non-toxicity may be extended to include the liver failure case. This was possible owing to the non-toxic surgical model not complicating the effects of the test substance, the cross-tabular design, the measurement of a large number of variables and the given ability to investigate the impact of the PFOB on the rate of liver regeneration following the injury.

The finding that PFOB did not impact the rate of liver regeneration following damage is of particular interest in view of the severity of liver failure in patients undergoing bioartificial liver treatments. In compromised livers, metabolic hypoxia may be significant. We found that PFOB actually decreased blood ammonia levels, possibly owing to improvements in blood oxygenation facilitating liver toxin clearance. A potential benefit may therefore lie in ameliorating the deleterious effects of ammonia. Of additional physiological interest: The decrease in albumin production associated with the PFC may have been due to the presence of the phospholipid lecithin surfactant in the emulsion. This may have activated a negative feedback mechanism

regulating blood albumin levels and thereby, blood viscosity. As previously stated, prior clinical studies [206,207] have demonstrated improvements in blood viscosity following PFOB injection. The 2nd phase flu-like symptoms post-operatively found [202] may also be correlated with the macrophage hypertrophic changes and leucocyte aggregations found in our 4-day lung histology specimens.

To conclude, this study did not provide any indication that IV injected PFOB was toxic at the concentrations employed in either healthy or severe liver injury scenarios. PFOB also had no impact on the rate of liver regeneration following the surgically induced damage. Bearing in mind the results of prior human clinical studies it is reasonable to assume the safety of using a PFOB emulsion in bioartificial liver support system treatments.

5.2 Standardization criteria for an ischemic surgical model of acute hepatic failure in pigs

Nieuwoudt M, Kunnike R, Smuts M, Becker J, Stegmann GF, Van der Walt C, Neser J, Van der Merwe S.

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5.2.1 Introduction

The verification of the efficacy of a supportive therapy for acute hepatic failure (AHF), such as a BALSS, requires the establishment of a reliable large animal model of AHF. Two categories of models have been identified as potentially useful in this regard: Firstly, toxic models involve inducing hepatotoxicity with substances such as galactosamine [222,223] and acetaminophen [224,225], and secondly, surgically induced AHF models involve either rendering the animal anhepatic [5,226] or devascularizing the liver by means of temporary [227] or permanent [228,229] hepatic artery ligation with portocaval anastomosis (PCA).

In this study a surgical ischemic model of AHF was investigated for the following reasons: First, total hepatectomy models lack the *in vivo* biochemical effects of a failing liver while devascularization models do not. Second, the total devascularization model used in this study is irreversible, without the potential for liver regeneration as may be present in certain toxic models, and culminates in the animal's demise. Having said this, surgical models are prone to instability and require the development of considerable surgical skill prior to standardization [230]. Several different species have been used in the development of these models; however, there is considerable difficulty involved in scaling a BALSS from a small animal to a large animal [220]. Thus, the porcine model is often used as these animals are available in large numbers and are metabolically and physiologically similar to humans [220,230]. While the model described in this study is not unique, the methods that have been used in standardizing it are.

The statistical analysis of the acquired data in this study, together with clinical experience, was used to determine trends in clinical change and to define model standardization criteria. The purpose of the criteria is to enable the exclusion of animals that present with systemic or biochemical characteristics other than that of AHF as defined by the findings of this study. The expectations were that several prognostic factors would simultaneously determine survival.

Historically, the criteria of Hickman and Terblanche (1991) [231], have been used to establish the characteristics that an ideal model of AHF should satisfy, including reversibility, reproducibility, death by liver failure, sufficient duration for clinical intervention, a large animal model and minimal risk to personnel. Although these criteria have helped direct the development of animal models in the recent past, they are not specific in terms of defining clinical or biochemical characteristics of AHF in any model. No prior studies have attempted to do this. In the human scenario the King's college criteria were defined to provide an estimation of prognosis in AHF, and thereby to aid decision making in terms of potential transplantation. The multivariate analysis of large quantities of hospital patient statistical data was used to establish these criteria [52,53]. No similar criteria exist for animal models.

The availability of specific criteria for each animal model of AHF is highly desirable since these will aid in the establishment of more reliable prognostic indices than have previously been available. Since animal AHF models are inherently complex, invariably use small groups, and are predisposed to inter-individual variation, the establishment of such criteria will also be useful for establishing uniform control and treatments arms in the evaluation of supportive therapies. Thus, more accurate comparisons will result and spurious conclusions may be prevented regarding benefits that occurred by chance. Additionally, clinical interventions are likely to improve and expenses may be limited through the earlier exclusion of non-ideal subjects.

5.2.2 Materials and methods

Study protocol

Experiments were conducted on 15 female Landrace pigs with a mean body weight of 29.2 kg. Each experiment started at a baseline preceding surgery, with T = 0

immediately following surgery and continued until each animal expired. Systemic measurements were made continuously (electroencephalogram (EEG), electrocardiogram (ECG) and arterial blood pressure (ABP)). Arterial blood-gas (ABG) and electrolyte analysis was performed hourly, while metabolic/biochemical sampling was performed every four hours using mixed venous blood, for the duration of each experiment.

Animal preparation, anaesthesia and catheter placement

Acclimated pathogen-free pigs living under environmentally controlled conditions were used in all experiments. These were anesthetized during the surgeries and kept sedated subsequently. Ventilation and volumetric management was as for human ICU patients. Carotid, jugular and urinary catheters were inserted for monitoring. For a complete description of these protocols refer to Nieuwoudt *et al* [213] and Appendix B.2.

Liver devascularization

A midline xyphopubic laparotomy was performed (Diathermy/Coagulator 2000, Electromedical systems). The portal vein was isolated and cleared from the bifurcation in the porta hepatus to the splenic vein and all other connecting veins were ligated and divided. All connecting ligaments and peritoneal attachments were transected. The lesser omentum was opened. The intestines were left inside the animal's abdomen. All structures in the hepatoduodenal ligament except the portal vein were ligated with number 2/0 Silk and divided. The hepatic artery and accessory branches to the liver were also ligated and divided. The infra-hepatic inferior vena cava (IVC) was isolated and cleared to the level of the renal veins. The infra hepatic supra renal IVC was then completely clamped with 2 vascular exclusion clamps $\pm$ 40 mm apart and a $\pm$ 15 mm by $\pm$ 7.5 mm window was made in a lengthwise direction by excising part of the wall of the IVC. The portal vein was cross-clamped with two vascular clamps $\pm$ 40 mm apart then transected. An end-to-side anastomosis of the extra-hepatic portal vein onto the IVC was performed and the period of portal occlusion (Ischemic_time] was recorded. A bolus dose of heparin (3-5 units/kg) was infused with 1000 ml Ringers lactate to prevent clotting at the anastomotic site. The perfusion of the intestinal organs was evaluated by observing capillary filling. A return to pink was regarded as normal. In the event of splanchnic congestion the pig was sacrificed. The abdomen was flushed with 1L of warmed Ringers lactate. The

linea alba was sutured with a simple continuous number 0 nylon. The skin and subcutaneous tissue was closed in one layer with number 2/0 nylon in a simple continuous suture pattern.

Note: In this study the data of animals that had only undergone a laparotomy with subsequent sedation is not presented. Although these 'sham-operated controls' have been claimed as a necessity for the establishment of surgical models of AHF [232,233], the animals in question demonstrated no significant alterations in the systemic or biochemical variables of interest. This is in apparent agreement with the findings of similar studies [227,229]. Thus, this data does not contribute to the definition of standardization criteria. It is also useful to bear in mind that the intensive care regimen aimed at actively maintaining cardiovascular and hemodynamic stability. This study was viewed as the standardization of surgical control data prior to the evaluation of a BALSS.

Intensive care and clinical measurements

Following surgery the animal was transferred to an intensive care unit (ICU) where continuous ventilation, sedation and hydrodynamic stability was maintained until the cessation of cardiac function, which was defined as the point of death in this study. Systemic and biochemical indices (table 5.2.1) were also measured for the total duration as described in Appendix B2.

Post Mortem

After termination complete necropsies were performed to establish the positioning of the catheters, the patency and integrity of the PCA as well as the macroscopic and histopathological changes induced by the procedure. Aerobic bacterial cultures were performed on the intestines and a wide range of other organs.

Statistical analyses

Microsoft Excel was used for general processing while Statistix 8 was used for data analysis. All values are presented as the mean $\pm$ the standard deviation. Linear trends were fitted to each data set, revealing a rate of increase or decrease in that variable. For homeostatic systemic variables, a mean value (average) was calculated. Mean and standard deviations were also calculated for all rates or averages. Since the purpose was to define standardization criteria derived from statistical trends and clinical

experience, only the analysed data is presented. Extensive raw data from the ischemic model has previously been reported [227,229] and for this reason only the absolute and homeostatic mean values and rates of change are provided.

Non-parametric (distribution-free) Spearman correlation coefficients (appropriate for small data sets) were calculated for each variable's rate of change or static mean with the animal's duration of survival. An average coefficient was then calculated from these, with a perfect correlation showing a magnitude of one. The quality of the linear fit, in the form of a coefficient of regression (R^2), is not presented below. These values are the numerical square of the (parametric) Pearson correlation coefficient, which bears some relation to the Spearman correlation coefficient. In general, Spearman correlations return more conservative values than those of Pearson. Paired t-tests ($p<0.05$) were calculated to indicate the significance of change of each variable from the mean T = 0 value until termination.

5.2.3 Results

Table 5.2.1 provides an explanation of the systemic and biochemical variables and their units, while table 5.2.2 presents absolute values, mean rates of change and significance of change. In the surgical period ($T<0$), systemic variables that were measurable *on-line* were correlated with the duration of survival (figure 5.2.1). These variables were useful at this time for three reasons: 1. the initial model was healthy, 2. Systemic indices change rapidly and 3. Only those animals that were stable were able to progress beyond the surgery. Thus, the experiment could potentially be terminated at this point.

Table 5.2.1 Measured variables

Variable name/s	Description	Units
Survival	survival duration from T=0	hours
Mass	animal body weight at baseline	kg
Ischemic_time	portal vein clamp duration	minutes
Bl.loss_Tot	total blood loss in experiment	ml
Urine, _Tot	volume of urine in experiment	ml
MAP, _pre, _isch, _post, r	mean arterial pressure	mmHg
Pulse, _pre, _isch, _post	pulse	beats/min
Temp, _post	temperature	^{o}C
pH, _I, ave_	pH	
pO_2, ave_, r	partial pressure of O_2.	mmHg
pCO_2, ave_, r	partial pressure of CO_2.	mmHg
HCO_3act, ave_	[activated bicarbonate]	mmol/l
Glu, ave_	[glucose]	mmol/l
EEG_Ff_a/d, T=0, r EEG_Fc_a/d, T=0, r	ratio of mean alpha to delta frequencies in frontal or central brain regions	
EEG_Pf_a/d, T=0, r EEG_Pc_a/d, T=0, r	ratio of relative power of alpha to delta spectra in frontal or central brain regions	
Lactate, r	[lactate]	mmol/l
Pyruvate, ave_, r	[pyruvate]	mmol/l
Lac/Pyr, r	lactate to pyruvate ratio	
Ammonia, r	[ammonia]	µmol/l
BilirubinTOT, r	[total Bilirubin]	µmol/l
Glutamine, r	[glutamine] in plasma	µmol/l
BcAA, r	[branch-chain amino acid]	µmol/l
AroAA, r	[aromatic amino acid]	µmol/l
BcAA/AroAA, r	ratio of above	
Total AA, r	[total amino acid]	µmol/l
LD, r	[lactate dehydrogenase]	IU/l
ALT, r	[alanine aminotransferase]	IU/l
AST, r	[aspartate aminotransferase]	IU/l
ALP, r	[alkaline phosphatase]	IU/l
GGT	[gamma glutamyl transferase]	IU/l
Na+, r	[sodium ion]	mmol/l
K+, r	[potassium ion]	mmol/l
Creatinine, r	[creatinine]	µmol/l
Urea, r	[urea]	mmol/l
Fluids_Tot	total volume of fluids provided IV	ml
Hb, r	[hemoglobin]	g/dl
Hkt, r	hematokrit	%
PT, r	prothrombin time	sec
APTT, r	activated thromboplastin time	sec
D-dimers	[D-dimer]	µg/l
Fibrinogen, r	[fibrinogen]	g/l
PLT, r	platelet count	10^9/l
WBC, r	white blood cell count	10^9/l
Factor II, r	percentage of normal factor II	%
Factor V	percentage of nomal factor V	%
Factor VII, r	percentage of normal factor VII	%
Factor IX	percentage of normal factor IX	%
Factor X, r	percentage of normal factor X	%
AntiThrombin, r	percentage of normal antithrombin	%

Notes: 1. Variables with suffices and prefixes: ***_pre*** = baseline, e.g. MAP_pre. ***_isch*** = during ischemic time in surgery, e.g. MAP_isch. ***_post*** = value at T=0, e.g. MAP_post. ***r*** = rate of change, e.g. rAmmonia. ***ave_*** = static mean, e.g. ave_Pulse. ***_I*** = initial value, e.g. K^+_I. ***_Tot*** = total, e.g. Bl loss_Tot. ***T=0*** = value mmediately after surgery.2. Square brackets [ ] indicate the concentration of the enclosed variable in blood.

Table 5.2.2 Values of measured variables

Variable name	Initial Values			*Terminal value*	Mean value (ave_)	Mean rate of change/hour (r)	Significance (p < 0.05)
	Baseline	Ischemic time	T=0				
Survival			17.1 ± 8.3	20.5 ±5.1			
Mass [weight]	29.2 ±6.2						
Ischemic time		18.6 ±8.2					
Bl.loss_Tot				174.4 ±243.2			
Pulse	83.8 ±16.1	160.3 ±29.3	139.2±34.3	128.5±48.1	117.7 ±23.1		0.839
MAP	83.8 ±15.9	43.5 ±14.0	72.4±17.5	45.18±11.66		-1.83 ±0.62	0.000
Urine			102.7 ±95.1	29.2±52.5		-7.86±6.65	0.003
Urine_Tot				927.3 ±533.8			
Fluids			893.58±714.9	627.08±384.36			
Fluids_Tot				3439.7±1136.5			
EEG_Ff_a/d	5.07±0.71		4.91±1.08	6.53±1.84		*-0.13±0.27	0.004
EEG_Fc_a/d	4.68±0.23		4.69±0.70	5.28±0.81		*-0.03±0.16	0.035
EEG_Pf_a/d	1.05±0.68		1.24±1.26	0.74±0.71		*-0.05±0.03	0.439
EEG_Pc_a/d	0.60±0.26		0.74±0.74	2.65±3.95		*-0.06±0.05	0.276
Hb			9.33±1.71	6.33±1.43		-0.18±0.11	0.001
Hkt			32.85±8.01	20.10±5.79		-0.70±0.32	0.002
Temp			35.6±0.5	37.61±1.3	37.41±1.3	0.09±0.06	0.000
pH			7.31±0.10	7.40±0.15	7.41±0.07	nd	0.160
pO_2			138.6±38.3	92.3±26.8	115.6±20.4	-2.1±2.1	0.012
pCO_2			61.1±21.7	47.7±17.9	47.6±5.2	-0.9±0.9	0.033
HCO_3act			27.6±3.6	23.4±6.0	26.9±3.1	nd	0.064
Glucose			8.4±5.5	6.4±4.0	7.2±1.8	nd	0.295
Lactate			1.95±1.48	6.61±6.33		0.21±0.53	0.024
Pyruvate			0.13±0.05	0.28±0.19	0.22±0.09	0.005±0.009	0.027
Lac/Pyr			16.36±11.31	18.30±5.63	16.15±5.04	0.04±0.3	0.056
Ammonia			86.6±41.8	1402.7±506.8		64.2±31.9	0.000
BilirubinTOT			4.53±1.86	43.25±43.25		1.76±1.61	0.009
Glutamine			152.1±34.4	314.5±122.3		9.03±5.3	0.000
BcAA			381.2±62.2	246.6±45.8		-5.80±4.0	0.000
AroAA			111.2±26.2	182.8±37.9		3.86±2.0	0.000
BcAA/AroAA			3.58±1.05	1.37±0.25		-0.10+0.06	0.000
Total AA			2344.0±430.2	2967.3±815.3		31.4±48.2	0.064
LD			330.0±163.3	4890.4±5286.3		281.0±273.5	0.017
ALP			107.3±44.8	544.7±276.4		24.6±12.5	0.000
ALT			39.7±16.6	281.5±210.6		14.07±11.77	0.002
AST			64.4±67.2	4821.6±4593.8		329.9±262.6	0.007
GGT			18.9±6.8	23.67±11.2		nd	0.031
Na+			138.07±6.03	133.81±7.63		-0.28+0.40	0.015
K+			4.09±0.52	6.59±2.31		0.13±0.13	0.003
Creatinine			86.92±31.45	237.78±106.66		7.99±7.21	0.000
Urea			3.03±1.16	2.23±0.99		-0.05±0.07	0.025
PT			10.28±0.75	44.21±43.81		0.88±0.47	0.049
APTT			79.90±57.86	93.87±61.39		3.04±2.23	0.140
D-dimers			65.22±20.7	52.2±5.0		nd	0.146
Fibrinogen			2.47±0.65	0.55±0.36		-0.11±0.03	0.002
PLT			302.00±207.25	168.25±65.36		-14.05±7.23	0.215
WBC			7.72±5.79	24.63±8.89		0.46±0.19	0.119
Factor II			51.65±11.31	15.78±4.09		-1.75±0.60	0.000
Factor V			196±0	171.61±50.5		nd	0.161
Factor VII			57.64±13.47	12.28±6.18		-2.19±0.75	0.000
Factor IX			254.8±0	169.96±62.4		nd	0.002
Factor X			74.59±24.67	6.10±3.16		-3.19±1.17	0.000
AntiThrombin			91.43±14.48	44.35±12.50		-2.19±0.82	0.000

Notes. 1. * Trends calculated toward the lowest value, 2-4 hours prior to death. 2. nd = not discernable. 3. Baseline and T=0 values were taken to be equivalent for slow changing biochemical variables. 4. If the (r) value was (+) the variable increased over time. If (–) then the variable decreased over time.

The sign of the correlations indicates whether survival is benefited or disadvantaged by the absolute value of each variable. For example, the coefficient of Ischemic_time with survival is negative, thus, as the portal clamping time increases, survival decreases. A multivariate picture of survival was revealed. Variables that strongly correlated with survival included the pulse rate during portal occlusion (Pulse_ischemic = –0.65) and the mean arterial pressure following surgery (MAP_post = +0.48). Thus, the maintenance of cardiovascular and hemodynamic stability through fluid and inotropic provision was important in terms of survival. The duration of portal clamping prior to anastomosis (Ischemic_time = -0.31) was important presumably as a result of splanchnic congestion. Since only successful experiments were included, it was felt that the importance of this variable was underestimated in the results. The intactness of the anastomosis was of importance in that it determined the amount of blood lost, as was subsequently measured (Blood loss_Tot = -0.37). Body weight was found to bear some relation to survival possibly due to greater hemodynamic or thermal sensitivity in smaller pigs and an increase in the requisite surgical skill. However, this result may be misleading in that unsuccessful experiments were eliminated. Initially, the finding was that there was some difficulty in maintaining body temperature. Thus, the abdominal organs were not subsequently exposed during surgery. The temperature value provided at T=0 (table 4.2) is reflective of that immediately after surgery, rather than the subsequent thermal baseline measured following stabilization in the ICU.

The ratio of the frequency and relative power of the alpha to delta spectra of the EEG measurements in the frontal and central regions of the brain during surgery did indicate some correlation with survival (EEG_Pf_ad_T0 = +0.62), (EEG_Ff_ad_T0 = –0.41). However, since isoflurane and pentobarbitone were used as anaesthetics in this study it was assumed that a degree of suppression of frontal activity had occurred. Electrodes placed in the temporal and occipital regions were also prone to artefacts (results excluded) presumably due to animal positioning. Using the exclusion criteria (table 4.2.3) the data from three animals was excluded after the surgical period. The criteria that were violated included a case of excessive blood loss (Blood loss_Tot), two of excessively long ischemic times and in all three, survivals of less than 6 hours respectively.

Thus, in the period following surgery (T>0), the animal data that was used for analysis reduced from 15 to 12. In this period correlating absolute values with survival became impossible due to the extended duration of the experiments. The rates of change in the predominantly *off-line* biochemical indices then improved in terms of prognostic value (figure 4.2.2). That is, the sign and magnitude of each coefficient indicates the impact of an increasing or decreasing trend in that variable on the duration of survival. For example, the higher the rate at which ammonia accumulated (rAmmonia), the shorter survival. Significant correlations included those of accumulating plasma branch chain amino acids (rBcAA= +0.89), ammonia (rAmmonia = -0.77), Total urinary excretion (Urine_tot = +0.80), mean blood pH (ave_pH = +0.65), the rate of change of body temperature (rTemp = -0.62) and the losses of Hemoglobin (rHb = + 0.60) and Hematocrit (Hkt = + 0.54). In other variables of interest in AHF lower correlations were noted: Prothrombin time (rPT = +0.30), potassium (rK^+= -0.49), the liver enzymes (rALP = -0.28, rAST = -0.22, rLD = -0.21), total bilirubin (rBilirubinTot = +0.01), the clotting factors (Factors X = +0.35, VII = +0.34, II = +0.21), creatinine (rCreat = +0.46) and lactate (rLactate = +0.22). All of these variables indicated significant changes over the duration of the experiments (table 4.2). Each variable indicates a different aspect of AHF, thus, all were considered useful. There were also significant correlations between survival and variables that may be monitored *on-line*. Thus, prognosis may potentially be evaluated in *real-time* as opposed to after-the-fact. It was apparent in the trends that there was considerable inter-individual variation and this validated the need for the establishment of standardization criteria (table 5.2.3).

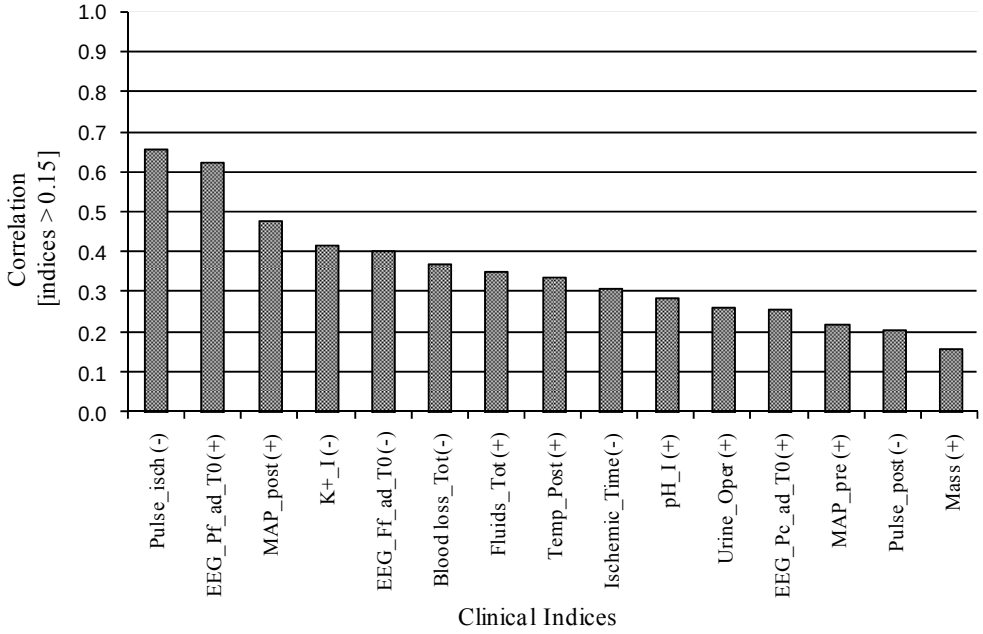

Figure 5.2.1 Magnitude of correlations of absolute values of systemic indices during surgery [T≤0] with duration of survival, N=15. Sign of correlation follows variable name, e.g. as ischemic pulse increases, survival decreases. Prognosis was multivariate

Post Mortem examinations:

A variable volume (60-200 ml) of serosanguinous abdominal effusion was present in most animals. In each case the PCA was found to be intact and patent with suspected complete ischemic necrosis of the liver extending from the edges and diaphragmatic surfaces to the hilus. There were smaller amounts of similar effusions within the thoracic cavity as well as a moderate to severe congestion and oedema in the lungs. The spleen, kidneys, and mesenteric lymph nodes showed variable congestion, while severe diffuse congestion and haemorrhage was observed in the small intestines in 7 of 12 animals. Histopathology revealed moderate to severe diffuse ischemic necrosis of hepatocytes characterised by cytoplasmic eosinophilia and nuclear pycnosis as well as nuclear dissolution in the severely affected foci. There was marked congestion and nephrosis in the kidneys with moderate to severe degeneration and necrosis of tubular epithelial cells. These findings are compatible with a severe functional renal failure of extended duration. No indications of DIC were found. Histopathological changes in the brain were characterised by moderate to severe congestion and oedema. Bacteriology: No unusual (significant) pathogens could be isolated from any of the cases.

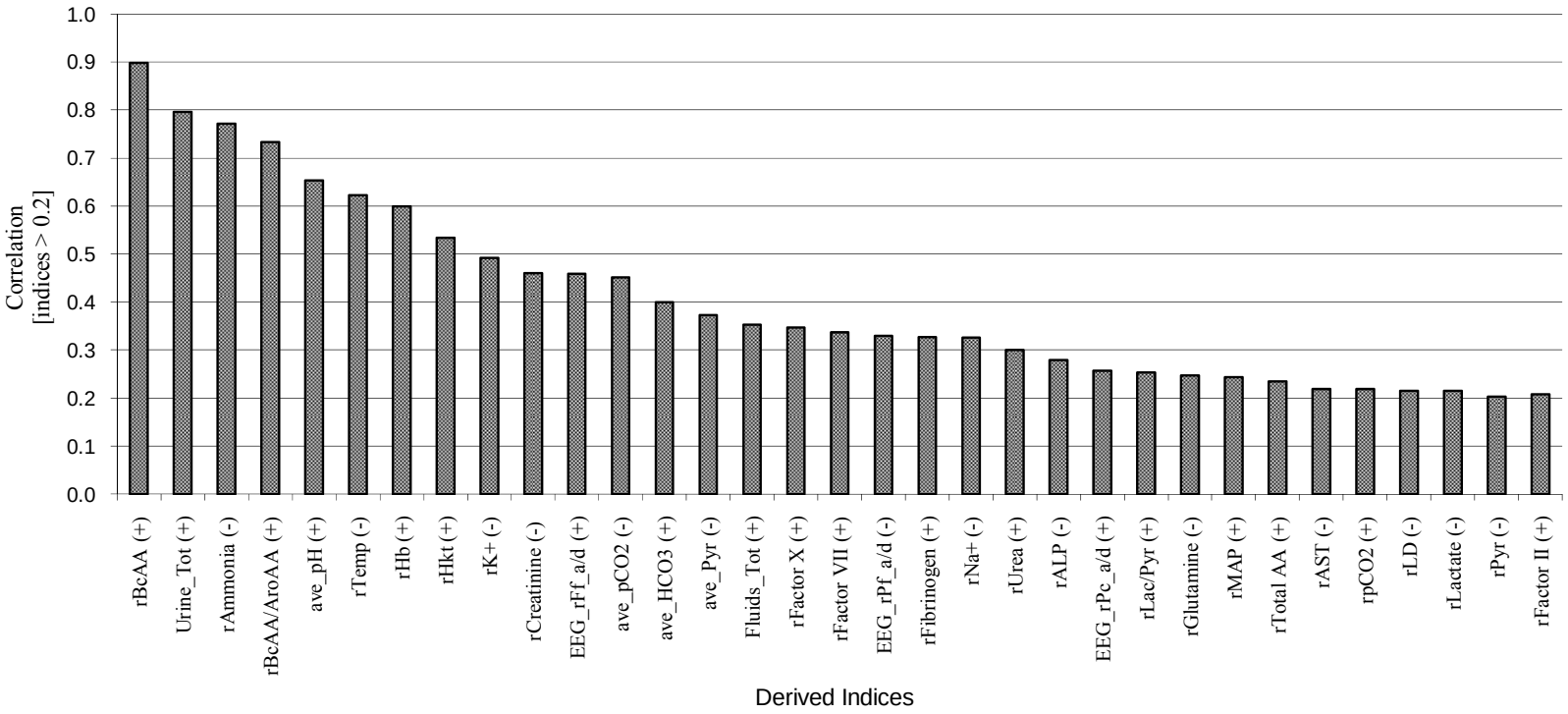

Figure 5.2.2 Magnitude of correlations of biochemical trends following surgery (T>0) with duration of animal survival, N=12. Sign of correlation follows variable name, e.g. As rAmmonia increases, survival decreases. A multivariate prognosis is apparent.

5.2.4 Discussion

Animal models that resemble the entire clinical syndrome of AHF in humans have not been successfully developed as yet [220]. Surgical or toxic animal models are often chosen to evaluate the efficacy of BALSS. Historically, two of the criteria of Hickman and Terblanche (1991) [231] that have proven the most difficult to meet include reversibility and reproducibility. Irreversible surgical models represent an attractive model for BALSS testing owing to the fact that they always end in death. However, AHF is intrinsically complex and the evaluation of support systems often relies on small sample sizes in experimental treatment or control arms. Thus, conclusively evaluating the benefit of the treatment may be difficult. This study describes systemic and biochemical parameters that may be used to define outcomes for an ischemic surgical model of AHF. These parameters facilitate the early detection and exclusion of compromised animals, whilst also allowing the identification of animals demonstrating characteristics predictive of longer survival, prior to BALSS connection.

The following clinical pattern was reproducibly observed in the above model: There was an initial hyperdynamic circulation followed by progressive anuria associated with rising serum creatinine values. EEG tracings revealed an initial slow decline in the ratio of alpha to delta activity, especially in the frontal part of the brain, succeeded by excitation towards the end. The increase in EEG activity was associated with muscular twitching, rigidity and finally gasping and convulsions. Hyperammonemia and lactic acidosis developed within 8 hours of the surgery. Clotting factors II, VII and X, antithrombin, prothrombin time and fibrinogen all declined significantly. On the other hand, the liver enzymes AST, ALP, ALT and LD, bilirubin and potassium all increased significantly. Finally, there was severe cardiovascular decompensation leading to hypotension, refractory shock and cardio-respiratory collapse. The clinical progress was multivariate, with renal failure, hyperammonemia and cardiovascular collapse all contributing to eventual death in these animals.

Since prognosis in this model was found to be dependent on surgical skill and since the disease etiology was complex, it was not desirable to further complicate the clinical

progress. For example, although routinely performed in human AHF treatment, intracranial pressure (ICP) was not monitored as complications have previously been recorded [234]. Similarly, hepatic encephalopathy was not treated with mannitol since it was desirable to gather data in a pure manner. Future studies may include these interventions.

In table 5.2.3 is summarized the systemic indices that are representative of clinical interventions during the surgical interval ($T \leq 0$) and in the blood biochemistry following that ($T > 0$). When these indices were present the animals demonstrated impaired survival. Important criteria that predicted survival during surgery ($T \leq 0$) were, amongst others, the ischemic clamping time (criterion 1), the presence of tachycardia (criterion 2), a loss of systolic blood pressure and the need for inotropic support. Fluid administration prior to portal clamping was shown to improve survival. Thus, by ensuring that the systemic indices reflected an adequate volume status, it was possible to avoid subsequent non-hepatic complications.

In the period following surgery ($T > 0$) the reasoning behind the criteria was as follows: Consensus indicates that AHF is unlikely to present in less than 6 hours following the surgical insult (criterion 8). The mean duration of survival in this study was 20.5 hours, which is in keeping with that observed in similar studies [227,229]. It seems reasonable to define the onset of AHF in terms of minimal or maximal limits in biochemical variables observed at 8 hours following the surgery (criteria 9-17). Animals failing to demonstrate these values (for whatever reason) can thereby be excluded. Fischer's ratio (criterion 9) and a raised ammonia concentration (criterion 10) confirm the onset of hepatic encephalopathy with hyperammonemia. The loss of clotting factors and the increased prothrombin time confirm coagulopathic effects (criteria 11 and 12) while the raised liver enzyme concentrations confirm increasing cellular membrane permeability and thus progressive hepatic necrosis (criterion 13). Loss of hepatic function is confirmed by an increased bilirubin concentration (criterion 14) while and renal failure by an increased creatinine concentration (criterion 15). Since the disease etiology was multifactorial it was felt that the relative importance of several biochemical indicators required definition in the criteria.

Table 5.2.3 Standardization criteria for a porcine surgical (ischemic) model of AHF

Interval	Limit	Criteria
During surgery $T \leq 0$	1. Ischemic clamping time > 15 mins. 2. Pulse > 200 bts/min during ischemic time. 3. Systolic Press. < 40 mmHg during ischemic time. 4. Metabolic acidosis pH < 7.2 following surgery. 5. If there was a small initial urinary volume [< 1 ml/kg body mass]. Alternately, total < 50 ml during the surgery. 6. Temperature < 35.0 ^{o}C immediately following surgery. 7. Total blood loss > 200 ml during surgery.	if affirmative to both 1 and 2, terminate after surgery. if affirmative to 1 or 2 and any two of 3-7, terminate after surgery.
After surgery $T > 0$	8. Duration of survival following surgery < 6 hours 9. Ratio BcAA / AroAA > 1.6 10. [Ammonia] < 500 µmol/L 11. Clotting factors:[Factor X] > 25 % [Factor VII] > 28 % [Factor II] > 30 % 12. PT < 15 secs 13. Liver Enzymes:[ALP] < 260 IU/L [AST] < 800 IU/L [LD] < 800 IU/L 14. [Total Bilirubin] < 12 µmol/L 15. [Creatinine] < 130 µmol/L 16. [Potassium] < 4.4 mmol/L 17. [Lactate] < 3.0 mmol/L	if affirmative to 8, exclude data. if affirmative to both 9 and 10, exclude data. if affirmative to either 9 or 10 and any one or more of 11-17, exclude data.

Notes:
1.T = 0 starts at the completion of surgery.
2. Criteria 9-17 are presented as limit values at 8 hours following the surgery, that is, the particular biochemical variable must be above or below that value at that time. These values are based on the mean values of the particular variables measured at 8 hours, and are in apparent agreement with similar studies [228,229].

Valuable procedural information was revealed by the data analysis in this study. For example, both haemoglobin and hematocrit declined significantly and this correlated

strongly with survival. Thus, it is reasonable to assume that the accumulated effects of loss of blood due to ABG and biochemical sampling, bleeding at surgical wounds and loss of hematocrit through continuous fluid provision, would contribute to the eventual hemodynamic instability and cardiovascular collapse that was observed. The data analysis also identified the variables that were of interest and allowed more selective sampling, improving prognosis and experimental reproducibility. Of interest was the high correlation of the rate of decline of branched chain amino acids (and by implication the branch chain to aromatic ratio), along with the rise in ammonia concentration, with the duration of survival. This finding serves to confirm the validity of Fischer's ratio as an indicator of HE [52,70]. This is the molar ratio of plasma concentrations of leucine, isoleucine and valine as numerator and tyrosine, phenylalanine and tryptophan as denominator. Changes in this ratio correlate with alterations in brain amino acid metabolism aided by an excess of plasma nitrogen in the form of ammonia. The synthesis of brain neurotransmitters is then affected, leading to HE. Alterations in this ratio also serve to confirm the value of providing antibiotics and fasting the animals prior to the surgery. Gut intestinal flora and protein content aid in the over-production of plasma nitrogen, thereby exacerbating HE and complicating the model of AHF.

Most of the biochemical indices are not measurable *on-line*, thus, their utility lies in confirming AHF after-the-fact. It is important to bear in mind that the initial model was healthy and that the biochemical indices started from a normal value. This was illustrated by clotting factor V; it started from a normal value and only declined at the end of the study period. Thus, these experiments were likely of insufficient duration to demonstrate large changes in this factor. In effect, the above criteria are the converse of the King's college criteria. The latter criteria are exercised on human patients already presenting with AHF [52,53] in which clotting factor V would have diminished *a priori*. In this study the criteria are designed to exclude experiments wherein the prognosis is insufficiently bleak, or due to factors other than AHF. Thus, while clotting factor V is of prognostic value in the human scenario, it was not useful in this model. Additionally, although our finding was that the mean arterial pH during an experiment displayed a strong positive correlation with survival, it was excluded as a criterion for two reasons: The animals were ventilated, thus, their pH could be controlled in this manner and due to

its measurement being complicated by the continuous provision of fluids. Since pH in AHF is strongly determined by the presence of lactate, it was felt that the latter would be a better index of AHF (criterion 17), despite the fact that lactate demonstrated a low correlation with survival.

In conclusion, the statistical analysis used in this study produced useful results in the following respects: Procedural information was generated that aided in the establishment of experimental reproducibility. Unnecessary determinations were identified, enabling the limiting of blood sampling, thereby improving prognosis and cutting laboratory costs. Specific standardization/exclusion criteria for discriminating between experiments that did or did not present a valid model of AHF, as defined by the settings of this study, were defined. These criteria are likely to allow more accurate comparisons between small treatment and control groups in evaluating supportive therapies for AHF. Variables were identified that may allow the evaluation of prognosis either in *real-time,* or after each experiment. Finally, the method of analysis and criteria presented in this study may be useful for standardizing AHF animal models prior to evaluating supportive therapies for AHF in the future.

5.3 Thoughts and recommendations

The above studies investigated two animal models to emulate respectively, the potential toxicity of PFC in a rat model of severe liver injury followed by recovery, and a surgically-induced ischemic liver failure model in pigs. These models were selected to determine if PFC would have a toxic effect and an impact on liver regeneration, and the efficacy in improving survival and biochemistry in severe irreversible ALF in a potential BALSS treatment scenario. The difficulty in these models obviously lies in the degree to which they emulate the intended disease process and this is exacerbated by the fact that controlled experiments require initially healthy animals. By comparison, human patients are often in the latter phase of a chronic liver failure or have abruptly progressed to ALF through, for example, drug or viral etiologies.

The rat model of liver regeneration following surgical injury was selected (effectively) by default. It was not possible to use a toxic model of ALF (e.g. CCl_4, galactoseamine, acetaminophen or lipopolysachiride) in that this would complicate the effects of an injected foreign substance, in this case PFC. Additionally, it was desirable to model the regenerative ability of an *in vivo* liver and to determine if the PFC would impact this or not. In both respects the study was successful, owing in all likelihood to the well-controlled study design.

The pig surgically-induced ischemic model of ALF was complex and haemodynamically unstable. Following the standardization experiments our group proceeded to evaluate the clinical efficacy of the BALSS in this model. It rapidly became apparent that the choice of animal ALF model may play a determining role in demonstrating the clinical efficacy of an experimental treatment system.

5.3.1 Clinical evaluation of the UP-CSIR BALSS using the ischemic model

In the course of the subsequent BALSS treatment experiments a large amount of knowledge was gained regarding the ischemic ALF model, the impact of the treatment on

the animal and consequently indicated BALSS-machine design alterations. Unfortunately, after embarking on this course the necessity of maintaining controlled circumstances precluded alterations in any part of the experimental design despite it becoming apparent that such changes may have resulted in improvements in the treatments. For these (and financial deadline) reasons the experiments were terminated prior to conducting the full planned number: 50 pigs were requested of which 29 were used.

As an example of the difficulties involved examine table 5.3.1 below. What was particularly noteworthy was the large range in duration of survival and the variety of causes of death, many of which were due to reasons unrelated to the treatments. Clearly, not all of the data was acceptable for inclusion into a subsequent analysis investigating BALSS clinical efficacy. A subset of the group was consequently selected. Table 5.3.2 below provides a summary and definitions of the variables used to make a comparison between the BALSS treatment group, the cell-free BALSS controls and the Surgical control group (the 'standardization data'). Table 5.3.3 provides the statistical results themselves.

Table 5.3.1 Record of large animal experiments

N	Comments	Survival (hrs)
1	Validation of surgical protocol changes. Ischemic time 13 mins.	36
2	Validation of surgical protocol changes. Ischemic time 12 mins.	32
3	BALSS treatment. Ischemic time 14 mins. High blood loss in surgery animal not connected to BALSS.	-
4	BALSS treatment. Ischemic time 11 mins. BALSS initiated 10hrs post operative. Pig died due to shock 45 mins after connection	11
5	BALSS treatment. Ischemic time 19 mins. Connected to BALSS 8 hours post operative.	20
6	BALSS treatment. Ischemic time 12 mins. Connected to BALSS 8 hours post operative.	29
7	BALSS treatment. Extended ischemic time, 30 mins.	26
8	BALSS treatment. Liver + multi organ failure	30
9	Exercise. Changes recommended by Prof R Hickman	terminated 6
10	BALSS treatment. Epidural omitted. Unsuccessful.	10
11	Haemodynamic instability. Not connected to BALSS	1
12	BALSS treatment. BALSS system unstable	24.5
13	Haemodynamic instability – Heart failure. Not connected to BALSS	9
14	Haemodynamic instability – Porcine circo virus detected– Not connected to BALSS	12
15	Control (-) cells. Haemodynamic instability after BALSS connection	16
16	Control (-) cells. Haemodynamic instability after BALSS connection	12
17	BALSS treatment. Haemodynamic instability after BALSS connection	10.2
18	Terminated prior to BALSS connection - extended ischemic time + blood loss	-
19	Haemodynamic instability after BALSS connection	8.5
20	Experiment without PFC in system. animal stable after connecting to BALSS	18.3
21	Control (-) cells. Haemodynamic instability after BALSS connection	7.5
22	Not connected to BALSS - heart failure	-
23	Control (-) PFC and (-) cells	18
24	Control (-) PFC and (-) cells. Tachicardia	6.5
25	Control (-) PFC and (-) cells Different perfusion circuits connected at different time intervals.	8.5
26	Control. BALSS not connected due to renal failure	-
27	Cardiac arrest in surgery	-
28	Machine failure (Air in plasma separator – machine disconnected after 8.5 h)	16.5
29	Machine failure due to excessive clotting (BALSS connected for 5h)	10

Table 5.3.2 Definition of variables and units

Variable	Definition	Units
Survival	duration from end of surgery to cardiac arrest	hours (hrs)
Treatment	duration of BALSS connection	hrs
Body weight	animal weight prior to surgery	kilogram (kg)
Ischemic time	duration of portal clamp	minutes (min)
Pulse_isch	mean pulse during ischemic time	beats/min
Pulse_post	mean pulse after release of portal clamp	beats/min
MAP_isch	mean arterial pressure during ischemic time	mmHg
MAP_post	mean arterial pressure after release of portal clamp	mmHg
Bl_loss	blood in peritoneal cavity following surgical procedure	milliliters (ml)
Urine_oper	urine volume measured following surgery	ml
pH_post	blood pH after surgery	
Temp_post	body temperature following surgery	degrees Celsius (^{o}C)
rMAP	rate of descent of mean arterial pressure for the duration of survival (i.e. in the ICU following surgery)	mmHg/hr
ave_pH	mean blood pH for the duration of survival	
ave_Lact	mean blood lactate concentration	mmol/l
rK+	rate of increase of blood potassium	mmol/l/hr
rHct	rate of descent of blood hematocrit	percentage (%)/hr
rAmmonia	rate of increase of blood ammonia concentration	µmol/l/hr
rBilirubin	rate of increase of blood total bilirubin concentration	µmol/l/hr
rCreatinine	rate of increase of blood creatinine concentration	µmol/l/hr
hrsFibrinogen > baseline	number of hours taken for fibrinogen to reach its lowest detectable limit (i.e < 0.5 g/l)	hrs and %
hrsPT < limit	number of hours taken for the prothrombin time to reach its detection limit (i.e > 150 seconds)	hrs and %
hrsClot II > baseline	number of hours taken for clotting factor II to reach its lowest detectable limit (i.e < 5 IU/dl)	hrs and %
hrsClot VII > baseline	number of hours taken for clotting factor VII to reach its lowest detectable limit (i.e < 5 IU/dl)	hrs and %
hrsClot X > baseline	number of hours taken for clotting factor X to reach its lowest detectable limit (i.e < 5 IU/dl)	hrs and %
hrsALP < max	number of hours taken for ALP to reach the maximum value for each animal	hrs and %
hrsALT < max	number of hours taken for ALT to reach the maximum value for each animal	hrs and %
hrsAST < max	number of hours taken for AST to reach the maximum value for each animal	hrs and %
hrsLD < max	number of hours taken for LD to reach the maximum value for each animal	hrs and %

Table 5.3.3 Comparison between BALSS treated and non-treated animals

Time period	Variable	†BALSS treatment group (E)	†Cell-free Control group (C)	∂Surgical control group (S)	#p value between E and C	#p value between E and S
Global	Number	6	3	12		
	Survival	23.5 ± 4.3	14.4 ± 3.5	20.5 ± 5.1	**0.018**	
	Treatment	13.8 ± 2.5	7.0 ± 4.6	none		
	Body weight	30.3 ± 3.2	29.7 ± 2.0	30.4 ± 5.1		
Surgery	Ischemic time	13.4 ± 3.2	11.3 ± 1.5	16.8 ± 4.1		**0.020**
	Pulse_isch	131.6 ± 28.5	149.7 ± 19.6	156.8 ± 29.9		
	Pulse_post	125.5 ± 16.3	120.7 ± 21.9	131.5 ± 30.0		
	MAP_isch	55.3 ± 4.1	56.5 ± 4.7	47.1 ± 11.8		
	MAP_post	99.9 ± 21.3	85.0 ± 8.0	78.3 ± 13.6		
	Bl_loss	96.0 ± 41.6	66.7 ± 28.9	96.3 ± 68.6		
	Urine_oper	402.2 ± 336.6	233.3 ± 231.8	122.9 ± 96.0		
	pH_post	7.40 ± 0.05	7.45 ± 0.03	7.32 ± 0.10		
	Temp_post	37.0 ± 1.4	35.8 ± 1.1	36.3 ± 1.6		
ICU	rMAP	-2.1 ± 1.1	-4.3 ± 2.4	-1.8 ± 0.6		
	ave_pH	7.35 ± 0.05	7.36 ± 0.04	7.41 ± 0.07		**0.020**
	ave_Lact	4.51 ± 2.33	4.85 ± 2.32	4.22 ± 3.25		
	rK+	0.11 ± 0.06	0.05 ± 0.04	0.13 ± 0.13		
	rHct	-0.73 ± 0.33	-1.08 ± 0.63	-0.70 ± 0.32		
	rAmmonia	85.8 ± 60.1	70.3 ± 35.4	64.1 ± 31.9		
	rBilirubin	0.55 ± 0.36	0.41 ± 0.43	1.76 ± 1.61		0.052
	rCreatinine	5.71 ± 3.59	6.80 ± 2.24	7.99 ± 7.21		
	hrsFibrinogen > baseline*	9.6 ± 3.3 41%	8.7 ± 2.5 60%	10.3 ± 4.5 50.2%		
	hrsPT < limit*	10.8 ± 2.7 46%	8.7 ± 2.5 60%	20.5 ± 5.1 100%	0.071	**0.0002**
	hrsClot II > baseline*	13.5 ± 5.7 57%	8.7 ± 2.5 60%	19.5 ± 4.7 95%		**0.035**
	hrsClot VII > baseline*	16.5 ± 3.0 70%	7.0 ± 1.0 49%	20.5 ± 5.1 100%	**0.029**	0.069
	hrsClot X > baseline*	10.5 ± 3.0 45%	7.0 ± 1.7 49%	20.5 ± 5.1 100%		**0.0005**
	hrsALP < max*	19.6 ± 6.4 83%	10.0 ± 6.9 69%	20.5 ± 5.1 100%	0.054	
	hrsALT < max*	19.6 ± 6.4 83%	8.0 ± 9.2 56%	20.5 ± 5.1 100%	0.054	
	hrsAST < max*	21.4 ± 3.1 91%	12.0 ± 6.0 83%	20.5 ± 5.1 100%		
	hrsLD < max*	18.8 ± 7.1 80%	12.0 ± 6.0 83%	20.5 ± 5.1 100%		

All values expressed as Mean ± standard deviation, and in all * as percentage of survival duration.
[#] Exact p values, only for significant differences (0.95 in **bold**, 0.90 in normal text), using the Wilcoxon Rank Sum test for non-parametric small populations.
[†] Only for those that survived the surgery (> 6 hrs) and were treated successfully for ≥ 3 hours starting > 6 hrs after the surgery.
[∂] Only for those that survived the surgery (> 6 hrs).

What was clear from the results was that, within the limited number of experiments, there was a significant difference ($p < 0.05$) in the duration of survival between the cell-free controls and the BALSS treated group. However, there was no significant difference in the duration of survival between the BALSS treatment group and the surgical control group, even though the treated group did survive for a longer period. The cell free controls also survived for a shorter period than the surgical group. This implied the following:

1. Adding cells to the bioreactor in the BALSS treatments did significantly improve survival.
2. Adding an extracorporeal circulation system to the surgical model hindered survival. This was to be expected if the haemodynamic instability that is intrinsic to this model of ALF is considered.
3. These results cannot be accepted as a conclusive demonstration of the efficacy of the BALSS mostly in view of the fact that more experiments are required.
4. The difference in the ischemic times reflected an improvement in the surgical technique over time. This was mirrored by differences in the Pulse and MAP after the procedures.
5. Although there was a significant difference in ave_pH between the BALSS and surgical control groups this was not due to lactate accumulation. It was likely as a result of non-lactic metabolic acidosis.
6. Since the rate at which ammonia and potassium accumulated similarly in the various groups, it indicated that an additional artificial clearance device should ideally be incorporated in the BALSS, assuming the use of an acutely toxic model.
7. Since the rate at which the MAP fell during the ICU period was insignificantly different between groups, it suggested that although the ALF model was associated with haemodynamic instability, it was not as a result of the BAL.
8. An impact of connecting the system can be seen in the various coagulation indices. Coagulation (as measured by increasing PT and coagulation factor losses) was increased in the treated groups when the period to coagulation failure was compared to the duration of survival (percentages in table 5.3.2). While these

figures are as expected, a re-evaluation in terms of coagulation of the mark II BALSS (as was used in these experiments) was indicated.

9. The difference between the liver enzymes in the BALSS and cell free groups was not a conclusive indication of differences between the interventions. Liver enzymes (ALP, ALT, AST, LD) are progressively released into the circulation by the ischemic damaged liver and are thus not impacted by the treatment. The differences highlighted the requirement of larger numbers of experiments.

In order to gain the maximum possible information, the unsuccessful experiments were also examined. Thus, a summary of *all* experiments are as follows:

A total of 29 experiments were performed. Of these, 8 were completely successful and 6 were used as controls, either of the surgical procedures (3) or of parts of the circulation system (3). Of the remaining 15, 8 were not connected to the BALSS owing to instability in the surgical model (4 died of cardiac arrest, 1 of renal failure, 1 of a detected circo virus, 1 of renal failure and 1 of instability as a result of the omission of the epidural in the anesthetic protocol). This result was taken as an indication of the many procedural and clinical difficulties associated with the employed model of ALF. 5 animals became haemodynamically unstable 30-45 minutes after connection to the BALSS and these survived a mean of 9.7 $\pm$ 1.4 hours. In the two remaining cases the circulation system failed due to complete coagulation, this resulted in survivals of 16.5 and 10 hours respectively. In these last two animals and in 3 of the 5 that became unstable following BALSS connection it was revealed that the epidural procedure had been omitted from the anesthetic protocol. Thus, the success rate for experiments was 8 out of a total of 15, i.e. 53 %, when only those animals that became unstable following connection or where the BALSS completely coagulated were taken to be failed experiments. This validated the stated clinical difficulty/instability of the ischemic surgical model and indicated the use of a more stable model in the future that will hopefully demonstrate greater differences between experimental and control groups.

As a consequence of the above:

1. Various improvements to the design of the mark II BALSS were planned, including: A simplification of the circulation system, an improvement of the operating methods, a minimization of coagulation effects in the system, the design of a new bioreactor (with better flow characteristics and more biocompatible substances) and the inclusion of an additional artificial toxin clearance device into the system.
2. Several coagulatory, immunological and cerebral variables, not measured in the ischemic experiments, were subsequently identified to have prognostic value in ALF. These new variables will require inclusion in any future experiments (and this issue is mentioned in greater detail in the mathematical modeling section 6.2 below).
3. Research into other animal models of ALF was indicated (see below). The above experiments demonstrated that the ischemic model is both highly acute and inherently toxic, potentially to the extent of 'clouding' any potential the benefit of the BALSS. The return to large animal clinical treatments would occur only once all of the above machine-design issues had been attended to.

5.3.2 Alternate animal models of ALF

Unfortunately, in that liver failure has remained the same, the possibilities for modeling it in animals have done so too. Thus, the issues identified in the second of the above two studies remains valid. Specifically, in evaluating a BALSS:

1. The porcine model should still be used as these animals are available in large quantities and are metabolically and physiologically similar to humans.
2. It is preferable to use surgical models as opposed to toxic ones due to inter-animal variations in response to the toxin/s.
3. Surgical models are prone to instability and require the development of considerable surgical skill prior to standardization.
4. Total hepatectomy models lack the *in vivo* biochemical effects of a failing liver while devascularization models do not.

However, having said the above, anhepatic models may be less *aggressive* than the ischemic model in that the rate of accumulation of endogenous toxins is less pronounced. That is, since there is no *in vivo* ischemic liver, accumulating ammonia and endotoxin in the blood are due only to *non-hepatic* causes. However, the pathophysiological course followed is then related to the absence of a liver, i.e. ammonia and endotoxins from the bowel may play a more important role in the outcomes. In a sense, the anhepatic is a 'control' for the ischemic: Subtracting the rates of accumulations, of the former from the latter, will indicate the contribution/extent of hepatic necrosis. For this reason, should anhepatic trials follow ischemic ones; the former acquired data will to some degree complement the latter. This is useful information in examining prognostic variables.

Other attractive aspects of the anhepatic model lie in its relative procedural simplicity and a decrease in the costs of the experiments. A considerable amount of prior studies have focused on standardizing the anhepatic model [5,220,221,226,230-238]. This, together with the historical experience gained by our group will hopefully function to decrease the experimental failure rate as previously demonstrated (table 5.3.1).

One variant of the anhepatic model seems particularly interesting: Engelbrecht *et al* (1999) [238] describe performing a side-to-side mesocaval shunt distal to the renal veins, followed by total hepatectomy with ligation of the portal vein, hepatic arteries and the bile duct. Their method was performed on rats and required the sacrifice of a donor animal to provide a section of vena cava for subsequent caval reconstruction. In a porcine model this may be performed using sterilizable tubing (e.g. silicon). The benefit of this surgical method lies in the absence of complete portal clamping and consequent splanchnic congestion. The duration of this clamping (ischemic time) was found to correlate strongly with survival in the ischemic model, most likely due to presumed endotoxin build up and then release from the splanchnic circulation.

Unlike in the ischemic model in which a donor animal is required for hepatocyte isolation procedures (to populate the BAL bioreactor), the liver of the anhepatic animal may autologously be used, decreasing the total cost of the experiments. On the other hand, assuming the inclusion of an artificial toxin clearance device into the BAL circulation

system, the experimental design must then control for the additional sub-group/s. Table 5.3.4 below provides an example:

Table 5.3.4 Control and experimental groups in animal trials

Group	Size (N)
Surgical control (no treatment)	5
System control (empty BR, no AL)	5
Experimental 1 (empty BR, with AL)	8
Experimental 2 (cells-BR, no AL)	8
Experimental 3 (cells-BR, with AL)	8
Unpredictables (assuming 30 % rejection rate)	12
TOTAL	46

Abbreviations: BR = bioreactor with or without cells.
AL = artificial toxin clearance system (e.g. dialysis and adsorption column)

5.3.3 Ammonia metabolism, measurement and reduction strategies

Accumulating blood ammonia levels in the ischemic surgical model were found to strongly correlate with the duration of survival of the animals. Additionally, while ammonia is clearly not the only variable of interest in ALF, there has been a growing consensus regarding its importance as an indicator of prognosis in human patients most likely owing to its causative role in hepatic encephalopathy (section 2.4.1, and returned to in section 6.2) [30-33,239-241]. There is consequently an incentive to find fast, convenient, accurate and inexpensive methods for its bedside detection and novel strategies for its reduction/minimization in the patient. A brief summary of ammonia metabolism is instructive,

1. Ammonia metabolism

Ammonia is produced mostly in the gut followed by the kidneys and muscle. The gut contribution is due to ammonia produced by protein catabolism and bacterial metabolism (i.e. from glycine and glutamine). The kidneys employ ammonia to buffer acids, i.e. ammonium is synthesized from glutamine in the proximal tubule then either released into the systemic circulation or used to facilitate proton excretion. Skeletal muscle produces ammonia during seizures or in intense exercise.

Ammonia degradation occurs primarily in the liver. Ammonia originating in the splanchnic circulation and in the muscle is metabolized to urea by means of the urea cycle. The enzymes participating in the urea cycle include carbamyl phosphate synthetase (CPS), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase and arginase. Arginine is a necessary raw material for the urea cycle. Owing to the healthy liver's efficiency in clearing ammonia, arterial and venous ammonia levels usually do not equate [31-36].

When the liver ceases effective functioning, the elimination of ammonia depends on the kidneys, muscle and brain. The kidneys decrease ammonia production and increase its urinary excretion. The muscle and brain metabolize ammonia to glutamine. Astrocytes normally support neurons with adenosine triphosphate (ATP), glutamine and cholesterol. The neuron metabolizes glutamine to glutamate which participates in neurotransmission by activating N-methyl-D-aspartate (NMDA) receptors. After release in the synapse glutamate is recycled to glutamine by the astrocyte. When ammonia levels rise acutely the astrocytes overproduce glutamine and this interrupts neurotransmission. Intracellular osmolytic changes also cause the astrocytes to swell and die [37].

Astrocytes release inflammatory cytokines (TNF-α, IL-1, IL-6 and interferon) and owing to ongoing oxidative and nitrosative stress additional astrocytes are lost due to apoptosis. In the remaining astrocytes ammonia inhibits alpha-ketoglutarate dehydrogenase and the depletion of carboxylic acids for glutamine synthesis causes the Kreb's cycle to shut down. ATP and reduced-nicotinamide adenine dinucleotide (NAD) production falls. As NADH levels rise in relation to NAD, the production of lactate from pyruvate is favoured (i.e. glycolysis). Thus, there is altered oxidative metabolism associated with mitochondrial dysfunction. With the loss of astrocytes, less receptors are available for glutamate and glutamate overload occurs resulting in seizures. Cerebral blood flow increases, cerebral autoregulation is lost and cerebral edema and intracranial hypertension develop [30-38].

2. Measurement

Historically the most common method of blood ammonia measurement has been routine enzymatic spectrophotometric laboratory approaches. However, this is problematic for a number of reasons. Firstly, the elapsed duration in awaiting results delays clinical decision making and this may be important in critically ill HE patients. Secondly, ammonia is unstable at room (and body) temperature. In the blood it exists in equilibrium between the ionic liquid ammonium (NH_4^+) and gaseous ammonia (NH_3) forms [31]. When blood samples are taken it is critical that they are frozen without delay in order to prevent the escape of the gas. Finally, the enzymatic kits used in the laboratory are exceedingly sensitive to atmospheric ammonia. Since detergents that are used to wash laboratory surfaces may contain ammonia, the blood results are at risk of spurious elevation [personal experience!].

Recently a portable device has become available, the PocketChem BA analyser (Arkay, Kyoto, Japan) which has demonstrated accuracy at least as high as that of the laboratory methods. However, blood samples should ideally be collected onto ice and analysed immediately. The sampling site is also important, levels in for example, the jugular vein or femoral artery are usually different due to the generation or uptake of ammonia by different tissues. Only arterial ammonia has been found to correlate with brain glutamine levels, the severity of HE and thus prognosis in ALF [32,241] and jugular levels were found to have a higher correlation with arterial ammonia than femoral levels [26].

3. Ammonia reduction strategies

In ALF interventions are usually aimed at treating brain edema, decreased cerebral metabolism, and raised cerebral blood flow and ICP rather than specifically the reduction of ammonia. However, ammonia is a critical part of this pathogenesis. Thus, current strategies, both conventional and not yet conclusively proven, are listed below in non-specific order (this is not intended as a review). The approaches fall into two groups [31,33]:

a. Biochemical strategies,

N-acetylcysteine infusion to improve cerebral edema and O_2 delivery and utilisation.
Mannitol to reduce cerebral edema.
Phenytoin to reduce cerebral edema and seizures.
Hypertonic saline to create a blood-brain osmotic (sodium) balance in opposition to that resulting from raised ammonia levels. This reduces the severity of ICP.
Indomethacin to prevent cerebral hyperemia and intracranial hypertension.
Propofol to prevent intracranial hypertension.
Dilantin or Phenobarbital to reduce seizures.
Lactulose to decrease ammonia.
L-ornithine-L-aspartate to enhance the muscular metabolism of ammonia.
Arginine administration to prevent protein catabolism.
Protein intake prevention to reduce ammonia production.
Sodium phenylacetate or benzoate to improve ammonia degradation through alternate metabolic paths.

b. Physical strategies, including,
Mild hypothermia to decrease the cerebral uptake of ammonia, to reduce CBF, to reduce the synthesis of lactate and to rectify alterations in brain osmolarity.
Blood purification/filtration approaches including bioartificial livers, high-volume plasmapheresis (e.g. continuous veno-venous or arterio-venous hemofiltration or hemodiafiltration) and albumin dialysis (MARS and Prometheus) to respectively provide hepatic synthesis/transformation functions, to remove water soluble and protein-bond toxins.

An appropriate combination of biochemical and physical approaches can successfully reduce blood ammonia levels to normal [33].

5.3.4 Which artificial toxin clearance system?

The complex tasks of regulation and synthesis have yet to adressed by the use of liver cells. For detoxification, e.g. the removal of bilirubin, bile acids and toxins, simple dialysis-like artificial detoxification technologies have been shown to be efficient [242]. As will become apparent in section 6.1 artificial toxin clearance devices have a greater capacity to remove blood-borne toxins than any current bioreactor/s. Since many different artificial systems have historically been developed (table 2.3) a difficulty lies in deciding which is most appropriate.

It should be stated that artificial liver support has been developed to a relatively advanced state and it is assumed that 'off-the-shelf' technology will be selected for inclusion into a bio-artificial liver system rather than development from scratch. The following discussion aims simply to identify trends in the literature and the factors that may affect the choice of technology.

Cell-free extracorporeal liver support technology can be divided into 'closed-loop' and 'open-loop' technologies. Closed-loop technology includes the molecular adsorbents recirculation system (MARS) and the Prometheus albumin dialysis system in which a minimal amount of exchanged treated plasma is discarded. Open-loop technology includes single-pass albumin dialysis and a variety of plasma exchange technologies in which large quantities of treated plasma are discarded. Both approaches have drawbacks including limited toxin removal rates, the non-selective removal of molecules (both *good* and *bad*) with open-loop systems, and limitations in perfusion time and thus total quantity removed with closed loop systems [243].

MARS works by an albumin diffusion gradient principle. The albumin is placed on the dialysate side of a high-flux membrane which maximizes the diffusive transport of albumin-bound toxins. The used dialysate is then regenerated in a secondary circuit by passage over adsorption cartridges (anion exchanger and activated charcoal) to remove the concentrated albumin-bound toxins and a low-flux dialyzer cartridge to remove water-dissolved toxins. The Prometheus (PROM) system works on the principle of

fractionated plasma separation and adsorption. A 100 kD cut-off polysulphone membrane allows partial filtration into a secondary circuit which then also cleans using sorbents, as above. Water-dissolved toxins are subsequently removed with a high-flux dialyser [244].

A clinical study comparing the above two systems demonstrated that the PROM system more efficiently removed blood borne toxins than MARS. Both systems caused increases in prothrombin time associated with loss of coagulation factors, however, both systems were well-tolerated and safe [245]. As yet, neither system has shown statistically significant benefits to patient survival in prospective randomized trials [246].

Single-pass albumin dialysis (SPAD) is a simple method of albumin dialysis using a standard renal dialysis machine and a high-flux hollow fiber hemodiafilter identical to that of the MARS system. On the outer side of the membrane (i.e. in respect of the patient's plasma) an albumin solution flows in the opposite direction and this is discarded after use. Continuous veno-venous or arterio-venous hemodiafiltration (CVVDHF or CAVDHF) also uses conventional dialysis equipment (e.g. a 35 kDa pulysulphone membrane) with a cross dialytic current of plasma, and potentially also the inclusion of ion-exchange and adsorption columns. The used dialysate is discarded after use [233]. In general, high-flow hemodialysis systems are superior to albumin dialysis (MARS) at removing (especially water soluble) toxins [247]. From a technical point of view this may suggest that CVVHDF is preferable for inclusion into a system with a biological component.

There are also selective plasma filtration technologies that have progressed through preclinical trials [248,249]. In these systems a high cutoff 100 kDa polysulphone membrane selectively filters toxins from mitogenic proteins in a continuous veno-venous configuration. The toxic ultrafiltrate is discarded followed by replacement with an equivalent volume of plasma. However, the authors believe this technology is superior to the albumin, sorbent and CVVHDF systems owing to the specificity of the toxin removal membrane. These systems are apparently also easy to use.

At present no particular system is ideal under all circumstances and developments in the technology are ongoing. The loss of coagulation factors (and beneficial mitogens) and the challenge of maintaining haemodynamic stability associated with an increase in the circulating extracorporeal volume is relevant to all systems. It would seem that the factors limiting the choice of system reduce to cost, ease of use, safety (haemodynamic stability) and obviously toxin clearance ability. Since CVVHDF and selective plasma filtration use conventional renal dialysis equipment and procedures, and are less costly, than for example MARS, it seems that at this point these may be the better choices. In terms of conducting animal trials a *well-known* and inexpensive control would also be preferable. It remains to be seen what will emerge as the best choice.