The Effect of Hyperbaric Oxygen Therapy (HBOT) on Liver Function and Fibrosis Using a Rat Model of Carbon Tetrachloride (CCl_4)-Induced Liver Injury: An Experimental Study

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Abstract

Significance: Hyperbaric Oxygen Therapy (HBOT) is an intervention in which an individual breathes in nearly 100% oxygen inside a hyperbaric chamber. Numerous studies support HBOT as an efficient therapeutic option to control progress of diseases due to its multi-modal properties. Currently, there is paucity of data with regard to the effect of HBOT on liver diseases. *Objective:* The objective of this study is to investigate the effect of HBOT on liver function and fibrosis using a rat model of carbon tetrachloride (CCl_4)-induced liver injury. *Methodology:* Fifty-one adult Sprague Dawley rats with CCl_4-induced liver injury were randomized into three groups: (1) Pilot (sacrificed immediately after liver injury induction), (2) Control (exposed to room air), and (3) Experimental (exposed to 12 consecutive 120-minute daily sessions of HBOT 2.8 ATA). Outcome measures are serologic parameters of liver function and histopathologic evaluation of liver fibrosis. *Results:* There is significant difference between control and hyperbaric oxygen-treated group in improving AST (p-value <0.001) and ALT (p-value <0.001) among rats with CCl_4-induced liver injury. On histopathologic evaluation, rats exposed to HBOT revealed very strong evidence of improving degree of hepatic fibrosis (p-value <0.001). Majority of rats (94%) exposed to HBOT revealed mild hepatic fibrosis. Rats in the control group showed 76% having moderate fibrosis and 24% having severe fibrosis. *Conclusion:* HBOT exhibited very strong evidence in improving ALT, AST and degree of hepatic fibrosis among adult Sprague Dawley rats with CCl_4-induced liver injury.

Keywords: experimental study, liver injury, liver fibrosis, HBOT

Introduction

Hyperbaric oxygen therapy (HBOT) is defined by the Undersea and Hyperbaric Medicine Society as an intervention in which an individual breathes in close to 100% oxygen intermittently while inside a hyperbaric chamber that is pressurized to greater than sea level pressure (one atmosphere absolute [ATA]).[1] Since its inception in 1662 and practical application in 1930, application of hyperbaric oxygen therapy has continued to elicit controversy.[2] During HBOT, the increased concentration and the partial pressure of oxygen provide increased oxygenation of the whole body.[3]

In recent decades, numerous studies supported HBOT as an efficient therapeutic option to control progress of various diseases due to its anti-inflammatory, anti-oxidant, anti-aging, and anti-bacterial properties, as well as its angiogenesis and regeneration effects. It is highly used in hypoxia-related injuries. HBOT has also been clinically established as a widely used therapy for patients with carbon monoxide poisoning, decompression sickness, arterial gas embolism and problematic wounds. HBOT is also an important adjunctive therapy to treat diseases accompanied by impaired oxygen delivery.

In the liver, HBOT has been studied in hepatic artery thrombosis, acute liver injury, non-alcoholic steatohepatitis, and liver-related cancer. The beneficial effects of HBOT in the liver are mainly attributed to its anti-oxidation, anti-inflammation, regeneration and heme oxygenase-1 (HO-1) properties, which seem to be closely involved in HBOT-mediated protection.[1]

Liver injury can be induced by diverse etiologies, which include hepatotropic viruses, chemicals, alcohol and drug abuse, autoimmune disorders, cholestasis, and metabolic diseases. Liver injury induced by carbon tetrachloride (CCl_4) causes oxidative stress via lipid peroxidation. It is metabolically activated by cytochrome p450 2E1, which produces trichloromethyl radicals. CCl_4 stimulates liver injury through hepatocellular DNA damage, inflammation, apoptosis and fibrosis. Further liver damage occurs from exposure to reactive oxygen radicals released from activated Kupffer cells.[1,3] Chronic insult to the liver through this mechanism can cause chronic liver disease.

Chronic liver disease (CLD) refers to a long-term pathological process of continuous destruction of liver parenchyma and its gradual substitution with fibrous tissue. It is a major cause of morbidity and mortality in many countries.[4,5] Liver fibrosis is a common result of the inflammation-damage-repair response following different types of chronic insult to the liver. In patients who develop liver fibrosis, the majority ultimately develop liver cirrhosis, decompensated liver disease, and hepatocellular carcinoma (HCC). HCC is a dominant complication of CLD and cirrhosis, with the third highest death rate among malignancies in the world.[5] Information on the stage of hepatic fibrosis is essential for making a prognosis and deciding on anti-fibrosis treatment.[4]

Currently, there is paucity of data with regard to the beneficial effects of HBOT on liver diseases. The main objective of this experimental study is to investigate the effect of HBOT on liver function and fibrosis, using a rat model of CCl_4-induced liver injury. Specifically, the study aims to (1) compare the serological parameters of liver function between experimental group rats (with CCl_4-induced liver injury exposed to HBOT) against controls (exposed to room air), particularly: alanine transaminase (ALT), alanine aspartate (AST), total bilirubin, conjugated bilirubin, unconjugated bilirubin, alkaline phosphatase, total protein, albumin, globulin, platelets, and prothrombin time; and (2) using the IASL (International Association for Study of the Liver) scoring system, compare the histologic stage for hepatic fibrosis of rat livers with CCl_4-induced injury exposed to HBOT (experimental group) as against those of rats with CCl_4-induced liver injury exposed to room air only (controls).

Sample Size Estimation

Sample size was computed using the formula $n=1+2C(s/d)^2$ based on the parameter assumptions of significance level at 0.05 with a power of 90%, and a constant C of 10.51 on a two-tailed alternative hypothesis. Sample size was calculated at 16.41 animals in each group. A total of 51 rats were used in the study, equally divided into three groups: 17 rats for the pilot study group (rats that were sacrificed immediately after carbon tetrachloride liver injury induction), 17 rats for the control group (rats with desired degree of hepatic injury and exposed to room air only), and 17 rats for the experimental group (rats with desired degree of hepatic injury and exposed to HBOT).

Methodology

Animal Maintenance and Regulatory Compliance

Rats were obtained from the Philippine Department of Science and Technology (DOST) in whose animal facility these animals were grown. The hospital Institutional Animal Care and Use Committee and the Bureau of Animal Industry approved the study in accordance with RA 8485 (Animal Welfare Act of the Philippines) and the institution's guide for use of laboratory animals.

During the course of the study the rats were maintained under St. Luke's Medical Center Research and Biotechnology (SLMC-RBD) animal testing quarantine protocol. They were housed in a separate area under standard conditions dictated by SLMC-RBD protocol, including cage cleaning method, humidity, ventilation, room temperature regulation at 22-24^{0}C and a 12-hour light/dark cycle.

All animals were fed with standard diet and water, but were fasted for eight hours prior to blood collection, hepatectomy and animal euthanasia.

Induction of Hepatic Injury

CCl_4-induced injury to the liver causes lipid peroxidation by trichloromethyl radicals leading to hepatocellular membrane damage. Liver injury was

produced according to the Ozdogan Protocol. CCl_4 was administered by the primary investigator together with the institution's resident veterinarian. Ten percent CCl_4 was dissolved in olive oil and given by intraperitoneal injection three times a week, according to the following schedule: 0.3 ml/kg in the first week, 0.7 ml/kg in the second week, and 1.0 ml/kg for the next two weeks.

Pilot Study Model to Establish Desired CCl_4-induced Liver Injury

To represent baseline liver function and establish histopathologic liver injury status prior to the start of experiment, rat model with CCl_4-induced liver injury was used. Seventeen adult rats weighing 250 to 300 grams comprised the pilot study group. After four weeks of hepatic injury induction, these rats underwent blood collection and hepatectomy followed by animal euthanasia. Blood and liver specimens were submitted for serologic and histologic evaluation.

Process flow for the study is shown in **Figure 1.**

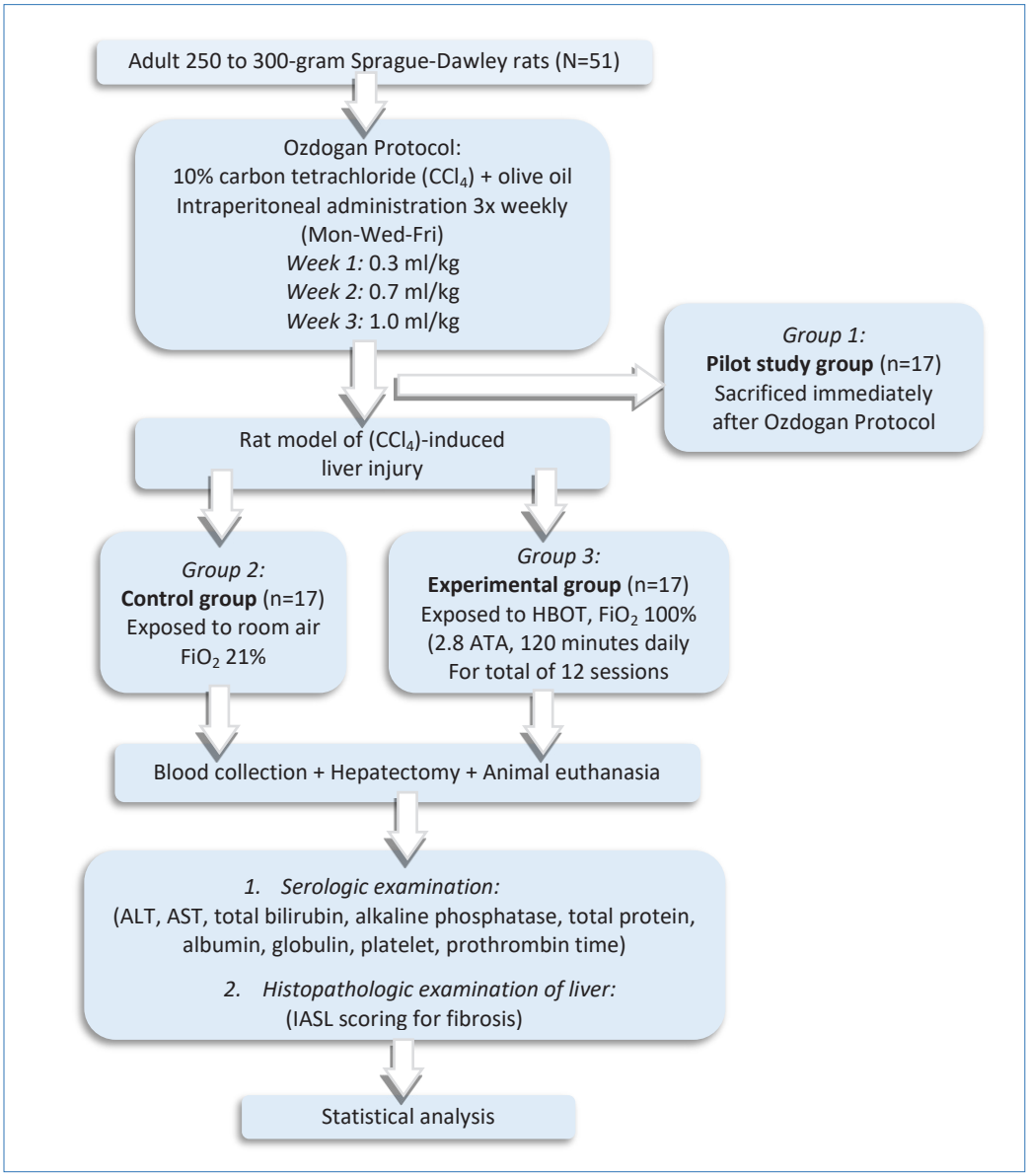

Figure 1. Process flow of study and distribution of subjects into (1) pilot study, (2) control group, and (3) experimental group

Description of Experiment Procedure

After four weeks of CCl_4 administration to induce the desired degree of hepatic injury, rats were assigned by simple randomization technique into two groups: 17 rats in the control group were exposed to room air; and 17 rats in the experimental group were exposed to HBOT.

Experimental and control rats were brought down from the Animal Facility to the St. Luke's Medical Center-Wound Care and Hyperbaric Oxygen Therapy Unit, where the HBOT monoplace chamber (*Perry Sigma 34*) (**Figure 2**) has been prepared and set up by the HBOT nurse. For the experimental group, treatment regimen with HBO was initiated (2.8 ATA for 120 minutes), done daily for a total of 12 sessions. Rats in the control group received no intervention.

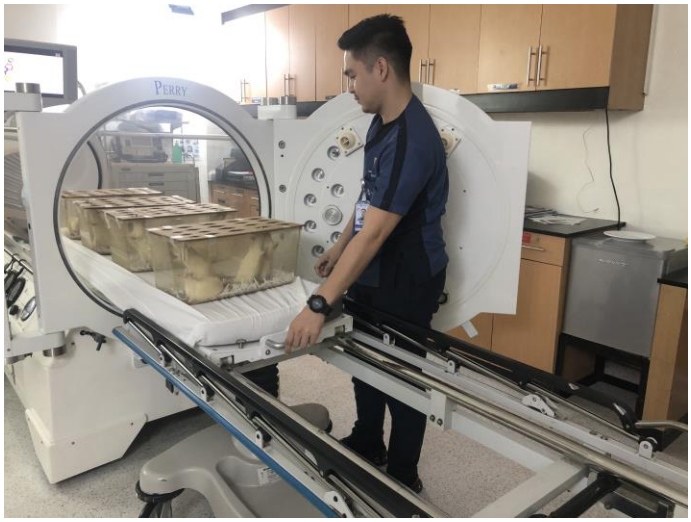

Figure 2. Experimental rats undergoing HBOT

After the 12th session, animals were ready to undergo blood extraction and hepatectomy followed by animal euthanasia. They were restrained in supine position and deeply anesthetized with tiletamine hydrochloride and zolazepam hydrochloride injection (Zoletil®) at a dose of 70 mg/kg, allowing them to breathe spontaneously during the procedure. Blood samples were obtained via intracardiac blood collection technique and sent to the laboratory for serologic evaluation of liver function. Hepatectomy was likewise performed by excision of the entire liver. Specimens were fixed in 10% formaldehyde and submitted for histopathological evaluation of liver fibrosis.

Hyperbaric Oxygen Therapy (HBOT)

The regimen dose used in this study (2.8 ATA, 120 minutes daily for total of 12 sessions), as patterned from previous HBOT studies in rats, is proven safe. This dose prevents animal mortality secondary to hyperbaric oxygen toxicity.[1,2,3] Previously reported hyperbaric oxygen toxicity in some earlier studies was due to limited clinical experience with hyperbaric oxygen application.[1] Toxicity was primarily due to the initiated free radical chain reaction by oxygen, which was then aggravated spontaneously with consequent lipid peroxidation, eventually leading to death. The condition leading to hyperbaric oxygen toxicity was observed with giving more than 3 ATA. In clinical application, however, HBOT is always controlled under 3 ATA.[1] Oxygen pressure is raised up to 10 to 15 times above its normal level when the patient breathes in 100% oxygen at 2.8 ATA.[3]

Animal Euthanasia

The pilot study group rats were immediately euthanized after the fourth week of induction of liver injury following Ozdogan protocol. The control group and experimental group rats were euthanized on the seventh week blood extraction and hepatectomy. The principal author together with the resident veterinarian

of St. Luke's Medical Center administered the carbon tetrachloride to induce liver injury, blood extraction, hepatectomy and animal euthanasia.

Blinding

A blinded medical technologist ran the blood samples for statistical analysis. A blinded veterinary pathologist performed the histopathologic evaluation of liver fibrosis using IASL Scoring. The biostatistician who analyzed the data was also blinded.

Description of Outcome Measures

Primary Outcome Measures:

1. Serological parameters of liver function

Blood samples were obtained using intracardiac blood collection technique before rats were euthanized. Serological parameters of liver function were the following: serum ALT (in *U/L*), AST (in *U/L*), total bilirubin (in *mg/dL*), conjugated bilirubin (in *mg/dL*), unconjugated bilirubin (in *mg/dL*), alkaline phosphatase (in *g/dL*), total protein (in *g/dL*), albumin (in *g/dL*), globulin (in *g/dL*), platelets (in 10^9/*L*), and prothrombin time (in *INR*). Test were done by a blinded medical technologist using the following commercially available machines of Diagnostic Veterinary Laboratories (DVL) as accredited by the Republic of the Philippines Department of Science and Technology:

- Hematology: *Mindray Vet Hematology Analyzer*, Mindray, Shenzhen, China;
- Clinical chemistry: *Fully Automated Biochemistry Analyzer*, E-lab Biological Science and Technology, Nanjing City, China;
- Coagulation factors: *Healvet Veterinary Coagulation Analyzer*, Guangzhou Wondfro Biotechnology, Guangzhou, China.

2. Histopathological evaluation of liver fibrosis

Rat livers were extracted and sent for histopathology using basic hematoxylin and eosin (H&E) staining. A blinded veterinary pathologist compared the histopathological features of the samples using the International Association for Study of the Liver (IASL) scoring system for histopathological stage of fibrosis; *Grade 0:* No fibrosis; *Grade 1:* Mild fibrosis (periportal fibrotic expansion); *Grade 2:* Moderate fibrosis (periportal septae, more than one septum); *Grade 3:* Severe fibrosis (portal-central septae); and *Grade 4:* Cirrhosis. Standard pictographs of the micro-sections were obtained and analyzed using the same image editing software (Adobe Photoshop 7.0; Adobe Systems, Inc.).

Data Analysis

Frequency data were reported as counts and percentages while continuous data were reported using means and standard deviations. Statistical analysis of frequency data was conducted using Chi-square test. All continuous data were first tested for normality, then *t*-test for two independent groups assuming equal variance was used. All computations were done using Microsoft Excel data calculator.

Ethical Considerations

The hospital Institutional Animal Care and Use Committee (IACUC) and Bureau of Animal Industry approved the study in accordance with the RA 8485 (The Animal Welfare Act of the Philippines) and the institution's guide for use of laboratory animals. All anesthetic agents were administered according to the approved rodent anesthesia and analgesia formulary. Animal euthanasia was conducted humanely, using deep anesthesia.

Biosafety

The Biosafety Review Committee (BRC) of St. Luke's Medical Center approved the study in accordance with universal recommendations when handling hazardous substances. All personnel who handled CCl_4 during aliquot preparation, handling and administration to mice underwent biosafety/biosecurity training and certification (**Table 1**).

Precautions and Biosecurity

During handling and preparation of the CCl_4 aliquot, wearing of proper personal protective equipment was necessary, including the use of mask, gown, gloves, and goggles/eye shield. Material safety data sheets (MSDS) were available and accessible.

Facility Management

The study was conducted at St. Luke's Medical Center Molecular Diagnostics Laboratory and Animal Laboratory. Biosafety cabinet was not needed, as the preparation of aliquot was handled using a fume hood. In case of spillage of carbon tetrachloride, personnel

protection (protective clothing, safety goggles, rubber gloves and respiratory protective device) was safeguarded. Small quantities CCl_4 were disposed of by evaporation in a fume cupboard or in a safe, open area. Hand washing facilities and eye wash stations were available within the work area. Access to the laboratory was limited only to persons advised of the nature of the CCl_4 in this research.

Transport of Hazardous Material

Handling and preparation of the aliquot of carbon tetrachloride were done using a fume hood located at the St. Luke's Medical Center Molecular Diagnostics Laboratory. Once prepared, the aliquot of CCl_4 was placed in labelled, airtight container in a well-ventilated place at a temperature below 30°C and protected from light. This was transported to the St. Luke's Medical Center Animal Facility/Laboratory using triple packaging system, consisting of three layers: the primary receptacle, the secondary packaging and the outer packaging.

Disposal of Animal Carcasses

Carcasses of rats used in the study were autoclaved prior to disposal. They were then disposed of using sealable yellow plastic bags (infectious waste) even if these were not treated with any infectious agents.

During Hyperbaric Oxygen Therapy Session

At the HBOT Unit, the animals were placed in clean cages with plastic liners to catch fecal material and urine. In the event of a spill of infectious or potentially infectious material (rat urine/feces), the following spill clean-up procedure was used (WHO recommendation):

1. Use of gloves and protective clothing;
2. Spill covered with cloth or paper towels to contain it;
3. Disinfectant poured over paper towels and immediate surrounding area.
4. Disinfectant applied concentrically from the outer margin of the spill area working toward the center;
5. After appropriate amount of time (e.g., 30 min), materials cleared away. Broken glass or sharps collected using dustpan or stiff cardboard and deposited into a puncture-resistant container;
6. Cleaning and disinfection of the area of spillage (if necessary, steps 2 to 5 repeated);
7. Contaminated materials disposed of into a leak-proof, puncture-resistant waste disposal container;
8. After disinfection, competent authority informed.

Table 1. Project hazard carbon tetrachloride (CCl_4) management

Handling, Storage and Disposal	
Handling / Storage	Stored in labelled, air-tight containers in a well-ventilated place protected from light, at a room temperature below 30^0C, stored separately from chemically active materials.
Disposal	Small quantities of CCl_4 disposed of by evaporation in a fume cupboard or in a safe open area.
Preparation and Handling of CCl_4 Aliquot	
Location of CCl_4	St. Luke's Medical Center, Molecular Diagnostics Laboratory
Biosafety Level	Level 1
Containment Device	Fume hood
During Administration of CCl_4 Aliquot to Rats	
Location of CCl_4	St. Luke's Medical Center, Animal Laboratory
Biosafety Level	Level 1
Containment Device	Not needed

Results

This experimental study was conducted to investigate the effect of hyperbaric oxygen therapy (HBOT) on liver function and fibrosis using a rat model of carbon tetrachloride (CCl_4)-induced liver injury.

It is of noted that 17 rats under the pilot study group revealed significant hepatic damage, with mean ALT that was 11-12 times elevated than the upper limit of normal and mean AST that was 4-5 times elevated than the upper limit of normal. All other liver parameters were noted to be within acceptable limits (**Table 2**).

Table 2. Serologic parameters of liver function after CCl_4 administration

Liver Parameters	Normal Limit *(range)*	Pilot Study *(mean ± SD)*
ALT, *U/L*	17.5 – 30.2	388.67 ± 30.50
AST, *U/L*	45.7 -80.8	413.71 ± 35.99
Total bilirubin, *mg/dL*	0.20 – 0.55	0.32 ± 0.08
Alkaline phosphatase, *g/dL*	56.8 - 128	110.18 ± 16.34
Total protein, *g/dL*	5.1 – 6.5	6.07 ± 0.18
Albumin, *g/dL*	2.6 – 3.5	3.32 ± 0.08
Globulin, *g/dL*	2.5 – 3.0	2.75 ± 0.15
Platelet, 10^9/*dL*	923 -1580	1169.65 ± 168.37
Prothrombin time, *INR*	0.8 – 1.2	0.85 ± 0.05

Histopathologic evaluation to establish the degree of induced liver injury was also done, which revealed that the greater majority, around 88% of rats' liver, developed severe liver necrosis and only a little more than 10% had moderate liver necrosis (**Figure 3**).

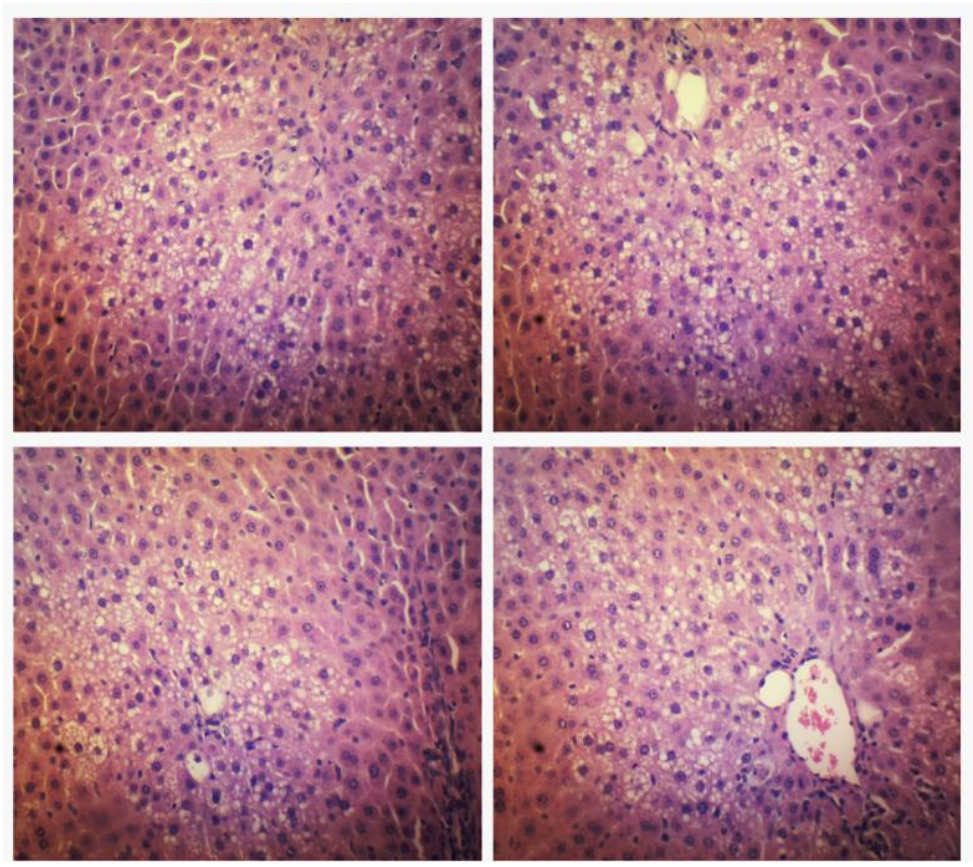

Figure 3. Histopathologic evaluation (H&E) of the liver after CCl_4 administration showing moderate (two out of 17 rats, 11.76%) and severe (15 out of 17 rats, 88.24%) liver necrosis.

This study showed that there is a significant difference between control and HBOT group in improving AST and ALT among rats with CCl_4-induced liver injury. Rats exposed to HBOT revealed very strong evidence of improved ALT and AST levels compared to controls. The mean ALT level of rats in the experimental group revealed 0-1 time more than the upper limit of normal, compared to controls which was 1-2 times

more than the upper limit of normal. On the other hand, the mean AST level of experimental group rats revealed 1-2 times elevated than the upper limit of normal compared to the controls which was 2-3 times elevated than the upper limit of normal. All other liver parameters revealed no significant difference between rats in the experimental and control groups (**Table 3**).

Table 3. Serologic parameters of liver function between experimental and control groups after CCl_4 administration

Liver Parameters	Normal Limit *range*	Experimental *mean ± SD*	Control *mean ± SD*	*p* value
ALT, *U/L*	17.5 – 30.2	55.28 ± 6.89	69.18 ± 7.90	**<.001**
AST, *U/L*	45.7 -80.8	195.53 ± 30.94	248.53 ± 28.31	**<.001**
Total bilirubin, *mg/dL*	0.20 – 0.55	0.33 ± 0.07	0.31 ± 0.12	0.293
Alkaline phosphatase, *g/dL*	56.8 - 128	81.29 ± 15.33	82.18 ± 10.57	0.435
Total protein, *g/dL*	5.1 – 6.5	6.10 ± 0.32	6.01 ± 0.27	0.176
Albumin, *g/dL*	2.6 – 3.5	3.21 ± 0.12	3.16 ± 0.11	0.162
Globulin, *g/dL*	2.5 – 3.0	2.89 ± 0.22	2.84 ± 0.22	0.235
Platelet, 10^9/*dL*	923 -1580	1313.00 ± 158.79	1288.06 ± 243.70	0.336
Prothrombin time, *INR*	0.8 – 1.2	0.81 ± 0.02	0.80 ± 0.00	0.166

Histopathologic evaluation to compare the degree of induced liver injury was also done. Rats exposed to HBOT revealed very strong evidence of improved liver fibrosis compared to controls. It can be noted that majority (94%) of rats exposed to HBOT revealed mild hepatic fibrosis. In contrst, 76% of rats in the control group developed moderate fibrosis, and 24% sustained severe fibrosis (**Table 4**).

Table 4. Histopathologic description of experimental and control groups

Fibrosis	Experimental *n (%)*	Control *n (%)*	*p* value
Mild	16 (94.12)	0 (0,00)	**<0.001**
Moderate	1 (5.88)	13 (76.47)	
Severe	0 (0.00)	4 (23.53)	

Discussion

Rats with hepatic injury induced by CCl_4 and exposed to HBOT revealed a very strong evidence of improved ALT, AST and degree of hepatic fibrosis compared to controls (*p*-value <0.001).

The above findings were compatible with the reported mechanism of CCl_4-induced liver injury, causing oxidative stress via lipid peroxidation by trichloromethyl radicals, which then leads to hepatocellular DNA damage, inflammation, apoptosis, and activation of hepatic stellate cells (HSCs) triggering fibrogenesis. This was comparable to the reported general mechanism scheme of oxidative stress induced by various factors on liver disease such as alcohol, drugs, viruses, environmental toxins, obesity and insulin resistance[1,3] (**Figure 4**).

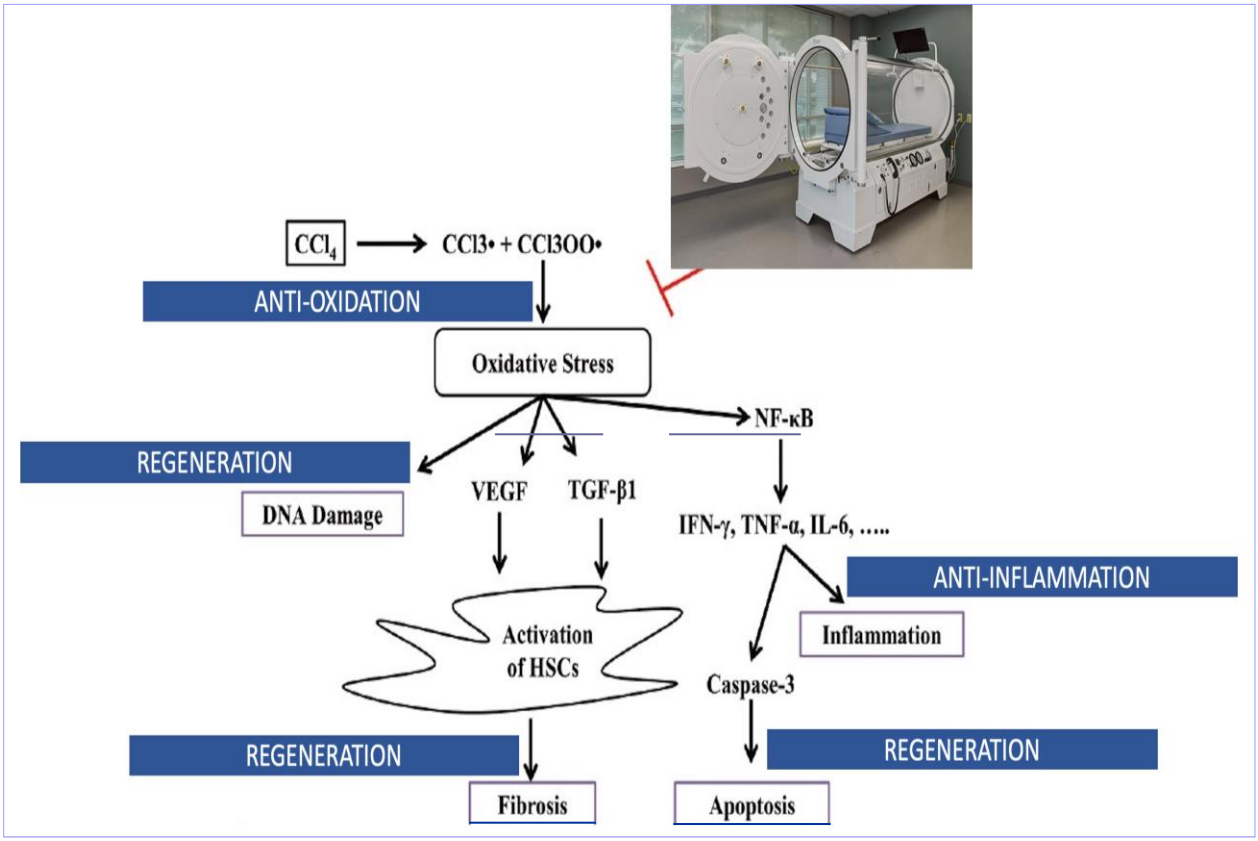

Figure 4. Mechanism of liver injury induced by CCl_4 and reported beneficial properties of HBOT (blue boxes)

Conclusion

HBOT exhibited very strong beneficial evidence in improving ALT, AST and degree of hepatic fibrosis among rats with CCl4-induced liver injury.

Authors' Contributions

MJH Navarro and EM Bondoc conceived and planned the experiment. MJH Navarro prepared the research proposal, methodology and performed needed calculations. JG Cervantes and IHY Cua verified analytical methods. MJH Navarro and EM Bondoc carried out the experiment. JG Cervantes and IHY Cua contributed to the interpretation of the results. MJH Navarro took the lead in writing the manuscript with support from EM Bondoc, JG Cervantes, and IHY Cua. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Conflict of Interest

All JPG peer reviews are blinded. Dr. EM Bondoc, as co-author and at the same time JPG's editor-in-chief, inhibited himself from the review process and acceptance of this paper.

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