

Early myocardial injury is an integral component of experimental acute liver failure – a study in two porcine models

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Abstract

Introduction: There is accumulating clinical evidence that acute liver failure may be regularly associated with myocardial injury. To test this hypothesis in a standardized experimental setting, we used two porcine models of ALF.

Material and methods: In 14 domestic pigs ALF was induced by either a) surgical devascularization of the liver (DV group, $n = 7$), or b) partial (70-75%) hepatectomy and ischaemia/reperfusion of the liver remnant for 150 min (I/R group, $n = 7$). Four additional animals constituted the sham operation group. All animals were monitored for a 12-h period, at the end of which their hearts were harvested. Plasma troponin I (cTnI) and malondialdehyde (MDA) were measured before the operation (baseline) and at 6 h and 12 h postoperatively. The harvested hearts were histologically analysed, appointing a score from 0 (no injury) to 3 (maximum injury) to selected injury indicators.

Results: In the sham group, all cTnI measurements and total myocardial injury score were zero in all animals. In both ALF groups, plasma cTnI levels increased by the 6th and remained elevated up to the 12th postoperative hour ($p < 0.01$ vs. sham animals). Total myocardial injury score and total histological score revealed some extent of myocardial injury. The rise of MDA levels suggests an underlying oxidative mechanism.

Conclusions: Our study provides direct evidence of early myocardial injury in the setting of acute liver failure in pigs. The mechanism of injury remains to be elucidated.

Key words: hepatectomy, troponin I, portacaval shunt.

Introduction

Multiorgan failure is a major complication of acute liver failure (ALF). Although circulatory disturbances which accompany acute liver failure were reported early on [1], direct effects upon the myocardium have not been studied, except for sporadic references of paracetamol overdose cases

accompanied by cardiac injury [2-4]. Recently, the US Acute Liver Failure Study Group [5] showed that elevations of serum troponin I (cTnI) levels are common (74% overall) in patients with ALF of various aetiologies. In the same study, higher troponin levels were associated with more severe clinical features and poorer outcomes. Despite the suspected high frequency of cardiac injury in ALF, very few experimental studies have examined the impact of liver injury upon the myocardium, mostly in the context of ischaemia-reperfusion injury [6-11]. Prompted by the clinical observation of the US Acute Liver Failure Study Group, we designed the present study using two different porcine models of surgically-induced liver failure in order to test the hypothesis that early myocardial injury is an integral part of ALF.

Material and methods

This study was approved by the Research and Bioethics Committee of our institution. All animals used in this study received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals", published by the National Institutes of Health.

Fourteen female Landrace pigs of mean weight 29.3 kg (28-32 kg) were randomly assigned to two groups, the liver devascularization group (group DV, $n = 7$) and the liver ischaemia/reperfusion group (group I/R, $n = 7$). Four additional animals constituted the sham operation (SH) group. Each animal was brought to the research facility at least 7 days prior to surgery, was housed separately and given free access to food and water. Twelve h prior to surgery, feeding was discontinued and only free access to water was allowed.

Anaesthesia and postoperative care

Induction to anaesthesia was made with 4 mg/kg ketamine, 0.2 mg/kg midazolam and 0.5 mg atropine administered intramuscularly, 30 min before general anaesthesia. The animal was secured in a supine position and a 20 G catheter was placed in an ear vein. General anaesthesia was induced with iv thiopental sodium 5 mg/kg and ketamine 2 mg/kg. The trachea was intubated with a cuffed tube of internal diameter of 5.5-6.0 mm and mechanical ventilation was started using a volume ventilator (Sulla 808 V, Dräger, Lübeck, Germany) with a fraction of inspired oxygen (FiO_2) of 0.4, tidal volume of 10-12 ml/kg and respiratory rate of 10-15 breaths/min. A bolus dose of fentanyl 10-20 μ g/kg and vecuronium bromide 0.5 mg/kg was given intravenously. Then, a nasogastric tube was inserted, the right external jugular vein was exposed and a central vein catheter 6.5 Fr (G986, Arrow International, Reading, PA, USA) was inserted

to obtain central venous pressure measurements. The right femoral artery was cannulated with a 20 G catheter for arterial pressure measurement. Intracranial pressure (ICP) was monitored using a subdural pressure transducer (Neuromonitor basic kit, Johnson and Johnson, Raynham, MA). Temperature was kept stable by means of a heating pad. Heart rate, arterial blood pressure, end tidal CO_2 , pulse oximetry and temperature were monitored continuously. Anaesthesia during the surgical procedure and throughout the monitoring phase was maintained with continuous intravenous infusion of fentanyl 15-20 μ g/kg/h, ketamine 5-8 mg/kg/h and vecuronium bromide 0.5 mg/kg/h. No volatile anaesthetics or vasoactive drugs were given. Intraoperatively and during the monitoring phase, dextrose 5% 20-100 ml/h, normal saline 100-300 ml/h, colloids (Voluvent® 6%, Fresenius Kabi, Germany) 50-150 ml/h and Ringer's lactated 100-300 ml/h were given as needed in order to maintain normoglycaemia, normal electrolytic values and the central venous pressure between 4 mmHg and 9 mmHg.

After completion of 12 h of monitoring, the abdominal incision was reopened and the abdomen was explored for signs of surgical complications. Liver biopsies were also taken. The animals were then euthanised using *i.v.* infusion of propofol 1% 20 ml, vecuronium bromide 20 mg and KCl 2 g. Immediately after euthanasia, sternotomy was performed, the heart was harvested *en bloc* and a $\sim 2 \times 2$ cm piece of the left ventricular wall was obtained, at a distance of 2-4 cm from the apex.

Surgical procedure

Devascularization protocol (group DV): A midline abdominal incision was performed, followed by cystotomy and insertion of a Foley catheter in the bladder. An end-to-side portacaval anastomosis was then created with a 5-0 polypropylene suture and the hepatoduodenal ligament with its contents (hepatic artery, bile duct) was ligated and transected. This marks the beginning of the monitoring period. To ensure interruption of blood inflow from small collaterals, all other ligamentous attachments of the liver were also transected. The duration of the procedure was 60 ± 12 min. Total blood loss was 70 ± 25 ml.

Ischaemia/reperfusion protocol (group I/R): A midline abdominal incision was performed, followed by cystotomy and insertion of a Foley catheter in the bladder. A side-to-side portacaval anastomosis was created using 5-0 polypropylene sutures. After completion of the anastomosis, the left hepatic artery was ligated and the hepatoduodenal ligament was clamped. A 65-70% hepatectomy was then performed by resection of the median and left liver lobes. The liver remnant was kept ischaemic for 150 min and then the

portacaval anastomosis was clamped and blood flow was redirected to the liver remnant by unclamping the hepatoduodenal ligament. This time point marks the start of the monitoring period. The duration of the procedure – excluding the 150 min of ischaemia – was 65 ± 15 min. Total blood loss was 120 ± 50 ml.

Animals in the sham-operation group underwent only laparotomy of 60 min duration.

Blood and tissue sampling

After placing the ear vein catheter (baseline sample) as well as at the time intervals of 6 h and 12 h of monitoring, blood samples were obtained, placed in anticoagulant-containing aliquots and centrifuged at 3500 rpm, 4°C for 20 min. The plasma was then immediately stored at -80°C . Twenty-four h blood samples were obtained immediately prior to euthanasia. Plasma samples were used to measure cTnI and malondialdehyde (MDA). Myocardial and liver biopsies were instantly fixed in 4% formaldehyde/phosphate buffered saline and stored at room temperature. Later the samples were dehydrated and embedded in paraffin. Routine sections (3-5 μm) were stained with haematoxylin-eosin. The rest of the heart was macroscopically inspected for possible infarct areas.

Biochemical measurements

cTnI measurement was done using the Dimension® system CTNI Flex® reagent cartridge kit (RF421C) (Dade-Behring, Greece). Reported sensitivity (from the manufacturer) is 0.04 ng/ml. Cardiac troponin I use has been evaluated in pigs and was found both sensitive and specific for myocardial damage [12].

The malondialdehyde (MDA) concentration was determined in plasma samples spectrophotometrically at 586 nm and expressed in nmol/ml using a commercially available kit (Oxford Biomedical Research, Oxford, MS, USA).

Briefly, 0.65 ml of 10.3 mM N-methyl-2-phenyl-indole in acetonitrile was added to 0.2 ml of plasma. After vortexing for 3-4 s and adding 0.15 ml of HCl 37%, samples were mixed well, closed with a tight stopper and incubated at 45 min for 60 min. The samples were then cooled on ice, centrifuged and the absorbance was measured spectrophotometrically at 586 nm. A standard curve comprised of accurately prepared standard MDA solutions (from 2-20 nmol/ml) was also run for quantitation.

AST, ALT, LDH, urea, creatinine and NH_3 were measured in the clinical laboratory.

Histological examination

Haematoxylin-eosin stained myocardium sections were blindly analysed by two pathologists

simultaneously in a semi-quantitative manner, using 250× and 400× magnification under a light microscope. Approximately 25 fields of view (FOV) were examined under each magnification. Each specimen received a score from 0 to 3, according to criteria outlined in Table I, for the following indicators: cell necrosis, presence of polymorphonucleocytes and eosinophils, loss of cross striation, oedema and microscopic bleeding. If more than one score criteria were met on the same slide (e.g. some FOV gave a score of 1, others a score of 2 or 3) the highest score was appointed. The total histological score for each specimen was determined by the sum of all the partial scores.

Statistical analysis

Data are reported as mean \pm SD. Differences inside the same group for every parameter were analysed using one-way repeated measures ANOVA followed by the Bonferroni *post hoc* test. Differences between groups were analysed with ANCOVA using baseline values as the covariate. All statistical analyses were made with SPSS v.15.0 software; *p* values < 0.05 were considered statistically significant.

Results

All animals were maintained in good haemodynamic condition, with no statistically significant differences of mean arterial pressure (MAP) measurements between the baseline, 6-h and 12-h time points of all groups (Figure 1). Also, haemoglobin values and renal function – as expressed by urea and creatinine – did not change significantly between the baseline and the 12th h in all groups (Table II).

Animals in both the DV and the I/R group developed ALF by the 6th h of monitoring, as evidenced by the intracranial hypertension, the significant increase in ammonia and the increase of markers of hepatocellular injury (Table II).

Cardiac troponin I and MDA

Baseline cTnI values were below the detection limit in all animals. No increase of cTnI above the detection limit was recorded in sham-operated animals, at either the 6th or the 12th h time-point. In contrast, all ALF animals had increased cTnI values at both the 6th and the 12th h time point (*p* < 0.05 compared to baseline). Differences between cTnI values of the DV and the I/R group were not statistically significant. Also the decrease of mean cTnI values recorded in both ALF groups between the 6th and the 12th h measurement was not statistically significant (Table III). The malondialdehyde levels were significantly increased in DV

Table I. Histological score

	0	1	2	3	Magnification
Necrosis	None or 1-3 dead cells in < 3 FOV	≤ 3 dead cells per FOV in at least 3 FOV or 4-6 cells in no more than 3 FOV	4-6 dead cells per FOV in at least 4 FOV or > 6 cells in no more than 3 FOV	> 6 dead cells in at least 4 FOV	400×
Polymorphonuclear leucocytes	None or 1-3 cells in < 3 FOV	≤ 3 cells per FOV in at least 3 FOV or 4-6 cells in no more than 3 FOV	4-6 cells per FOV in at least 4 FOV or > 6 cells in no more than 3 FOV	> 6 cells in at least 4 FOV	400×
Eosinophils	None or 1-3 cells in < 3 FOV	≤ 3 cells per FOV in at least 3 FOV or 4-6 cells in no more than 3 FOV	4-6 cells per FOV in at least 4 FOV or > 6 cells in no more than 3 FOV	> 6 cells in at least 4 FOV	400×
Loss of striation	None or 1-5 cells in < 3 FOV	≤ 5 cells per FOV in at least 3 FOV or 5-10 cells in no more than 3 FOV	5-10 cells per FOV in at least 4 FOV or > 6 cells in no more than 3 FOV	> 10 cells in at least 4 FOV	400×
Oedema	None	< 10% of FOV in at least 3 FOV or > 10% in < 3 FOV	10-30% of FOV in at least 3 FOV or > 30% in < 3 FOV	> 30% of FOV in at least 3 FOV	250×
Microscopic bleeding	None	Present in < 10% of FOV in at least 3 FOV or > 10% in < 3 FOV	Present in 10-30% of FOV in at least 3 FOV or > 30% in < 3 FOV	Present in > 30% of FOV in at least 3 FOV	250×

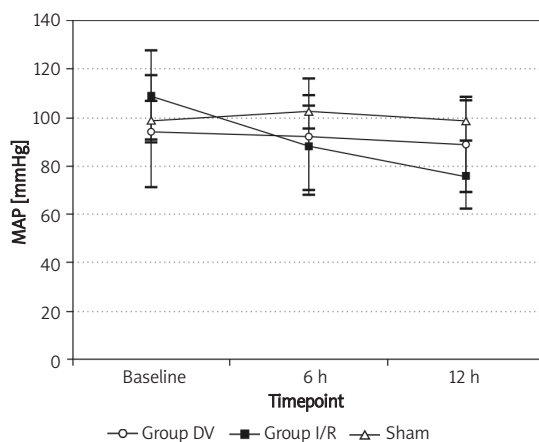


Figure 1. The differences of mean arterial pressure (MAP) measurements between the baseline, 6-h and 12-h time points

but not in I/R animals compared to the sham group at the 6th and the 12th h time points (Table IV).

Histological findings

Haematoxylin-eosin stained liver sections revealed, in both groups, disorganization of hepatic lobules, widened portal spaces, inflammatory cell infiltration and dilated sinusoidal spaces. Haemorrhagic necrosis was also present in both groups but was more pronounced in DV animals (Figure 2). The histological scoring of myocardial injury for DV and I/R animals is presented in Table V. Myocardial cell necrosis and oedema were the most prominent changes. The total injury score in both the DV and the I/R group was significantly increased ($p < 0.05$) compared to the sham group, in which almost all animals received a score of 0 for all parameters

(one animal received a score of 1 for microscopic oedema). Scoring differences between the DV and the I/R group were not statistically significant.

Discussion

Our study provides direct evidence of early myocardial injury in two different big-animal models of surgically induced ALF. All animals experienced a rise in troponin I as early as 6 h after the induction of ALF – a biochemical finding that corresponded to the histological alterations seen in myocardial biopsies obtained 12 h after the induction of ALF. This finding cannot be attributed to haemodynamic factors, since all animals were maintained in a good haemodynamic condition, with stable haemoglobin. Furthermore, the normal findings in sham-operated animals exclude anaesthesia, mechanical ventilation and invasive monitoring procedures as aetiological factors of myocardial injury.

For this study we selected two established surgical models of ALF which involve either complete liver devascularization (DV animals) or a combination of extensive hepatectomy with I/R injury of the small liver remnant (I/R animals). Both animal models have been shown in previous studies by our group and others [13, 14] to result in a rapid development of ALF, which is irreversible in DV animals and potentially reversible in I/R animals. We avoided toxin-induced ALF models because of poor reproducibility in large animals and the possible side effects of hepatotoxins on the myocardium.

An important observation in our study is the subtlety of myocardial injury. A histological score of 3 (the maximum for each histological parameter examined) was only once appointed, with all parameters ranging from 0 to 2 for each specimen. Necrotic cells were mostly dispersed with very little clustering (Figure 2). This indicates that the factor which caused cell death probably affected the whole heart and that it was not regional microcirculatory ischaemia, at least not per se. No necrotic, haemorrhagic or otherwise abnormal regions were observed macroscopically in any of the hearts.

The early appearance of cTnI in plasma and the fact that as time passed its levels did not rise indicates that cell necrosis must have started early on, before the 6 h time point in both protocols. This is confirmed by the study of Chen *et al.* [11]. cTnI can be detected in the bloodstream within minutes after cell damage [15]. Parekh *et al.* [5] report that 74% of patients with acute liver failure had elevated cTnI levels, while in our groups all animals (100%) exhibited elevated cTnI levels. This discrepancy could be due to interspecies differences and a considerable heterogeneity of patient groups regarding the cause of acute liver failure.

Table II. Biochemical and ICP measurements

Parameters	Group DV			Group I/R			Sham group		
	Baseline	6 h	12 h	Baseline	6 h	12 h	Baseline	6 h	12 h
AST	45 ±30	820 ±590 [†]	1107 ±640 ^{††}	34 ±6	608 ±340 ^{††}	600 ±431 ^{††}	55 ±6	54 ±7	80 ±21
LDH	488 ±208	1383 ±343 ^{††}	2277 ±154 ^{††}	624 ±30	1215 ±586 ^{††}	1322 ±387 ^{††}	555 ±187	403 ±188	381 ±175
Urea	20 ±5	11 ±2	10 ±3	13 ±5	13 ±4	10 ±2	18 ±4	13 ±3	11 ±5
Cr	1.08 ±0.17	0.81 ±0.13	0.87 ±0.08	0.95 ±0.38	0.92 ±0.45	0.8 ±0.52	1.12 ±0.12	1.32 ±0.15	1.34 ±0.13
NH ₃	45 ±24	332 ±37 ^{††}	463 ±116 ^{††}	29 ±22	189 ±64 ^{††}	239 ±128 ^{††}	32 ±12	40 ±11	35 ±15
Hb	9.7 ±0.5	9.3 ±0.6	8.8 ±0.5	9.5 ±0.3	10.7 ±0.5	9.7 ±0.5	10.3 ±0.2	11.1 ±0.4	10.7 ±0.5
ICP	9 ±3	20 ±6 ^{††}	25 ±7 ^{††}	10 ±1	16 ±3 ^{††}	20 ±3 ^{††}	11 ±4	13 ±3	9 ±4

^{††}p < 0.05 vs. baseline, [†]p < 0.05 vs. Sham at same time point

Table III. Troponin I (ng/ml)

Time point	Group DV (mean ± SD)	Group I/R (mean ± SD)	Sham group	Statistical significance between groups (p)	
				DV vs. Sham	I/R vs. Sham
Baseline	< 0.04	< 0.04	< 0.04	NS	NS
6 h	1.40 ±0.91	0.85 ±0.68	< 0.04	0.025	0.044
12 h	0.93 ±0.44	0.47 ±0.20	< 0.04	0.007	0.002

Table IV. MDA (nmol/ml)

Time point	Group DV (mean ± SD)	Group I/R (mean ± SD)	Sham group	Statistical significance between groups (p)	
				DV vs. Sham	I/R vs. Sham
Baseline	1.88 ±0.07	1.86 ±0.25	1.59 ±0.26	NS	NS
6 h	3.20 ±0.20	1.69 ±0.48	1.47 ±0.04	< 0.001	NS
12 h	2.90 ±0.28	1.83 ±0.81	1.52 ±0.05	0.001	NS

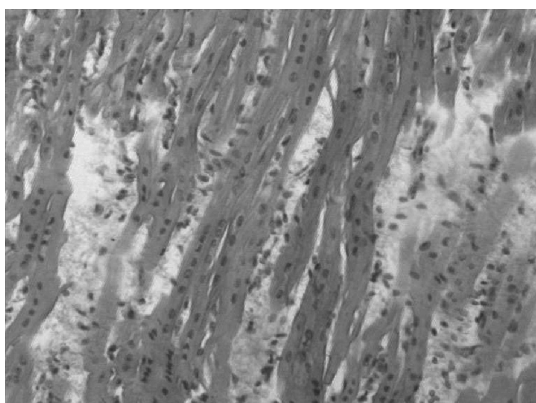


Figure 2. The presence of haemorrhagic necrosis in DV groups

Table V. Histological score

	Group DV (mean ± SD)	Group I/R (mean ± SD)
Necrosis	1.25 ±0.5	1 ±0.63
Polymorphonuclear leucocytes	0.75 ±0.5	1.66 ±0.51
Eosinophils	0.25 ±.5	1 ±0.89
Loss of striation	0.75 ±0.5	1.16 ±0.40
Oedema	1.25 ±0.95	1.16 ±0.75
Microscopic bleeding	0.25 ±0.5	0.5 ±0.83
Total score	4.5 ±1.73	6.5 ±1.87

During the experiments, we did not observe any arrhythmias or other major electrocardiographic abnormalities in any animal. This indicates that there was no major functional compromise of the heart.

There is little evidence to suggest a causative mechanism, or that there is a single mechanism involved in both our experimental groups. Cardiovascular collapse and sepsis, which could potentially be the cause of elevated cTnI levels in at least some humans with ALF [5], were not observed in our setting and therefore can be effectively ruled out. Oxidative stress is a possible candidate in the DV group, as indicated by the rise in plasma MDA. However, in the I/R group a similar rise is not seen. It is unclear whether this is due to lower levels of oxidative stress in this setting or to MDA kinetics. Early studies have shown that MDA is, mainly at least, metabolized by the liver [16, 17] and that it is rapidly eliminated [18]. If we accept that the main oxidative event in the I/R group was reperfusion,

theoretically plasma MDA could have been metabolized by the remaining liver by the first 6-h time point. Parekh *et al.* [5] propose that the effect of high cytokine levels or other factors leading to multi-organ failure in humans could play a significant role. This is a plausible theory, but one would expect that, as ALF progresses, cTnI levels would continue to rise – a trend that was not observed in our setting. The basic limitation of our study is the lack of an in-depth investigation of the pathophysiological mechanisms involved in myocardial injury. In our opinion, oxidative mechanisms should be a primary target of future investigation. Serial heart biopsies and the use of anti-oxidants could be useful tools in such an investigation.

In conclusion, both our experimental models verify that myocardial injury is an integral part of experimental acute liver failure. However, the precise mechanisms leading to this injury, as well as possible targets for intervention, remain elusive.

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