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Generation of a Rat Model of Acute Liver Failure by Combining 70% Partial Hepatectomy and Acetaminophen

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The Editor,
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Dear Editor,

Thank you for providing us comments on our revision entitled "A rat model of acute liver failure by combining 70% partial hepatectomy and Acetaminophen" JoVE60146.

We are submitting the revised manuscript and the edited video.

In the revised manuscript we have addressed all the comments.

The accompanying video has also been edited to incorporate made suggestions.

Trust you will find the revision in order.

With regards,

Pramod Upadhyay
Scientist, NII

TITLE:

Generation of a Rat Model of Acute Liver Failure by Combining 70% Partial Hepatectomy and Acetaminophen

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KEYWORDS:

acute liver failure model, ALF, partial hepatectomy, acetaminophen, APAP, intrasplenic transplantation, Wistar rats, hepatocyte transplant

SUMMARY:

The acute liver failure animal model developed in the current study presents a feasible alternative for the study of potential therapies. The current model employs the combined effect of physical and drug-induced hepatic injury and provides a suitable time window to study the potential of novel therapies.

ABSTRACT:

Acute liver failure (ALF) is a clinical condition caused by various etiologies resulting in the loss of metabolic, biochemical, synthesizing, and detoxifying functions of the liver. In most irreversible liver damage cases, orthotopic liver transplant (OLT) remains the only available treatment. To study the therapeutic potential of a treatment for ALF, its prior testing in an animal model of ALF is essential. In the current study, an ALF model in rats was developed by combining 70% partial hepatectomy (PHx) and injections of acetaminophen (APAP) that provides a therapeutic window of 48 h. The median and left lateral lobes of the liver were removed to excise 70% of the liver

mass and APAP was given 24 h postsurgically for 2 days. Survival in ALF-induced animals was found to be severely decreased. The development of ALF was confirmed by altered serum levels of the enzymes alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP); changes in prothrombin time (PT); and assessment of the international normalized ratio (INR). Study of the gene expression profile by qPCR revealed an increase in expression levels of genes involved in apoptosis, inflammation, and in the progression of liver injury. Diffused degeneration of hepatocytes and infiltration of immune cells was observed by histological evaluation. The reversibility of ALF was confirmed by the restoration of survival and serum levels of ALT, AST, and ALP after intrasplenic transplantation of syngeneic healthy rat hepatocytes. This model presents a reliable alternative to the available ALF animal models to study the pathophysiology of ALF as well as to evaluate the potential of a novel therapy for ALF. The use of two different approaches also makes it possible to study the combined effect of physical and drug-induced liver injury. The reproducibility and feasibility of current procedure is an added benefit of the model.

INTRODUCTION:

Acute liver failure (ALF) is defined by the American Association for the Study of Liver Diseases as rapid development of acute liver injury without any prior signs of damage and is characterized by severe impairment of the synthetic, metabolic, and detoxifying functions of the liver¹. ALF differs from chronic liver failure where the failure occurs as a result of liver injury caused over a long period of time and from acute chronic liver failure (ACLF), where abrupt liver damage takes place as a result of chronic liver diseases²⁻⁴. The only available cure for ALF is orthotopic liver transplant (OLT), or death may occur. Due to the shortage of liver donors, the rate of mortality in patients suffering from ALF is very high.

To study the potential of alternative therapeutic approaches and to better understand the pathophysiology of ALF, animal models that can reflect the ALF occurring in human beings are needed. Many of the already available ALF animal models have several shortcomings. Acetaminophen (APAP) effects are difficult to reproduce but have the closest similarities in terms of temporal, clinical, biochemical, and pathological parameters. APAP-induced animal models frequently encounter problems due to the presence of methemoglobinemia caused by the oxidation of hemoglobin by APAP and its intermediates⁵⁻⁷. Another problem is the lack of reproducibility reflected by unpredictable dose responses and the time of death. The ALF animal models produced using carbon tetra chloride (CCl₄) have poor reproducibility⁸⁻¹¹. Concavalin A (Con A) and lipopolysaccharide (LPS)-induced ALF animal models do not reflect the clinical pattern of the human disease, though they have advantages in the study of cellular mechanisms involved in autoimmune liver diseases and in the study of sepsis respectively¹²⁻¹⁵. Similarly, thioacetamide (TAA) also requires biotransformation to an active metabolite thioacetamide sulfoxide and shows species variation¹⁶⁻¹⁹. D-galactosamine (D-Gal) produces some biochemical, metabolic, and physiological changes similar to ALF but is not able to reflect the whole ALF pathological condition²⁰⁻²³. There have been very few attempts to combine two or more of these methods to develop an ALF model that is able to reflect the ALF syndrome in a better manner¹³. Therefore, further studies are required to develop a model that can reflect the disease parameters, has better reproducibility, and provides enough time to study the effects of a therapeutic

intervention.

In the current study, an alternative ALF model in rats has been created by combining the effects of partial hepatectomy (PHx) and lower doses of a hepatotoxic reagent. APAP has a well-established role in causing liver injury^{5,24,25}. It is a widely used analgesic and is toxic to the liver at supratherapeutic doses by forming toxic metabolites. APAP is the cause of many deaths in developed countries. Physical injury caused by partial hepatectomy initiates activation of various processes involved in inflammation as well as liver regeneration. Injection of the hepatotoxic agent APAP causes a hostile environment in the liver, preventing the proliferation of hepatocytes. This reduces the stress period on the animal, which when combined with smaller doses of hepatotoxin, leads to better reproducibility of the procedure. Therefore, using this model, a combinatory effect of two types of liver injuries has been studied. To characterize the developed ALF animal model, physiological and biochemical parameters have been studied. Successful reversibility of ALF was confirmed by transplantation of syngeneic healthy rat hepatocytes.

PROTOCOL:

The procedure described below has been approved by the Institutional Animal Ethics Committee of National Institute of Immunology, New Delhi. The serial reference number of the approval is IAEC#355/14.

1. Preparation

1.1. Prepare for the surgical procedure as described earlier by Barun Das et al.²⁶.

1.2. Use 6–8-week-old inbred Wistar rats with a body weight of 200–250 g.

1.3. House the animals under standard animal care conditions and feed them with rat chow and libitum before and after the procedure.

1.4. When performing 70% PHx, use a standard cocktail mix of ketamine hydrochloride (100 mg/kg body weight) and xylazine (10 mg/kg body weight), which is injected intraperitoneally.

NOTE: The type of anesthetic used can have postoperative effects on the mortality and morbidity.

1.5. During the cell transplant, use inhalant anesthesia Isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane) to reduce the time of recovery of the animal after the transplantation surgery.

1.6. Induce and maintain the inhalational anesthesia using a customized anesthesia system. Maintain the oxygen flow at 4 L/min. For induction use isoflurane at 4% and for maintenance use 2–3% during the surgical procedure.

2. Preoperative procedures

2.1. Anesthetize the rat by injecting the ketamine-xylazine mixture described in step 3.1 intraperitoneally. Confirm the complete anesthetization by pinching the toe of the animal. Further procedures are carried out only when there is no pedal reflex.

2.2. To prevent corneal desiccation, apply a carboxy methyl-cellulose based eye drop to both eyes.

2.3. Restrain the anesthetized animal to a surgical board using white tape. Place the animal with the abdominal side facing up, ensuring the mouth is on the distal side from the person performing the operation.

2.4. Remove hair from the upper right abdominal surgical area using an electric clipper.

2.5. Disinfect the surgical site by three alternating scrubs of povidone iodine and 70% ethanol using sterilized cotton pads in circular motion.

3. Partial hepatectomy (PHx) to remove 70% of the mass of the liver

NOTE: Perform the entire surgical procedure under a sterile environment in a laminar flow hood. Use only sterile surgical instruments to minimize the risk of infection postsurgically. Removal of 70% of the liver mass, named 70% partial hepatectomy (70% PHx), was performed as described by C. Mitchell and H. Willenbring, 2008²⁷.

3.1. Prior to the start of the surgery, confirm the complete anesthetization of the animal by pinching its toe. Further procedures are carried out only when there is no pedal reflex.

3.2. Mark the skin to be cut just beneath the sternum, perpendicular to the xiphoid, and parallel to the ribcage.

3.3. Place a sterile drape sheet having an opening of around 3 cm x 1 cm over the marked skin.

3.4. Perform a transverse incision of around 2–3 cm along the marked line with a scalpel. Use surgical blade No. 22. Gently remove the attachment of the skin to the underlying muscle layer in the vicinity of the incised area using sterile moistened cotton tips.

3.5. Next, make a transverse incision through the peritoneal layer just beneath the xiphoid process.

3.6. With the help of two saline moistened cotton tips, expose the left lobe of the liver by applying gentle pressure on the thorax. Place one cotton tip on the diaphragmatic region of the incised portion and the other cotton tip below the incised region to lift the liver lobe up.

3.7. Slip an 8–10 cm long sterile nylon thread loop (size 4–0, 0.15 mm diameter) around the exposed liver lobe. Take the loop to the base of the lobe close to the hilum with the help of microdissecting forceps or moistened cotton buds.

3.8. With the help of the microsurgery needle holder and microforceps, tie the two ends of the loop, placing the knot as close to the base of the lobe as possible to constrict the blood vessel and reduce bleeding after the liver lobe is removed. Tie two additional knots on the other side.

3.9. Take precaution not to tie the knot too close to the nearby blood vessels, which may otherwise cause venous obstruction (stenosis).

3.10. Use microsurgery scissors to cut the tied lobe just above the knot, which leaves a discolored mass of tissue called an ischemic stump in place of the lobe.

NOTE: The rat liver, like those of mice, is divided into four distinct lobes: the median lobe, right lateral lobe, left lateral lobe, and caudate lobe, which represent about 40%, 20%, 30%, and 7% of the total liver mass, respectively. Any combination of these lobes can be removed to excise 70% liver mass. In the current study, the median lobe and left lateral lobes were removed.

3.11. Carefully locate the median lobe without damaging the remaining stump of left lateral lobe. Gently pull it out of the abdominal cavity, and at the base of the lobe tie an 8–10 cm long nylon thread (size 4–0) knot as mentioned earlier. Tie two additional knots on the other side. Carefully excise and remove the tied median lobe taking all the precautions mentioned.

3.12. After removing the lobes, suture the peritoneum using an absorbable chromic 4–0 suture with continuous stitches followed by skin suturing with an interrupted suture.

3.13. Apply povidone iodine on the skin surrounding the sutures to prevent infection.

3.14. Remove the drape sheet and remove the animal from the surgery board.

4. Postoperative care in animals

4.1 Intraperitoneally inject the animal with a dose of 12 mg cefotaxime antibiotic in 1 mL of 5% glucose solution with a 1 mL syringe to protect it from the risk of postoperative infection.

4.2 Administer a subcutaneous injection of analgesic meloxicam (1 mg/kg body weight) for pain relief after surgery and follow it up by two more doses, keeping the regimen as one dose per day.

4.3 House the operated animals under standard conditions of 12 h light/dark cycle and monitor at regular intervals.

5. Injection of drug in partially hepatectomized animals to induce liver failure

5.1 After 24 h postsurgery, when the animals have successfully recovered from 70% PHx, measure the body weight of the animal followed by the injections.

5.2 Inject 750 mg/kg body weight of APAP intraperitoneally in partially hepatectomized animals 24 h after the 70% PHx following the animals' successful recovery from the surgical procedure. Repeat the dose again after 24 h.

NOTE: Two doses of APAP are administered intraperitoneally to the animal (i.e., 24 h and 48 h post the 70% PHx procedure, respectively).

5.3 At each time point after the injection of APAP, measure the body weight of the recovering animal.

NOTE: APAP (biocetamol) is injected in animals as a 150 mg/mL solution in 2% benzyl alcohol.

6. Transplantation of healthy hepatocytes in ALF animal models

NOTE: To study the reversibility of ALF in rats, transplant healthy syngeneic rat hepatocytes intrasplenically in the ALF-induced animals along with the 1st dose of APAP. In the current study, to provide ample time to the transplanted cells for homing and engraftment, the transplantation was done just after giving the 1st dose of APAP. Rat hepatocytes are isolated by a protocol first published by Berry and Friends et al.²⁸ and later adapted in various other studies^{29–31} with some modifications. For intrasplenic transplantation of cells in the ALF animal model, follow the steps mentioned below.

6.1. Place the rat into a poly (methyl methacrylate) chamber for induction of anesthesia with 4% isoflurane and 4 L/min oxygen flow for a rat of 250–350 g body weight. Check for the depth of anesthesia by the lack of pedal reflexes when pinching the toe of the animal.

6.2. Place the anaesthetized rat on the surgical board such that its left lateral portion is facing up. Maintain anesthesia at 2–3% isoflurane inhalation through a suitable mouthpiece.

6.3. Shave the skin on the left lateral region and sterilize it by povidone iodine solution.

6.4. Make a transverse incision on the shaved region of skin.

6.5. Make a 1–2 cm cut in the peritoneal layer to expose the spleen.

6.6. Gently take out the spleen of the peritoneal cavity and lift it up with the help of two moistened cotton tips.

6.7. Keep the cells (typically 10⁷ per animal) to be transplanted suspended in 50 µL IMDM media in a 1 mL insulin syringe with a 29 G needle.

6.8. Gently pierce the needle into the spleen cortex and release the cell suspension into the spleen within 2–3 min.

6.9. After the cell transplantation is completed, carefully take out the needle and dab the area of the needle puncture with a moistened cotton tip to avoid leakage of the cell suspension from the site.

6.10. Close the peritoneum and skin by a 4–0 absorbable suture with continuous and discontinuous suturing respectively.

6.11. Apply povidone iodine solution on the skin at the place of the sutures to prevent infection on the operated site.

6.12. Intraperitoneally inject 1 mL volume of 12 mg/mL of antibiotic (e.g., cefotaxime) solution and subcutaneously inject analgesic (e.g., meloxicam) 1 mg/kg body weight to the animal as a part of postoperative care. Move the animal to a warm recovering cage.

6.13. Keep the operated animal in isolation under normal conditions of 12 h light/dark cycle until the surgical wounds are completely healed. This may take 3–4 days.

7. Characterization of ALF development

7.1. Euthanize the animals by overdose of ketamine-xylazine solution 2 h after the 2nd dose of APAP treatment and collect blood and tissue samples.

7.2. Collect serum from blood for biochemical studies³².

7.3. Process liver tissue samples for histological and gene expression studies^{33–35}.

REPRESENTATIVE RESULTS:

Survival percentage in animal models of ALF

The optimum dose of APAP to cause ALF in combination with 70% PHx was standardized as 750 mg/kg body weight. The treatment regimen started 24 h after 70% PHx, when the animals had completely recovered from surgery, and consisted of two APAP doses at 24 h intervals. Mortality was observed at the rate of 80% after the administration of the second dose of APAP, 48 h post-surgery. The survival percentage was analyzed and plotted via the Kaplan-Meier method (**Figure 1**). The reproducibility in time of death and time period provided by this model makes it a suitable candidate for studying a therapeutic intervention against ALF.

Four groups of animals were considered for the study: Group 1 (control group, only saline treatment), Group 2 (only APAP at 750 mg/kg body weight), Group 3 (saline treatment with 70% PHx) and Group 4 (APAP at 750 mg/kg body weight with 70% PHx). Three animals each were included in all the groups and two representative images are included from each group. Animals from all four groups were euthanized 2 h after the administration of the 2nd dose of the respective

treatment. Liver samples taken from all four groups showed distinct morphology from each other. Group 1 showed the reddish-brown morphology of a healthy rodent liver. Liver samples of Group 2 showed no apparent signs of damage at a morphological level and showed similar appearance to the healthy livers of Group 1. Liver samples taken from Groups 3 and 4 did not appear healthy and had discoloration and a patchy appearance. The discoloration corresponds to the damage in the liver tissue and was more apparent in Group 4. Representative data are presented in **Figure 2**.

The extent of liver injury was determined by checking the serum levels of ALT, AST, and ALP enzymes^{36–38}. The levels of ALT, AST, and ALP showed marked differences among the four groups corresponding to the extent of liver damage. Group 4 showed a significant increase in the AST and ALP levels compared to Group 1 (**Figure 3**).

To compare the gene expression profile in control and ALF-induced groups, q-PCR analysis of genes involved in cell death (*Bax2*, *Caspase3*, *Fas*) in liver tissue samples was performed^{35,39}. These were found to be upregulated in Group 3 in comparison to control Group 1, whereas no difference was seen in Group 2 and Group 4. Genes which are known to be overexpressed in response to liver injury (*Mcp1* and *Mmp2*)^{33,40} were found to be upregulated in all three groups in comparison to control Group 1. *Mmp9* was upregulated in Groups 3 and 4 whereas Group 2 showed no marked difference from control Group 1. Expression levels of *Timp1* and *Timp2*³⁴ were found to be higher in Group 3 but showed no marked overexpression in Group 4 (**Figure 4**).

Genes involved in inflammation of the liver (*Tnfa*, *IL1α*, *Tgfbr1*, and *IL1b*)^{41–44} were found to be overexpressed in Group 3 in comparison to control Group 1 but their expression level was decreased in Group 4. ALR is overexpressed in liver tissue after the induction of liver injury. The downstream cascades of this gene help in liver regeneration. RIP1 protein is necessary in APAP-induced toxicity. *ALR* and *RIP1* were found to be upregulated in Groups 2 and 3 in comparison to control Group 1, whereas their levels remain similar or lower in Group 4. Alpha smooth muscle actin (*αSMA*), a marker of excessive ECM deposition that further leads to fibrosis, was found to be upregulated in Group 3 and 4 in comparison to control Group 1 (**Figure 4**). Gene expression data suggests that the above-mentioned biomarkers of inflammation and cell death that are known to be overexpressed during liver injury are upregulated in liver tissue samples of animals in which ALF was-induced by proposed method, thus confirming the occurrence of liver injury at the molecular level.

Hematoxylin and eosin (H&E) staining

Hematoxylin and eosin (H&E) staining was done to assess the severity of liver injury by observing the extent of hepatocyte degeneration. The liver tissue sections stained with H&E were subjected to blind analysis by a third party. In the 70% partial hepatectomy group vesicular fatty degeneration was observed and in the acetaminophen group periportal inflammation and necrosis was seen along with mild sinusoidal dilatation. Vesicular fatty degeneration was observed mostly in the ALF group (**Figure 5**).

Prothrombin time and blood glucose levels

Prothrombin Time (PT) is a parameter that depends on the activity of the liver-synthesized tissue thromboplastin and is used to characterize the efficiency of the blood coagulation mechanism⁴⁵. Generally, an increase in PT is observed in the conditions of liver-related diseases. PT was found to be significantly higher in the ALF-induced group in comparison to the control group. The international normalized ratio (INR)⁴⁵ was also found to be higher, representing a defect in the mechanism of blood coagulation as a result of inefficient liver physiology (**Figure 6**).

Evaluation of survival after cell healthy syngeneic rat hepatocytes transplantation

An important criterion of an ALF model is the reversibility of liver failure in the presence of a therapeutic intervention. In the current study, 10 million healthy syngeneic rat hepatocytes were transplanted intrasplenically to observe the effect of cell transplant therapy on liver failure. The appropriate number of cells to be transplanted was determined by observing the reversibility of liver failure after transplantation of different cell numbers (data not shown). Cells were transplanted after administration of the 1st dose of APAP, and survival was found to be restored in animals after the transplantation of cells (**Figure 7**). The serum levels of enzymes ALT, AST, and ALP were found to be normalized after 10 days of cell transplantation (**Figure 8**).

FIGURE AND TABLE LEGENDS:

Figure 1: Survival percentage in the ALF-induced group. Kaplan-Meier Survival Curve showing the survival percentage of animals after induction of ALF with the combination of 70% PHx and APAP dose (750 mg/kg body weight). Time 0 h denotes the time of 70% PHx. The 1st and 2nd doses of APAP were administered 24 and 48 h postsurgically (n = 12, total animals).

Figure 2: Representative images of liver morphology in the four different groups included in the study. Image panels A–D show the morphology of the livers of different groups after being euthanized 2 h after the 2nd dose of the respective treatment. (A) The liver morphology of control Group 1 in which only saline was given. (B) The liver morphology of Group 2 in which only APAP was given at the dose of 750 mg/kg body weight. (C) The liver morphology of Group 3 in which saline was given following 70% PHx. (D) The liver morphology in Group 4 in which APAP was given following 70% PHx at the dose similar to Group 3 (n = 3 animals per group).

Figure 3: A comparison of the biochemical profile of serum in the different groups. Bar graphs represent the mean value of ALT, AST, and ALP in serum samples of the four groups included in the study. Serum was collected 2 h after the 2nd dose of the respective treatment. Error bars = S.E.M; *** = $p < 0.001$; * = $p < 0.05$; n = 3.

Figure 4: Gene expression profile of liver tissue samples. Bar graphs represent the relative quantification of the mRNA expression of various genes involved in inflammation and cell death in the four groups subjected to different treatments. All genes were normalized against the expression of control *Gapdh*. Error bar = standard error mean of the three different samples (n = 3).

Figure 5: Histological study of liver tissue samples. Representative hematoxylin and eosin staining images of liver tissue samples from the four groups included in the study, as observed

under 200x magnification in bright field microscopy. There were mostly normal hepatocytes in control Group 1 with mild periportal infiltration (green arrows). In Group 3 (70% partial hepatectomy) vesicular fatty degeneration was observed (white arrows) and in the acetaminophen Group 2 periportal inflammation (red arrows) and necrosis were seen along with mild sinusoidal dilatation (yellow arrows). Macrovesicular fatty degeneration was mostly observed in the ALF Group 4 (orange arrows). Scale bar = 50 μ m.

Figure 6: Comparison of prothrombin time (PT) and international normalized ratio (INR). (A) Comparison of PT between control Group 1 (saline injection post 70% PHx) and ALF-induced Group 4 (APAP injection post 70% PHx). Blood was collected 2 h after the 2nd dose of the respective treatment and time (in seconds) required for fibrin clot formation is shown on the Y axis (**** = $p < 0.0001$, $n = 5$). **(B)** The INR in the ALF-induced Group 4 in comparison to control Group 1. The mean value of INR in Group 4 was found to be 2.28, which falls in the Warfarin-induced anti coagulating effect ($n = 5$).

Figure 7: Survival percentage in ALF-induced group after cell transplant. Kaplan-Meier survival curve showing the survival percentage after healthy hepatocyte transplantation in the ALF-induced Group 4 in comparison to control Group 1 ($n = 5$).

Figure 8: Serum biochemical profile of ALF-induced animals after cell transplant. Serum levels of enzymes ALT, AST, and ALP 10 days after cell transplantation in comparison with the serum levels of ALF-induced animals euthanized after a 2nd dose of APAP administration. Error bar = standard error of mean of five different samples; **** = $p < 0.0001$; ** = $p > 0.01$; $n = 5$.

DISCUSSION:

The development of an appropriate animal model for ALF is paramount for the better understanding of pathogenesis and progression of ALF. A well characterized ALF animal model also provides the opportunity for the development and trial of new therapeutic approaches against ALF. Many attempts have been made to develop a clinically relevant model of ALF^{6,12,21,23,46–48}. Most of these studies either utilize surgical procedures or induce liver injury by hepatotoxic chemicals.

It is difficult to create an ALF model by PHx alone because PHx would not reflect the pathology of ALF due to the absence of inflammation caused by the necrotic and apoptotic tissues. At therapeutic doses, APAP is metabolized completely by the processes of glucoronidation and sulphation. At higher doses, when these pathways are saturated, APAP is metabolized by cytochrome p450 enzymes which are primarily synthesized by hepatocytes, resulting in the formation of a toxic intermediate, NAPQI (N-acetyl-p-benzoquinone imine). Under normal conditions NAPQI is neutralized by the glutathione (GSH) reserves of cells, whereas at supratherapeutic doses it causes oxidative stress in hepatocytes, resulting in hepatocyte death^{4,24,25,46}. Hence, by combining the physiological effects of 70% PHx and APAP-induced liver injury, an ALF rat model was created in the current study. A smaller therapeutic window reduces the stress period on the animal. When combined with smaller doses of APAP, this leads to better reproducibility of the procedure.

The dose of APAP was selected to provide a suitable therapeutic window during which a therapeutic intervention can be given to the animal (i.e., within 48 h of the 1st dose and 24 h of 2nd dose). Within 24 h after the 2nd dose of APAP, 100% mortality was observed. However, the time of death within this period was variable for each animal. Hence, for the purpose of studying the development of ALF, the animals were euthanized at a standard time point of 2 h after the 2nd dose of APAP.

The time of therapeutic intervention can be decided depending upon the individual study and the type of therapy being studied, which in the current study was transplantation of syngeneic rat hepatocytes just after the 1st dose of APAP, allowing the cells enough time to remain and engraft. In the current model, transplantation of at least 10 million cells is required for a successful rescue from ALF.

To ensure the success of the surgical procedure, a few important points should be considered. It is recommended to use different types of anesthetics in different surgical procedures. To perform partial hepatectomy, a cocktail of ketamine-xylazine should be used in the recommended doses because it gives ample time to complete the surgical procedure. During cell transplant, inhalable anesthesia isoflurane should be used because it reduces the physical stress on ALF-induced animals.

In conclusion, due to a uniform mortality rate and a convenient therapeutic window, an APAP and 70% PHx combined model was used to study the reversibility of ALF by cell transplant. To assess the reversibility of ALF, 10 million healthy syngeneic rat hepatocytes were transplanted intrasplenically in the ALF-induced animals. After transplantation, the survival percentage was found to be increased in ALF-induced animals. Improvement in serum levels of ALT, AST, and ALP was also observed in animals after cell transplant, suggesting the restoration of liver metabolism. This ALF animal model presents an alternative to evaluate the therapeutic potential of cells bridging the gap between acute liver failure and liver transplantation. It also provides the opportunity to study liver damage caused by physical injury and hepatotoxic agent.

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DISCLOSURES:

The authors have nothing to disclose.

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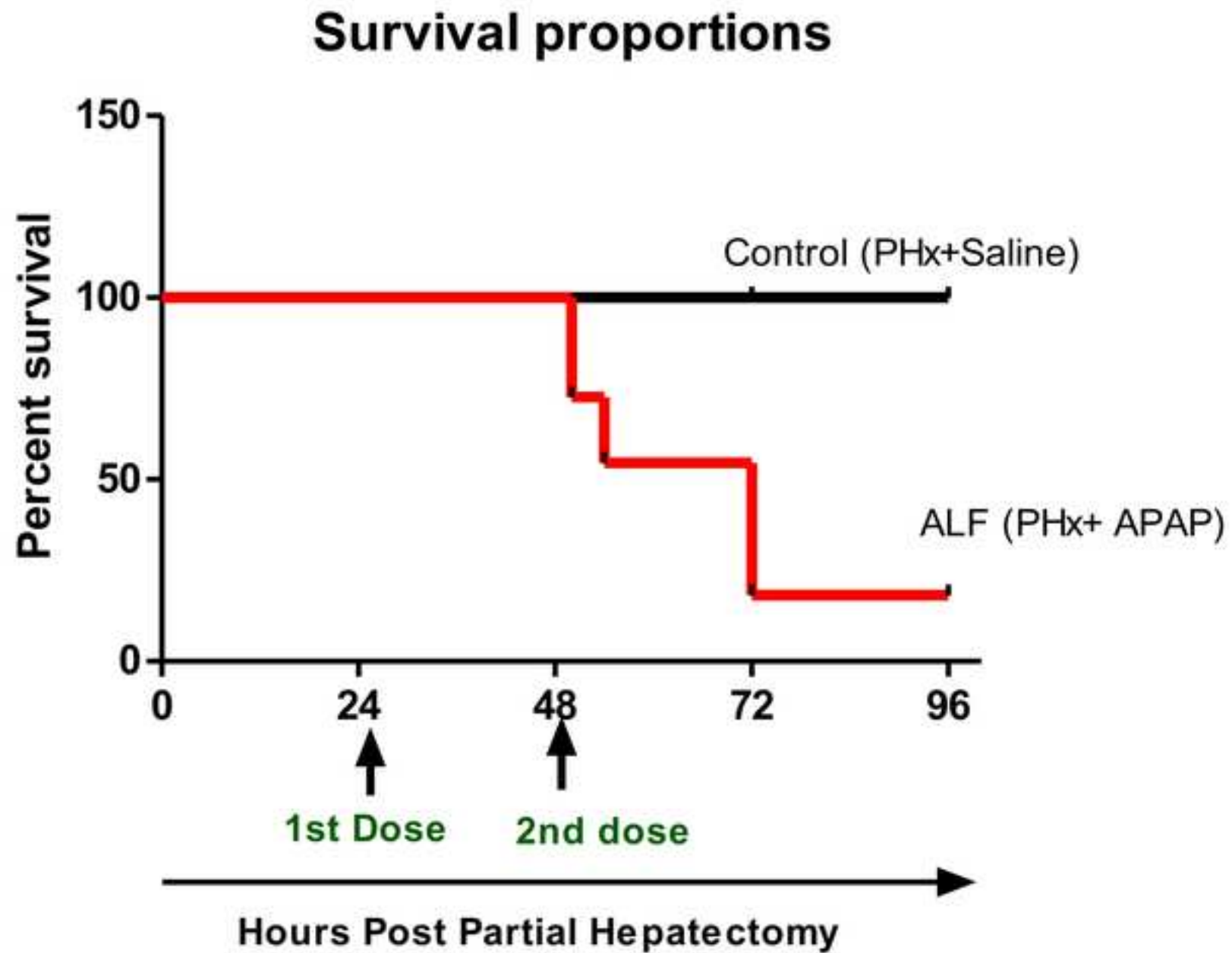
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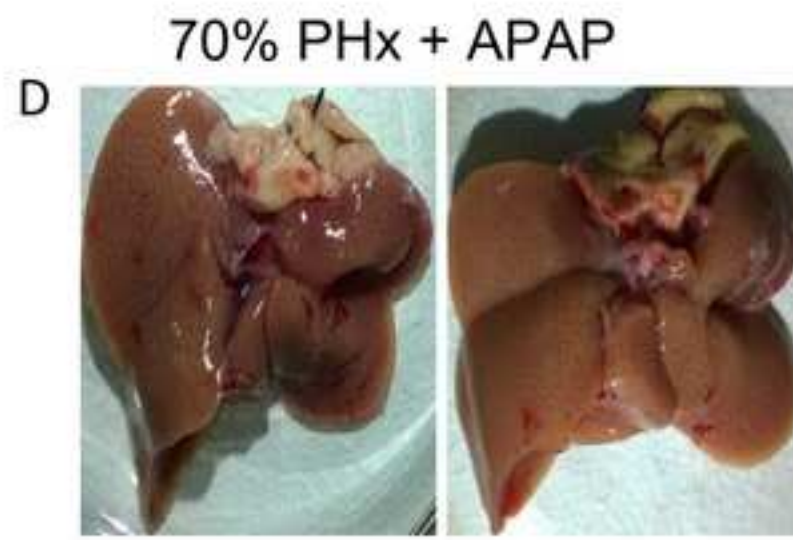
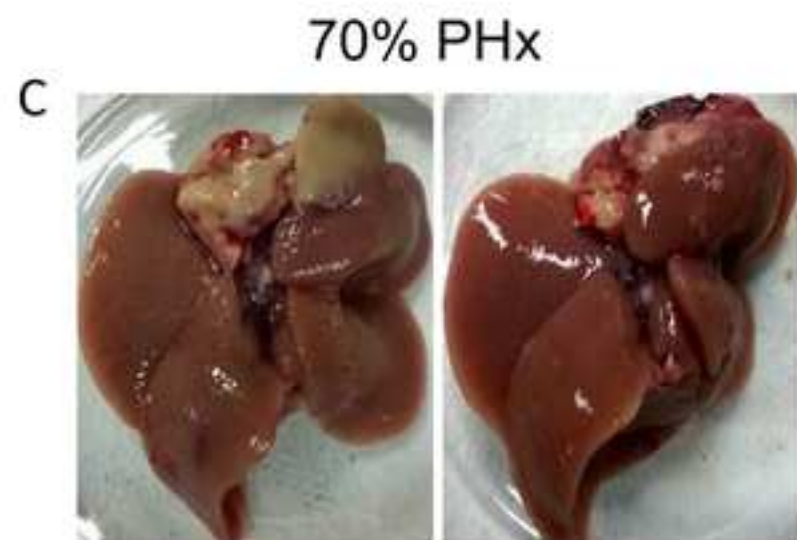
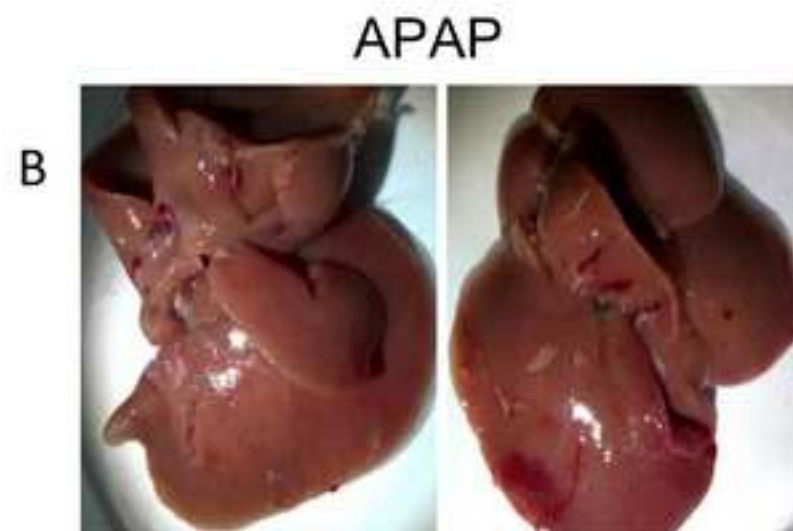
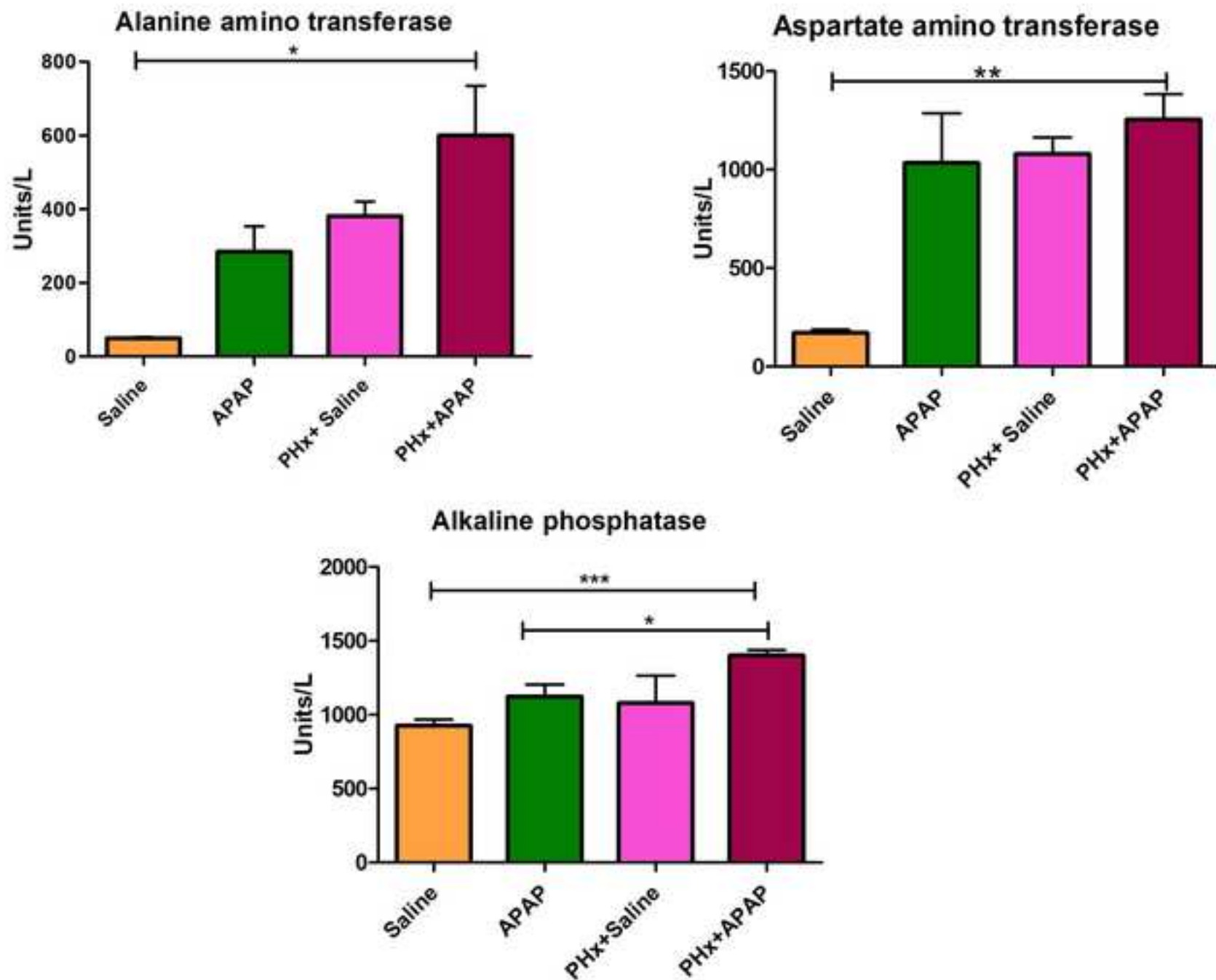
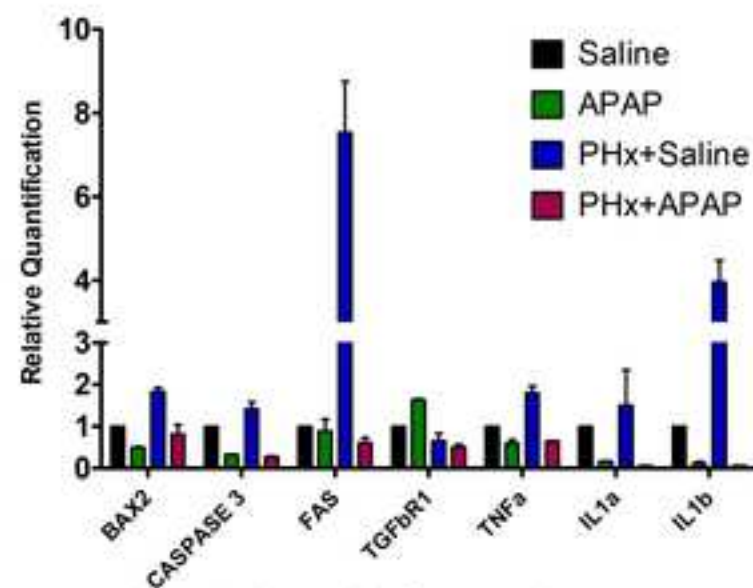
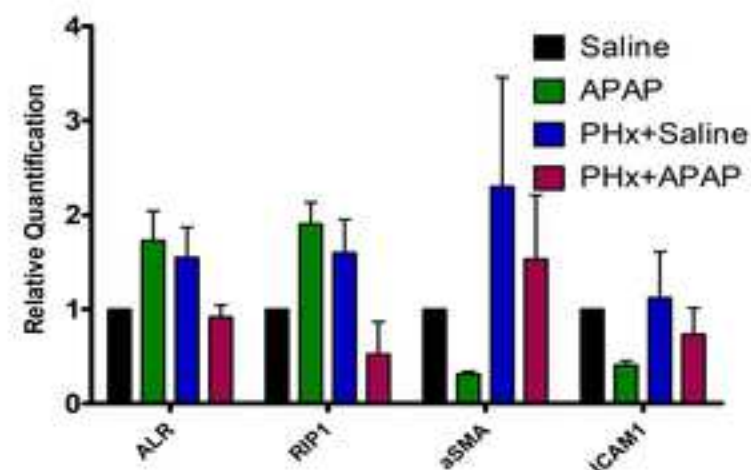


Figure 3

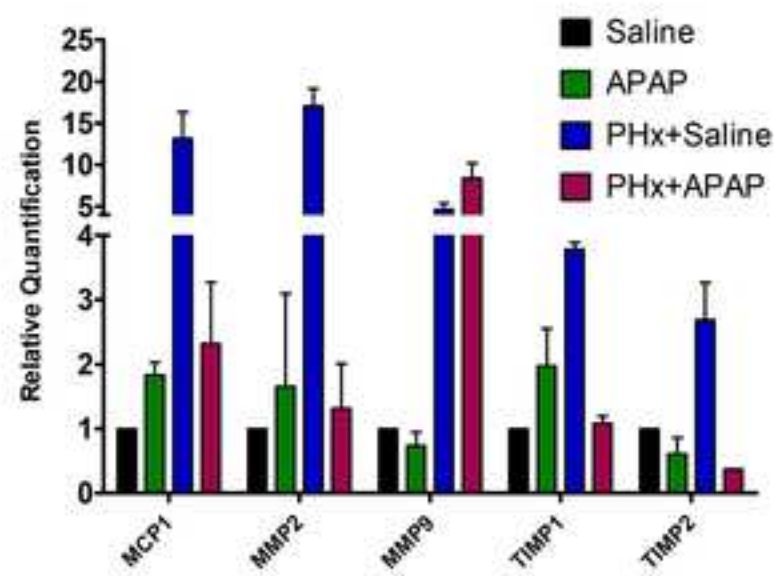




Apoptosis and inflammation genes

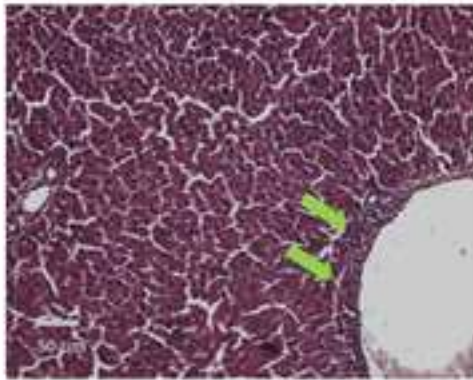


Apoptosis and inflammation genes



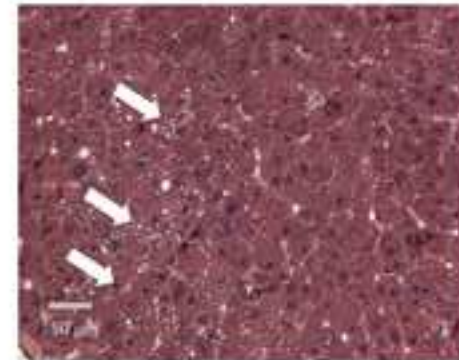
Progression of liver injury genes

Saline (Control)



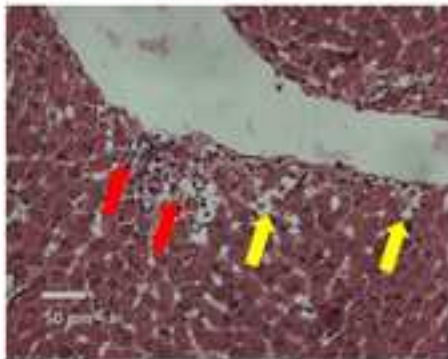
Mostly normal hepatocytes,
mild peri portal infiltration of
inflammatory cells

70% PHx + Saline



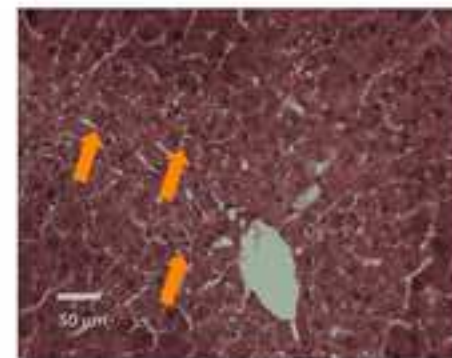
Micro and macro vesicular
fatty degeneration

APAP

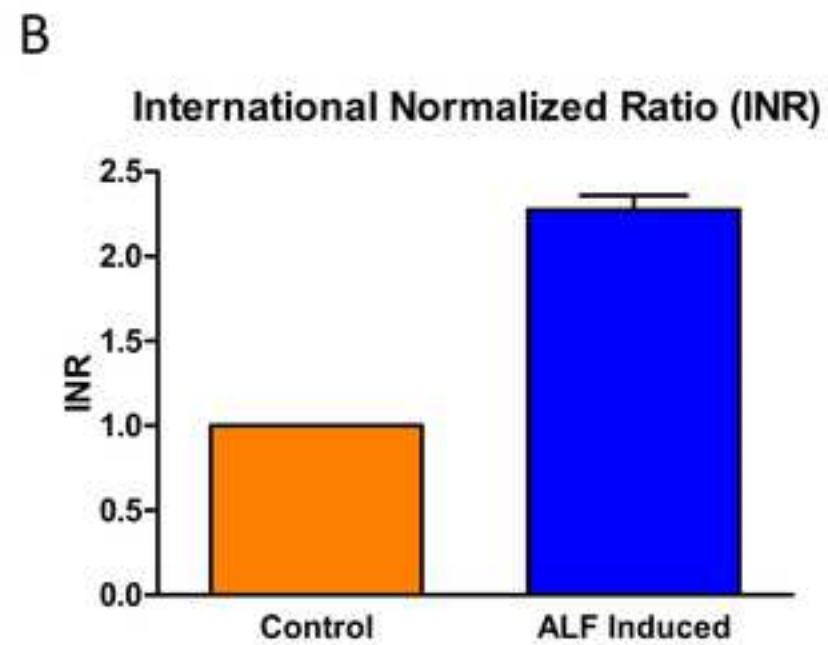
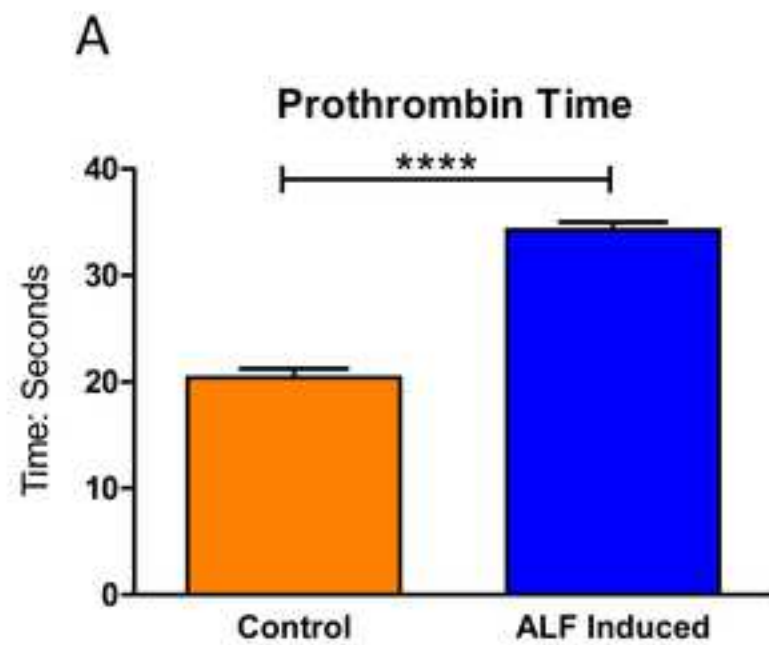


Peri portal inflammation and
necrosis (red), mild
sinusoidal dilatation (yellow)

70% PHx + APAP



Moderate macro vesicular
fatty degeneration



Survival percent after cell Transplantation

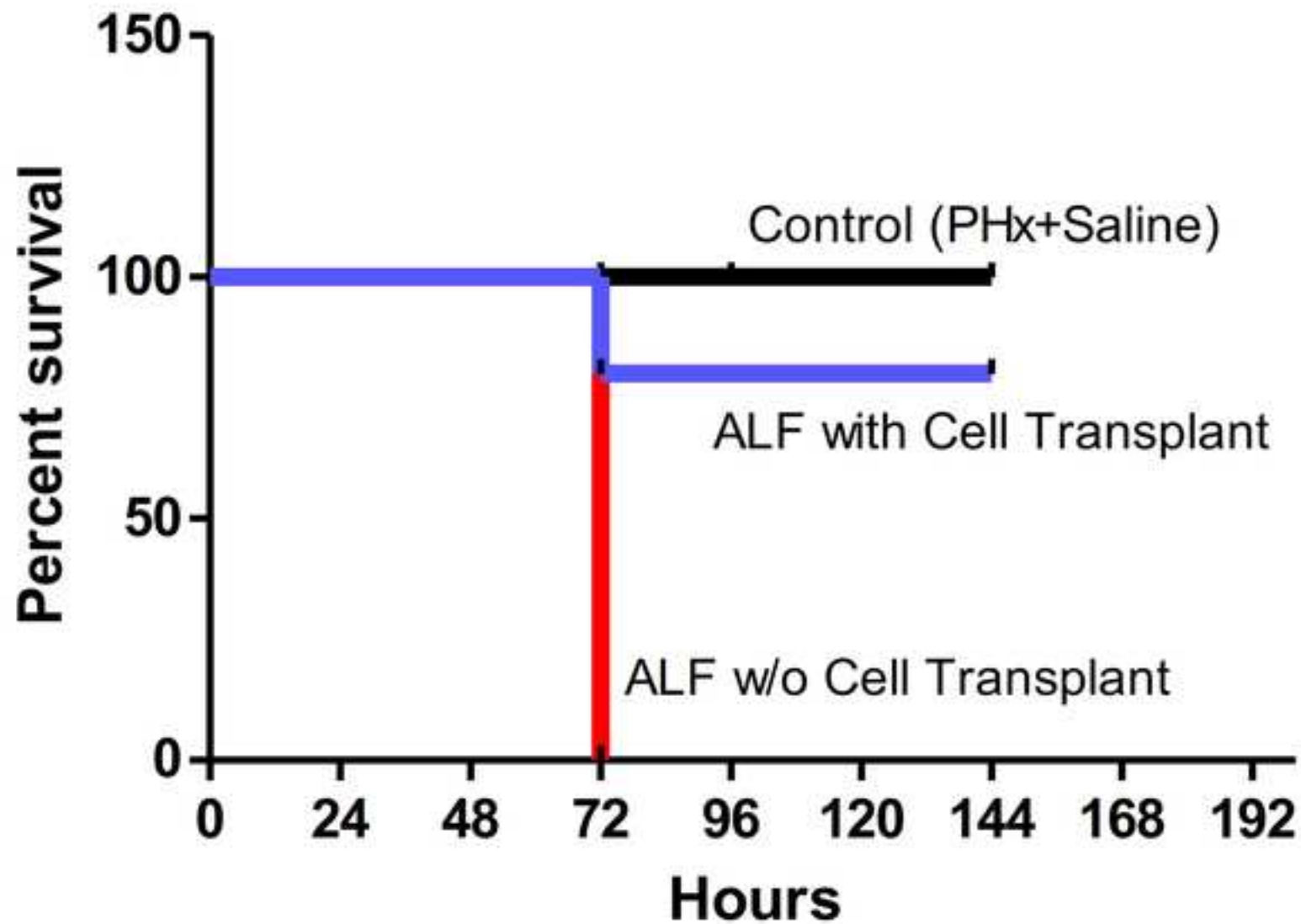
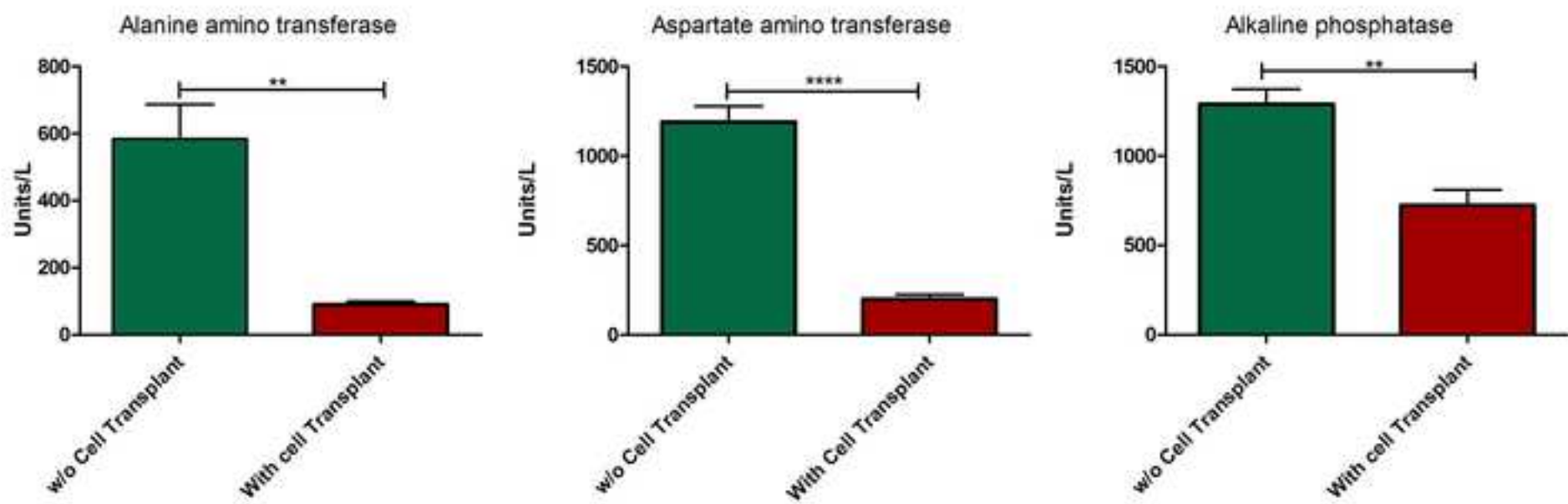


Figure 8



Name of Material/Equipment	Company	Catalog Number
Acetaminophen (Biocetamol)	EG Pharmaceuticals	
Alkaline Phosphatase Kit (DEA)	Coral Clinical System, India	
Automated analyser	Tulip, Alto Santracruz, India	Screen Maaster 3000
Betadine (Povidon-Iodine Solution)	Win-Medicare; India	
Biological safety cabinet (Class I)	Kartos international; India	
Bright Field Microscope	Olympus, Japan	LX51
Cefotaxime (Taxim®)	ALKem ; India	
Cell Strainer	Sigma ; US	CLS431752
Collagenase Type I	Gibco by Life Technologies	17100-017
Cotton Buds	Pure Swabs Pvt Ltd ; India	
Drape Sheet	JSD Surgicals, Delhi, India	
DPX Mountant	Sigma ; US	6522
Eosin Y solution, alcoholic	Sigma ; US	HT110132
Forceps	Major Surgicals; India	
Gas Anesthesia System	Ugo Basile; Italy	211000
Glucose	Himedia, India	GRM077
Hair removing cream (Veet®)	Reckitt Benckiser , India	
Hematoxylin Solution, Mayer's	Sigma ; US	MHS16
Heparin sodium salt	Himedia; India	RM554
Hyaluronidase From Sheep Testes	Sigma ; US	H6254
I.V. Cannula (Plusflon)	Mediplus, India	Ref 1732411420
Insulin Syringes	BD ; US	REF 303060
Isoflurane (Forane®)	Asecia Queenborough	No B506
Ketamine (Ketamax®)	Troikaa Pharmaceuticals Ltd.	
Meloxicam (Melonex®)	Intas Pharmaceuticals Ltd; India	
Micro needle holders straight & curved	Mercian ; England	BS-13-8
Micro needle holders straight & curved	Mercian ; England	BS-13-8
Microtome	Histo-Line Laboratories, Italy	MRS3500
Nylon Thread	Mighty ; India	
Paraformaldehyde	Himedia; India	GRM 3660

Percoll®	GE Healthcare	17-0891-01
Refresh Tears/Eyemist Gel	Allergan India Private Limited/Sun Pharma, India	P3060
RPMI	Himedia; India	
Scalpel	Major Surgicals; India	
Scissors	Major Surgicals; India	
SGOT (ASAT) KIT	Coral Clinical System, India	
SGPT (ALAT) KIT	Coral Clinical System, India	
Shandon Cryotome E Cryostat	Thermo Electron Corporation ; US	
Sucrose	Sigma ; US	S0389
Surgical Blade No. 22	La Medcare, India	
Surgical Board	Locally made	
Surgical White Tape	3M India ; India	1530-1
Sutures	Ethicon, Johnson & Johnson, India	NW 5047
Syringes (1ml, 26 G)	Dispo Van; India	
Trimmer (Clipper)	Philips	NL9206AD-4 DRACHTEN QT9005
Weighing Machine	Braun	
William's E Media	Himedia; India	AT125
Xylazine (Xylaxin®)	Indian Immunologicals Limited	

Comments/Description

No specific Catalog Number (Local Procurement)

No specific Catalog Number (Local Procurement)

Biochemical analyser for liver functional test

No specific Catalog Number (Local Procurement)

No specific Catalog Number (Local Procurement)

cefotaxime sodium injection, No specific Catalog Number (Local Procurement)

No specific Catalog Number (Local Procurement)

No specific Catalog Number (Local Procurement)

No specific Catalog Number (Local Procurement)

No specific Catalog Number (Local Procurement)

Inhalation Anaesthetic

Ketamine hydrochloride IP, No specific Catalog Number (Local Procurement)

No specific Catalog Number (Local Procurement)

No specific Catalog Number (Local Procurement)

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No specific Catalog Number (Local Procurement)

Micropore Surgical Tape

No specific Catalog Number (Local Procurement)

No specific Catalog Number (Local Procurement)

Sedative, Pre-Anaesthetic, Analgesic
and muscle relaxant

Reply to comments:

• *0:00-2:12, 9:09-9:34- There are very thin black borders on the left and right sides of the frame during this time. The clips' scale should be increased slightly so that the video fills the frame entirely.*

Done.

• *Please increase the homogeneity between the written protocol and the narration in the video. It would be best if the narration is a word for word from the written protocol text. For the text, please use American English throughout.*

We have tried our best to keep the homogeneity between the written protocol and the narration except in situations where only a few words could fit in the video clip.

• *Please number the affiliations as 1,2, as done in the text to bring out consistency.*

Done.

• *The protocol should be divided into sections as done in the text. Please divide the protocol into sections by placing the section title cards similar to that in the text; preparation, Pre-operative procedures, Partial hepatectomy (PHx) in the rats to remove 70% mass of the liver, post-operative care, Injection of the drug in partially hepatectomized animals to induce liver failure.*

Done.

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Done.

• *2:48- 3:15: Please do not show the face of the mouse in this clip.*

Done.

• *3:05: Please change the VO to state alternating scrubs of 70% ethanol and povidone iodine-based scrub to bring out clarity.*

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