Journal of Visualized Experiments

Inducing Acute Liver Injury in Rats via Carbon Tetrachloride (CCl4) Exposure Through an Orogastric Tube

--Manuscript Draft--

Article Type:	Invited Methods Article - Author Produced Video
Manuscript Number:	JoVE60695R4
Full Title:	Inducing Acute Liver Injury in Rats via Carbon Tetrachloride (CCl4) Exposure Through an Orogastric Tube
Section/Category:	
Keywords:	Acute liver injury (ALI); carbon tetrachloride (CCl4); hepatic injury; liver damage; rat model; Toxin
Corresponding Author:	Matthew Bokyo, PhD Soroka Medical Center, Ben-Gurion University of the Negev Beer Sheva, Beer Sheva ISRAEL
Corresponding Author's Institution:	Soroka Medical Center, Ben-Gurion University of the Negev
Corresponding Author E-Mail:	matthewboykoresearch@gmail.com
Order of Authors:	Dmitry Frank
	Shiri Savir
	Benjamin Fredrick Gruenbaum
	Israel Melamed
	Julia Grinshpun
	Ruslan Kuts
	Boris Knyazer
	Alexander Zlotnik
	Max Vinokur
	Matthew Bokyo
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Beer Sheva, Israel
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TITLE:

Inducing Acute Liver Injury in Rats via Carbon Tetrachloride (CCl_4) Exposure Through an Orogastric Tube

AUTHORS AND AFFILIATIONS:

Dmitry Frank[1,*], Shiri Savir[1,*], Benjamin F. Gruenbaum[2], Israel Melamed[3], Julia Grinshpun[1], Ruslan Kuts[1], Boris Knyazer[4], Alexander Zlotnik[1], Max Vinokur[1], Matthew Boyko[1]

[1]Department of Anesthesiology and Critical Care, Soroka Medical Center, Ben-Gurion University of the Negev, Beer-Sheva, Israel
[2]Department of Anesthesiology, Yale University School of Medicine, New Haven, CT, USA
[3]Department of Neurosurgery, Soroka University Medical Center and the Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel
[4]Department of Ophthalmology, Soroka University Medical Center and the Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel

*These authors contributed equally.

Corresponding Author:

Matthew Boyko (matthewboykoresearch@gmail.com)

Email Addresses of Co-Authors:

Dmitry Frank (frdima16@gmail.com)
Shiri Savir (yona.shiri@gmail.com)
Benjamin F. Gruenbaum (bengruenbaum@gmail.com)
Israel Melamed (melamedi@bgu.ac.il)
Ruslan Kuts (ruslanKo@clalit.org.il)
Max Vinokur (max.vinokur@gmail.com)
Boris Knyazer (Knyazer@bgu.ac.il)
Julia Grinshpun (juliag7648@gmail.com)
Alexander Zlotnik (AleksZl@clalit.org.il)

KEYWORDS:

acute liver injury (ALI), carbon tetrachloride (CCl_4), hepatic injury, liver damage, rat model, toxin

SUMMARY:

This protocol describes a common and feasible method of inducing acute liver injury (ALI) via CCl_4 exposure through an orogastric tube. CCl_4 exposure induces ALI through the formation of reactive oxygen species during its biotransformation in the liver. This method is used to analyze the pathophysiology of ALI and examine different hepatoprotective strategies.

ABSTRACT:

Acute liver injury (ALI) plays a crucial role in the development of hepatic failure, which is characterized by severe liver dysfunction including complications such as hepatic

encephalopathy and impaired protein synthesis. Appropriate animal models are vital to test the mechanism and pathophysiology of ALI and investigate different hepatoprotective strategies. Due to its ability to perform chemical transformations, carbon tetrachloride (CCl_4) is widely used in the liver to induce ALI through the formation of reactive oxygen species. CCl_4 exposure can be performed intraperitoneally, by inhalation, or through a nasogastric or orogastric tube. Here, we describe a rodent model, in which ALI is induced by CCl_4 exposure through an orogastric tube. This method is inexpensive, easily performed, and has minimal hazard risk. The model is highly reproducible and can be widely used to determine the efficacy of potential hepatoprotective strategies and assess markers of liver injury.

INTRODUCTION:

The frequency of toxic insults to the liver, especially due to alcohol and drug abuse, is increasing. Acute liver injury (ALI) is associated with high mortality rates and has caused clinical concerns[1,2]. Toxic injury leads to death signaling pathways in the liver, resulting in hepatocyte apoptosis, necrosis, or pyroptosis. ALI plays a crucial role in the development of hepatic failure, which is characterized by severe liver dysfunction including complications such as hepatic encephalopathy and impaired protein synthesis[3,4]. Although recent research has increased our knowledge about the physiological and pathological changes accompanying hepatic failure, it has not completely explained the pathomolecular features that affect the mechanisms of cell death. Furthermore, no medications are currently available to reverse the progressive deterioration in ALI patients. Currently, the only significantly effective treatment is liver transplantation[5,6].

In order to investigate the mechanism and pathophysiology of ALI and to test different hepatoprotective strategies, different animal models are used to induce ALI. A preferable animal model of ALI should mimic the pathological process of the disease via a reliable, validated, inexpensive and easy to apply method. Examples of experimental models include hepatotoxic agents, surgical procedures such as total or partial hepatectomy, complete or transient devascularization, and infective procedures[7-9]. Known hepatotoxic substances include galactosamine, acetaminophen, thioacetamide, azoxymethane and CCl_4. Of these, CCl_4 is widely used although it has not yet been well characterized[10-13].

CCl_4 is an organic colorless liquid compound with a sweet smell and almost no flammability at lower temperatures. Exposure to high concentrations of CCl_4 can cause damage to the central nervous system, including deterioration of the liver and kidneys. CCl_4 induces ALI through its biotransformation in the liver, which forms reactive oxygen species. This occurs via the P450 cytochrome enzyme 2E1, forming an active metabolite and resulting in cell damage by macromolecule binding, enhancement of lipid peroxidation and disturbance of intracellular calcium homeostasis[14]. In addition, the CCl_4 model can be used to stimulate the astrocytes at the level of RNA synthesis[15]. This hepatotoxin has been administered by the intraperitoneal, intraportal, oral, and intragastric routes[16].

In this protocol, we describe in detail CCl_4-induced ALI in rats via an orogastric tube. This method induces robust and reproducible ALI that can be used to investigate the pathogenesis of ALI. Determination of liver disease severity is monitored by measurement of serum glutamate-

pyruvate transaminase (GPT), glutamic oxaloacetic transaminase (GOT) enzymes and total bilirubin (TB) as well as definitive histological diagnosis by hematoxylin and eosin (H&E) stained liver tissues. Exposure to CCl_4 through an intragastric access allows for a practical, inexpensive, minimally invasive method with minimal hazard risk.

PROTOCOL:

The experiments were conducted according to the recommendations of the Declarations of Helsinki and Tokyo and to the Guidelines for the Use of Experimental Animals of the European Community. The experiments were approved by the Animal Care Committee of Ben-Gurion University of the Negev.

NOTE: The CCl_4 model has been generated and used in a previous study[17]. The protocol timeline is demonstrated in **Table 1**.

1. Preparing rats for the experimental procedure

NOTE: Select adult male Sprague Dawley rats weighing 300−350 g.

1.1. Obtain approval for experiments from Institutional Animal Care and Use Committee (IACUC).

1.2. Maintain rats at room temperature (22 °C ± 1 °C), with 12 h light and 12 h dark cycles. Provide rat chow and water ad libitum.

1.3. Perform all experiments between 6:00 a.m. and 12:00 p.m.

1.4. Shave the rat and disinfect the skin with alcohol.

2. Determination of serum GOT, GPT, and TB baseline levels

2.1. Anesthesia

2.1.1. Prepare a continuous isoflurane administration system to induce anesthesia. Make sure the vaporizer system is filled with isoflurane.

2.1.2. Anesthetize the rat with 2% isoflurane. Confirm that the rat is fully anesthetized by observing the movement and pedal reflex in response to external stimuli.

NOTE: Use 1−5% isoflurane for anesthesia induction and maintenance.

2.2. Cannulate the tail vein with a 22 G catheter.

2.3. Collect a 0.5 mL blood sample at baseline. Ensure that the blood volume retrieved does not exceed IACUC guidelines.

2.4. Perform blood biochemical analysis including the measurements of serum GOT, GPT and TB, as previously described[18].

NOTE: Examinations of liver enzymes and TB level were carried out in the biochemical laboratory of Soroka Medical Center. Blood samples were analyzed using a fluorescence method on a chemistry analyzer (**Table of Materials**).

3. Induction of acute liver injury in rats

CAUTION: Exposure to high concentrations of CCl_4, including absorption through vapor or skin, can have negative effects on the central nervous system and cause degeneration of the liver and kidneys. Prolonged exposure can cause coma or death.

3.1. Prepare a 50% solution of CCl_4 (**Table of Materials**) by mixing CCl_4 with olive oil as a vehicle in a 1:1 ratio.

NOTE: The solution should be prepared according to IACUC guidelines for non-pharmaceutical grade compounds.

3.2. Induce hepatotoxicity in vivo by CCl_4 administration via an orogastric tube.

3.2.1. Insert a 16 G orogastric tube (3 inches deep) through the oral cavity of the rat.

3.2.2. Expose the rat to different doses of CCl_4 by injecting a syringe with one of the following diluted solutions into the rat's stomach: 1 mL/kg (mild ALI), 2.5 mL/kg (moderate ALI), or 5 mL/kg (severe ALI) of the 50% solution. For the sham-operated control group, expose the rat to 5 mL/kg olive oil only.

4. Determination of serum GOT, GPT, and TB levels after 24 h

4.1. Anesthesia

4.1.1. Prepare a continuous isoflurane administration system to induce anesthesia. Make sure the vaporizer system is filled with isoflurane.

4.1.2. Anesthetize the rats with 2% isoflurane. Confirm that the rat is fully anesthetized by observing the movement and pedal reflex in response to external stimuli.

4.2. Collect blood samples at 24 h from CCl_4 exposure.

4.3. Perform blood biochemical analysis including measurements of serum GOT, GPT and TB.

5. Liver collection for histological examination

5.1. Euthanize the rat by replacing the inspired gas mixture with 20% O_2/80% CO_2. Ensure that CO_2 is delivered at a predetermined rate in accordance with IACUC guidelines.

5.2. Ensure death by checking for lack of heartbeat and confirm by a secondary method in accordance with IACUC guidelines.

5.3. Place the rat on a dissecting board with its dorsal surface facing down and abdomen facing up. Shave the abdomen of the rat.

5.4. With a scalpel, incise the full length of the ventrum skin from the anus to the chin. Separate the skin. Incise the abdominal wall with a scalpel from the anus to the xyphoid cartilage, exposing the abdominal viscera.

5.5. Using scissors and forceps, isolate the liver by dissecting it from its ligaments and attachments. Starting at the liver hilum, carefully perform a hepatectomy by releasing all the liver lobes from attachments. Dissect and cut away all ligaments and blood vessels.

5.6. Transfer the liver into a Petri dish. Fix the liver in a 4% buffered formaldehyde solution (**Table of Materials**) for at least 24 h.

6. Histological examination

6.1. Sample preparation

6.1.1. After fixation, cut the sample into 5 µm thick slice series by microtome sectioning.

6.1.2. Gently place the slices on glass slides with a soft brush, 1 slice per slide.

6.1.3. Perform H&E staining as previously described[19].

6.2. Examine the slices under a microscope at 200x magnification using a 20 mm objective lens (**Table of Materials**).

NOTE: The liver sections should be graded by a specialized pathologist blinded to the treatment protocol. A score of 0 indicates no liver abnormalities, 1–2 indicates mild liver injury, 3–4 indicates moderate liver injury, and 5–6 indicates severe liver injury[20-22].

REPRESENTATIVE RESULTS:

The TB, GOT, and GPT levels significantly increased 24 h after inducing ALI (more at higher CCl_4 doses) compared to sham-operated controls ($p < 0.001$) (**Figure 1**). The levels of TB, GOT, and GPT at baseline were normal and were not significantly different than sham-operated controls. At 24 h, all three interventional groups, 1 mL/kg CCl_4 (1, 1−2), 2.5 mL/kg CCl_4 (3, 3−4), and 5 mL/kg CCl_4 (4, 4−5.75), had a significantly higher histological grading score than the sham-operated

control group (0, 0−0) ($p < 0.05$, data presented as median, 25−75% range). The H&E images of a sham-operated control (**Figure 2A**) and groups exposed to different CCl_4 doses (**Figure 2B–D**) show histopathological changes 24 h after CCl_4 exposure. Disruption of hepatocellular architecture by CCl_4 was demonstrated by a high grade of tissue injury with large fibrous septa deformation, extension of fibers, collagen accumulation, and pseudo lobe separation in liver sections (**Figure 2**).

FIGURE AND TABLE LEGENDS:

Figure 1: Serum TB (A), GOT (B), and GPT (C) levels in blood samples 24 h after exposure to different CCl_4 doses compared to sham-operated controls. Blue bar: control; red bar: 24 h after CCl_4 exposure. The significance of comparisons between CCl_4-exposed rats and unexposed rats are determined using the Mann–Whitney test. A p-value of <0.05 was considered significant.

Figure 2: Histopathological changes in liver tissue stained with H&E after 24 h CCl_4 intoxication in various doses. (**A**) sham-operated control, **(B)** 1 mL/kg CCl_4, (**C**) 2.5 mL/kg CCl_4, and (**D**) 5 mL/kg CCl_4. Scale bar = 50 μm. The distribution of the histological outcomes was predicted by linear regression.

Table 1: Demonstration of the protocol timeline. The various groups of rats at different times include a sham-operated control group, mild ALI (exposure to 1 mL/kg CCl_4), moderate ALI (exposure to 2.5 mL/kg CCl_4), and severe ALI (exposure to 5 mL/kg CCl_4). At time 24 h, serum GOT, GPT and TB levels were measured, and histological examination was performed for all four groups.

DISCUSSION:

In this protocol, CCl_4 is used as a liver toxin to induce ALI in rats. ALI is characterized by loss of hepatic parenchyma and subsequent dysregulation of the liver's metabolic and synthetic functions. Drugs, viruses, toxins, autoimmune diseases, metabolic diseases, and vascular disorders all induce hepatocyte death, and the subsequent inflammatory response contributes to the pathogenesis of ALI.

The initial insult to the liver leads to cytokine production, chemokine release, and subsequent infiltration of inflammatory cells into the liver. Three of the commonly tested biomarkers for ALI evaluation are GPT, GOT and TB levels. GPT and GOT are enzymes measured by activity level while TB level measures liver function by serum concentration. When elevated, serum enzyme activity levels denote injury to hepatocytes or cholangiocytes[23]. Rapid spectrophotometric method was first reported in the work of Arthur Karmen in 1955[24], which allowed for the widespread clinical application of serum enzyme measurement. Since then, GOT and GPT measurements have also been applied to detect hepatocyte injury. GPT is used more frequently, and simultaneous GPT testing usually reveals redundant results. The increase in activity levels of GOT and GPT between the release rates and the clearance rates from injured cells can be used to measure approximately the rate of injury to the cells. When the injured liver cells cause the liver to fail in its normal activities, such as processing and removing bilirubin as bile, this indicates that the ALI is more severe.

There are several steps in the protocol that are critical and merit careful consideration. Most protocols require serum biomarker testing before and after exposure to the investigative agent, as elevations in serum enzyme concentration levels are common. However, due to fluctuations in the timing of elevated ALT, several tests should be conducted periodically to detect any elevation. In this protocol, we chose to test GOT, GPT and TB levels at baseline and 24 h after exposure to the toxin. According to recent studies, the levels of these biomarkers correlated well with the severity of ALI during this time interval[17]. As shown in **Figure 1**, levels of blood GOT, GPT, and TB were elevated in all samples 24 h after inducing ALI. This indicates that the model has quantified outcomes in a very short time interval since exposure to CCl_4. One should take into account that in severe ALI the liver loses its ability to synthesize GOT and GPT. Therefore, in these cases these enzymes may lack their predictive value as demonstrated in the literature.

Histological findings of rats exposed to CCl_4 are characterized by ballooning of cells, centrilobular necrosis and the presence of Councilman bodies[25]. In this model there was widespread damage shown to be proportional to the dose of CCl_4 administered.

This method of inducing ALI via orogastric CCl_4 exposure has numerous advantages. It is simple, inexpensive, and with minimum hazard risk. The protocol provides significant results in a very short time interval. The model is highly reproducible and can be commonly used to determine the efficacy of potential hepatoprotective strategies and assess markers of liver injury.

It is important to note that CCl_4 mainly affects the central zone of the liver, which does not match the massive necrosis typically seen in human liver failure. Moreover, CCl_4 is not completely metabolized in the liver, and some of the nonmetabolized CCl_4 can damage other organs, including lungs and kidneys[16]. In addition, due to different levels of cytochrome P450 development and efficacy, there is a large variation in sensitivity depending on species and age[26]. Despite these limitations, the method of orogastric CCl_4-induced ALI still serves as a valuable rodent model.

ACKNOWLEDGMENTS:
The authors gratefully acknowledge Bertha Delgado, Department of Pathology, Soroka Medical Center, Faculty of Health Sciences, Ben-Gurion University of the Negev, for her help in the laboratory as well as in the histology analysis.

DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

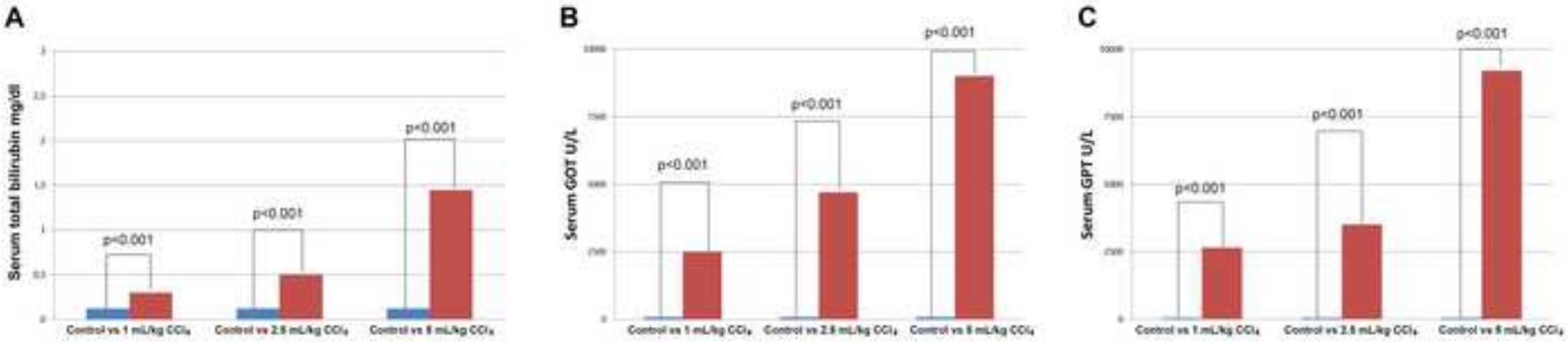
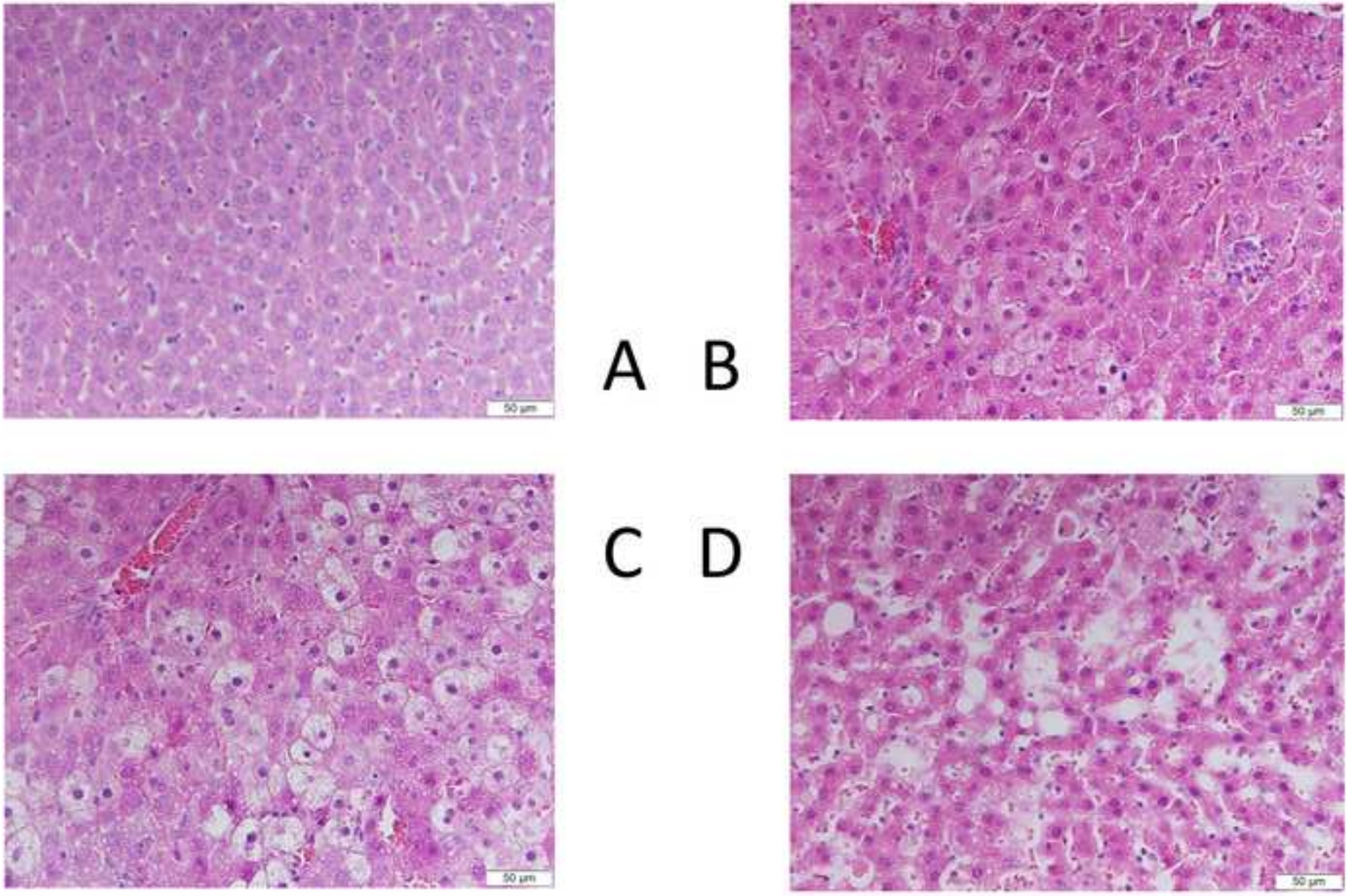


Figure 2



Groups	0 hours	24 hours
Sham (15 rats)	GOT, GPT, TB baseline level	GOT, GPT, TB level
Mild ALI (15 rats)		
Moderate ALI (15 rats)	CCl_4 exposure for ALI groups and olive oil for sham group	Histological examination
Severe ALI (18 rats)		

Name of Material/Equipment	Company	Catalog Number
22 G catheter BD Neoflon TM	Becton Dickinson Infusion Therapy AB	
4% buffered formaldehyde solution	Sigma - Aldrich lab materials technologies	
BD Microtainer SST TM Tubes	Becton Dickinson and Company	
Carbone tetrachloride	Sigma - Aldrich lab materials technologies	CAS 56-23-5
Isofluran, USP 100%	Piramamal Critical Care, Inc	
Olympus AU2700 Chemistry-Immuno Analyzer	Olympus (MN, USA)	
Olympus BX 40 microscope	Olympus	
RAT Feeding Needles	ORCHID SCIENTIFICS	
SYRINGE SET 1 and 2 ml MEDI - PLUS	Shandong Zibo Shanchuan Medical Instruments Co., Ltd	

Comments/Description

Analysis of blood samples was done by the fluorescence method

Attn: Xiaoyan Cao, Ph.D.

Review Editor

Journal of Visualized Experiments (JoVE)

JoVE60695R3

Title: Inducing acute liver injury in rats via carbon tetrachloride (CCl4) exposure through an orogastric tube

Dear Dr. Cao,

Please find attached a revised version of the manuscript JoVE60695R3, with changes highlighted in yellow. Below is a point-by point response to each of the reviewer's comments. We very much hope that this revised manuscript is now suitable for publication in the JoVE.

We thank you and the reviewers for your consideration.

Best regards,

Matthew Boyko, PhD

Editorial comments:

Changes to be made regarding the manuscript:
1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested.

Thank you. This has been done.

2. Please address specific comments marked in the attached manuscript (results and figure legends).

All of these comments have been addressed and incorporated into the manuscript.

3. Figure 1: Please change "ml" to "mL" and include a space between numbers and their units (1 mL/kg, 2.5 mL/kg, 5 mL/kg). Please change "CCL4" to "CCl4 (4 should be subscript)".

Done.

4. Please upload Table 1 as an .xlsx file.

Done.

Changes to be made regarding the video:
1. @5:00: Please replace with the updated Figure 1.

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