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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Significance: Hyperbaric Oxygen Therapy (HBOT) is an intervention in which an individual breathes near 100% oxygen while inside a hyperbaric chamber. Numerous studies supported HBOT as an efficient therapeutic option to improve progress of diseases due to its multi-modal properties. Currently, there is paucity of data with regards to the effect of HBOT on liver diseases. 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:

Study Population: Fifty-one, adult, Sprague Dawley rats with CCl_4-induced liver injury

Intervention: Rats were randomized into groups: *Pilot* (sacrificed immediately after liver injury induction), *Control* (exposed to room air) and *Experimental* (exposed to HBOT - 2.8 ATA, 120 minutes per session, daily, for total of 12 sessions).

Outcome Measures: Serological parameters of liver function and histopathological evaluation of liver fibrosis

Results: This study showed that there is a 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 in improving degree of hepatic fibrosis (p-value <0.001). Majority (94%) of rats exposed to HBOT revealed mild hepatic fibrosis, and on the other hand 76% of the control group revealed moderate fibrosis, with 24% revealing severe fibrosis.

Conclusion: HBOT revealed a very strong beneficial 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

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I. INTRODUCTION

Hyperbaric Oxygen Therapy (HBOT) was defined as "an intervention in which an individual breathes near 100% oxygen intermittently while inside a hyperbaric chamber that is pressurized to greater than sea level pressure (one atmosphere absolute [ATA])" by the Undersea and Hyperbaric Medicine Society (3). Since its inception in 1662 and practical application in 1930, application of hyperbaric oxygen therapy has continued to elicit controversy (4). During HBOT, the increased concentration and the partial pressure of oxygen provide increased oxygenation of the whole body. Oxygen pressure is raised to 10 to 15 times above its normal level when the patient breathes 100% oxygen at 2.8 ATA (1).

In recent decades, numerous studies supported HBOT as an efficient therapeutic option to improve progress of lots of diseases due to its anti-inflammation, anti-oxidation, anti-aging, anti-bacterial, promotes angiogenesis and regeneration properties. It is highly used especially in cases involving hypoxia-related injuries. Furthermore, HBOT has been clinically established as a widely used therapy for patients with carbon monoxide poisoning, decompression sickness, arterial gas embolism, problematic wound, and HBOT was 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, nonalcoholic steatohepatitis and liver-related cancer. The beneficial effects of HBOT in the liver are mainly attributed to its anti-oxidation, anti-inflammation and heme oxygenase-1 (HO-1) properties, which seems to be closely involved in HBOT-mediated protection (3).

Liver fibrosis is a common result of the damage–repair response following different types of chronic insult to the liver. In patients who develop liver fibrosis, the majority ultimately develops liver cirrhosis, decompensated liver disease, and hepatocellular carcinoma. Information on the stage of hepatic fibrosis is essential for making a prognosis and deciding on anti-fibrosis treatment (2). Currently, there is paucity of data with regards to the beneficial effects of HBOT on liver diseases. The main objective of this experimental study is 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. Specifically, the study aims:

1. To compare the serological parameters of liver function (i.e. ALT, AST, Total Bilirubin, Conjugated Bilirubin, Unconjugated Bilirubin, Alkaline Phosphatase, Total Protein, Albumin, Globulin, Platelets and Prothrombin Time) of rats with carbon tetrachloride induced liver injury exposed to the hyperbaric oxygen therapy (HBOT) against control

2. To compare the histopathogical examination of liver using International Association for Study of the Liver (IASL) scoring system for fibrosis histologic stage of rats with carbon tetrachloride induced liver injury exposed to the hyperbaric oxygen therapy (HBOT) against control

II. METHODOLOGY

Description of study group

The experimental study was approved by the Institutional Scientific Review Committee (ISRC), Biosafety Review Committee (BRC), Institutional Animal Care and Use Committee (IACUC) of St Luke's Medical Center and Bureau of Animal Industry. Sample size was computed according to the following parameter assumptions: Alpha error was at 0.05 with a P of 95% on a 2-tailed alternative hypothesis. Sample size was calculated at 34 rats for the study.

Total of fifty-one rats were used in the study, equally randomized into three groups: seventeen rats for Pilot Study Group (rats that were sacrificed immediately after carbon tetrachloride liver injury induction), seventeen rats for Control Group (rats with desired degree of hepatic injury + exposed to room air), seventeen rats for Experimental Group (rats with desired degree of hepatic injury + exposed to HBOT).

Once the desired degree of hepatic injury was established after the 4-week administration of carbon tetrachloride following the Ozdogan protocol, thirty-four adult, 250-300 grams Sprague Dawley rats were randomly assigned into control and experimental group. All those rats were obtained from the Philippines' Department of Science and Technology (DOST) and grown in its animal facility. The rats were maintained under St. Luke's Medical Center Research and Biotechnology (SLMC-RBD) animal testing quarantine protocol. Cage cleaning method, room temperature, humidity, ventilation, and lighting conditions were followed as per SLMC-RBD protocol.

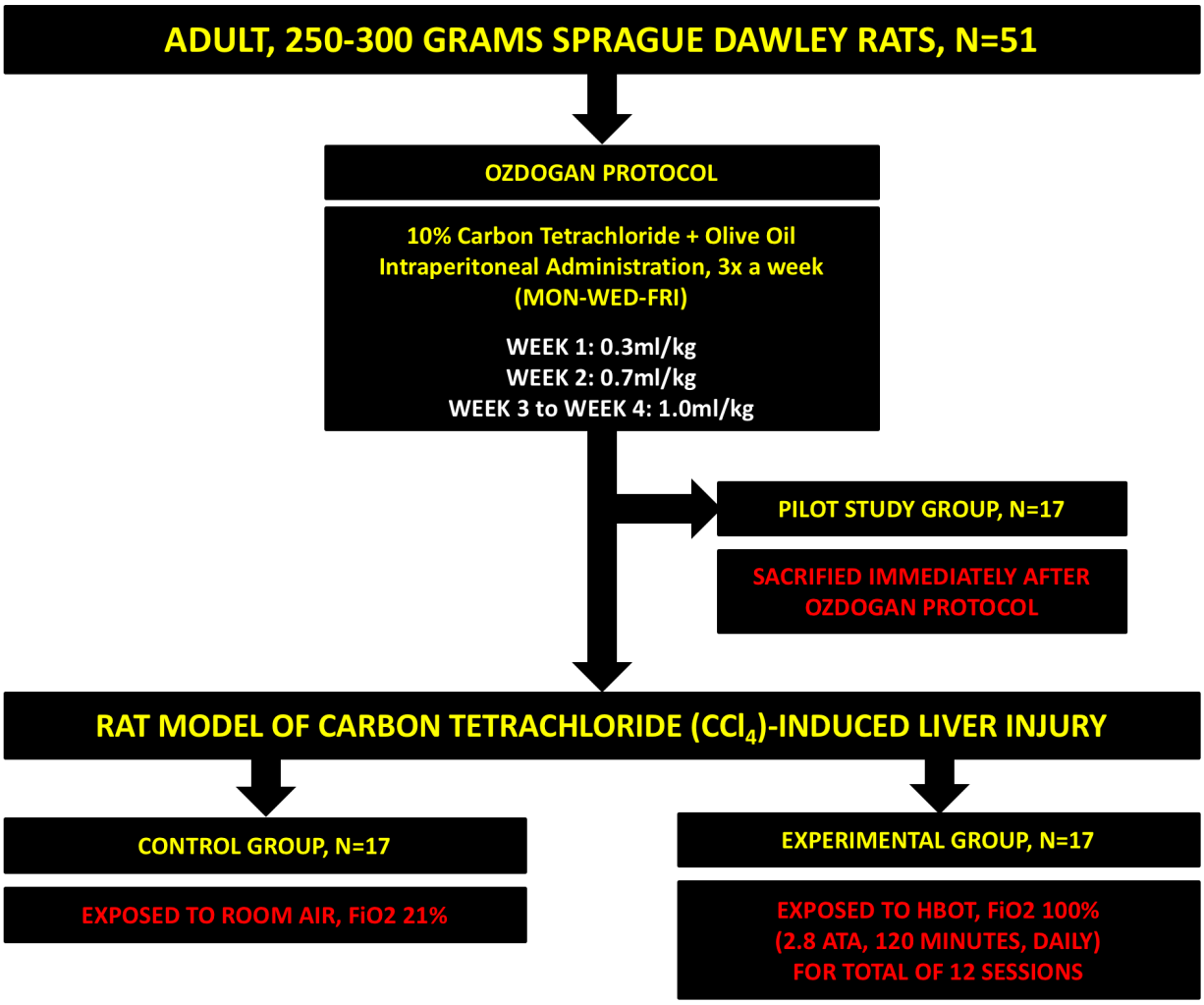

Figure 1. **Distribution of sample into pilot study, control and experimental groups**

Pilot study to establish desired Carbon Tetrachloride (CCl_4) induced liver injury model

The effect of HBOT on liver function and fibrosis were studied using a rat model with carbon tetrachloride induced liver injury. Liver injury induced by carbon tetrachloride causes lipid peroxidation by trichloromethyl radicals, which leads to hepatocellular membrane damage. Further liver damage occurs from exposure to reactive oxygen radicals released from activated Kupffer cells. In this study, hepatic injury was produced following Ozdogan Protocol by injecting the following doses of 10% Carbon Tetrachloride dissolved in olive oil, intraperitoneally three times a week: 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.

In order to establish the desired degree of carbon tertrachloride (CCl_4) induced liver injury, seventeen adult rats, weighing 250 to 300 grams were subjected first to a pilot study following Ozdogan protocol. After 4 weeks of hepatic injury induction, pilot rats underwent blood collection and

hepatectomy followed by animal euthanasia. Blood and liver specimens were submitted for serological and histopathological evaluation to represent and establish baseline liver function and histopathologic liver injury status prior to the start of experiment.

The rats were fed with standard diet and water during the experiment. All animals were housed separately and were kept under standard conditions of room temperature at 22 to 24 degrees Celsius and within 12-h light/ 12-h dark cycle.

Animals were fasted for 8 hours before blood collection, hepatectomy and animal euthanasia. 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.

Description of procedure for the study group

After 4 weeks of carbon tetrachloride (CCl_4) administration to induce the desired degree of hepatic injury, randomization was done. Rats were divided into two groups. Control Group: seventeen rats with desired degree of hepatic injury that were exposed to room air; Experimental Group: seventeen rats with desired degree of hepatic injury that were exposed to hyperbaric oxygen therapy (2.8 ATA, 120 minutes per session, daily, for total of 12 sessions). After 12 sessions of HBOT, all animals underwent blood extraction and hepatectomy followed by animal euthanasia. Animals were deeply anesthetized with tiletamine hydrochloride and zolazepam hydrochloride (ZOLETIL ® for Injection, 70mg/kg) and restrained in supine position. They were allowed to breathe spontaneously during the procedure. Blood samples were obtained via intracardiac blood collection technique and hepatectomy was performed by excision of the entire liver. The blood samples were sent to laboratory for serological evaluation of liver function. Likewise, the excised livers were fixed in 10% formaldehyde and were submitted for histopathological evaluation of liver fibrosis.

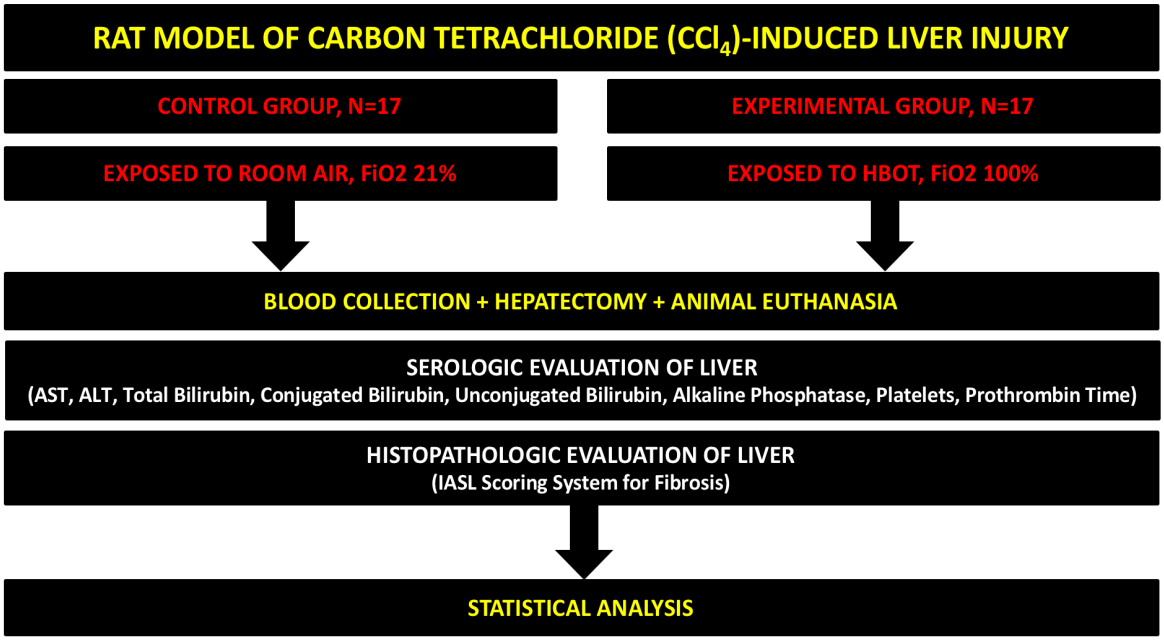

Figure 2. **Procedure for the study group**

Hyperbaric Oxygen Therapy (HBOT)

The experimental and control groups were brought down to the St. Luke's Medical Center – Wound Care and Hyperbaric Oxygen Therapy Unit from the Animal Facility. Hyperbaric Oxygen Therapy was performed using a monoplace chamber at St. Luke's Medical Center (*Perry Sigma 34*), treatment with HBO was started after the desired degree of carbon tetrachloride induced liver injury was established. The HBOT nurse was the one to set-up the chamber (2.8 ATA, 120 minutes per session, daily) for total of 12 sessions. For the control group, no intervention was done.

Animal Euthanasia

The pilot study group rats were immediately euthanized after Week 4 of Ozdogan protocol of induction of liver injury. The control group and experimental group rats were euthanized on Week 7. They were deeply anesthetized with tiletamine hydrochloride and zolazepam hydrochloride (ZOLETIL ® for Injection, 70mg/kg). After which, blood extraction, hepatectomy and animal euthanasia were facilitated for studies.

Blinding

On Week 5, the rats were randomly assigned into two groups. Control Group: seventeen rats with desired degree of hepatic injury that were exposed to room air; Experimental Group: seventeen rats with desired degree of hepatic injury that were exposed to hyperbaric oxygen therapy (2.8 ATA,

120 minutes per session, for total of 12 sessions). The main author together with St. Luke's Medical Center resident veterinarian administered the carbon tetrachloride to induce liver injury, performed the blood extraction, hepatectomy and conducted the animal euthanasia. A blinded medical technologist run the blood samples for statistical analysis. A blinded veterinary pathologist performed the histopathological evaluation of liver fibrosis using IASL Scoring. The biostatistician who analyzed the data was also blinded.

1. DESCRIPTION OF OUTCOME MEASURES

Primary outcome measure:

1.1 Serological parameters of liver function

Blood samples were obtained using intracardiac blood collection technique before rats were euthanized. ALT, AST, Total Bilirubin, Conjugated Bilirubin, Unconjugated Bilirubin, Alkaline Phosphatase, Total Protein, Albumin, Globulin, Platelets and Prothrombin Time levels were measured by a blinded medical technologist using commercially available machine.

1.2 Histopathological evaluation of liver fibrosis

Livers sent for histopathology were stained using basic hematoxylin and eosin (H&E) staining. A blinded pathologist compared the histopathological features of the samples. Standard pictograph of the micro-sections were obtained and analyzed using the same image editing software (Adobe Photoshop 7.0; Adobe Systems, Inc.).

2. SAMPLE SIZE ESTIMATION

Sample size was computed according to the following parameter assumptions: Alpha error was at 0.05 with a P of 95% on a 2-tailed alternative hypothesis. Sample size was calculated at 34 rats for this study. Thirty-four adult, 250-300 grams Sprague Dawley rats, were randomly assigned into control and experimental group.

3. DATA ANALYSIS

Frequency data was 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 was 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.

4. 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.

5. DESCRIPTION OF BIOSAFETY

The Biosafety Review Committee (BRC) of St. Luke's Medical Center approved the study in accordance to universal recommendations when handling hazardous substances.

Project Hazard: Carbon Tetrachloride (CCl_4)

Handling/ Storage: Carbon tetrachloride was stored in labeled, airtight containers in a well-ventilated place protected from light and at a temperature below 30°C. It was stored separately from chemically active metals.

Disposal: Small quantities of carbon tetrachloride was disposed of by evaporation in a fume cup board or in a safe, open area.

Personnel

All personnel who handled the carbon tetrachloride during aliquot preparation, handling and administration to mice underwent biosafety/ biosecurity training and certification.

Location of Hazardous Material, Biosafety Level, Containment Device

St. Luke's Medical Center, Molecular Diagnostics Laboratory
Biosafety Level 1
Containment Device: Fume Hood
For the preparation and handling of Carbon Tetrachloride aliquot

St. Luke's Medical Center, Animal Laboratory
Biosafety Level 1
Containment Device: Not needed
For the administration of Carbon Tetrachloride aliquot to rats

Precautions and Biosecurity

During handling and preparation of the carbon tetrachloride aliquot, wearing of proper personal protective equipment is 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 in St. Luke's Medical Center, Molecular Diagnostics Laboratory and Animal Laboratory. Biosafety cabinet was not needed, 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 of carbon tetrachloride 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. The access to the laboratory was limited to persons advised of the nature of the carbon tetrachloride in this research.

Transport of Hazardous Material

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

Disposal of Animal Carcasses

Animal carcasses that were used in the study were autoclaved prior to disposal. Then it was disposed using sealable yellow plastic bag (infectious waste) even if it is not treated with any infectious agents.

During Hyperbaric Oxygen Therapy Session

The animals were placed in clean cages with plastic liners to catch rats' feces and urine during the hyperbaric oxygen therapy session. In the event of a spill of infectious or potentially infectious material (rats urine/ feces), the following spill clean-up procedure was used (as recommended by WHO):

1. Wear gloves and protective clothing, including face and eye protection if indicated.

2. Cover the spill with cloth or paper towels to contain it.
3. Pour an appropriate disinfectant over the paper towels and the immediately surrounding area.
4. Apply disinfectant concentrically beginning at the outer margin of the spill area, working toward the center.
5. After the appropriate amount of time (e.g. 30 min), clear away the materials. If there is broken glass or other sharps involved, use a dustpan or a piece of stiff cardboard to collect the material and deposit it into a puncture-resistant container for disposal.
6. Clean and disinfect the area of the spillage (if necessary, repeat steps 2–5).
7. Dispose of contaminated materials into a leak proof, puncture-resistant waste disposal container.
8. After successful disinfection, inform the competent authority that the site has now been decontaminated.

III. 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. The following are the results:

The effect of HBOT on liver function and fibrosis were studied using a rat model with carbon tetrachloride induced liver injury. In order to establish the model of carbon tertrachloride (CCl_4) induced liver injury, seventeen adult rats were subjected first to a pilot study based on Ozdogan protocol. After 4 weeks of liver injury induction, pilot study group rats underwent blood collection and hepatectomy, followed by animal euthanasia. Blood and liver specimens were submitted for serological and histopathological evaluation to establish the degree of liver injury after carbon tetrachloride administration prior to the start of actual experiment.

It can be noted that 17 rats under the Pilot Study Group revealed significant hepatic damage, with mean ALT that is 11-12x elevated than the upper limit of normal for ALT and mean AST that is 4-5x elevated than the upper limit of normal for AST. All other liver parameters were noted to be within acceptable limits, (Table 1).

Table 1. Serological parameters of the liver function after carbon tetrachloride 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, $10^9/L$	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 greater majority of rats' liver revealed severe liver necrosis and only a little more than 10% revealed moderate liver necrosis, (Figure 1).

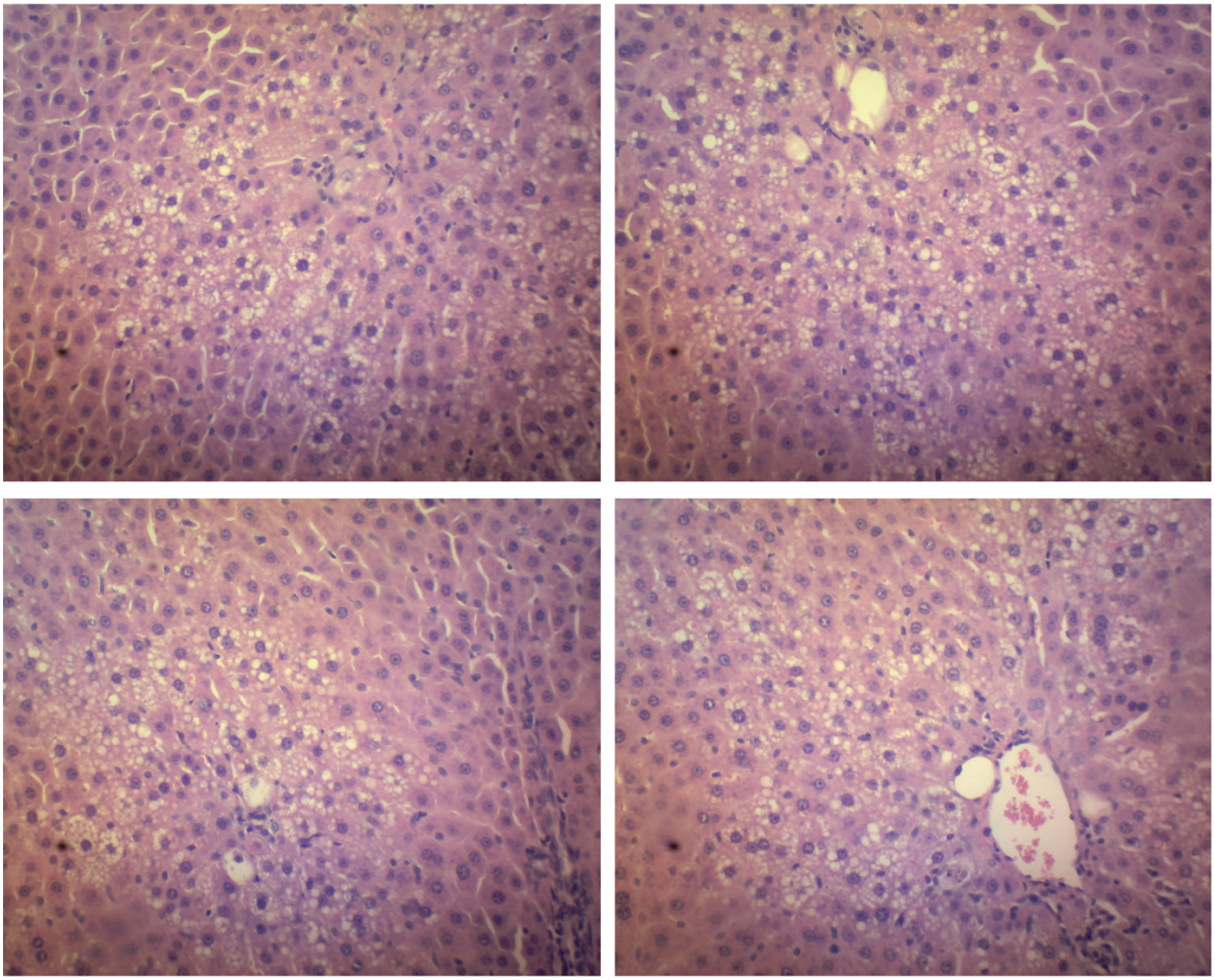

***Figure 3*. Histopathologic evaluation (H&E) of the liver after carbon tetrachloride administration, showing moderate (2 out of 17 rats, 11.76%) and severe (15 out of 17 rats, 88.24%) liver necrosis.**

After 4 weeks of carbon tetrachloride (CCl_4) administration to induce the desired degree of hepatic injury, thirty-four adult, Sprague Dawley rats were randomly assigned into control and experimental. Group I (Control): 17 rats without any treatment, Group II (Experimental): 17 rats that received hyperbaric oxygen therapy, 2.8 ATA, 120 minutes per session, daily, for total of 12 sessions.

This study showed that there is a significant difference between control and hyperbaric oxygen treated group in improving AST and ALT among rats with carbon tetrachloride (CCl_4)-induced liver injury. Rats exposed to HBOT revealed very strong evidence in improving ALT and AST

compared to the control. The mean ALT level of experimental group revealed 0-1x elevated than the upper limit of normal compared to control which is 1-2x elevated than the upper limit of normal. On the other hand, the mean AST level of experimental group revealed 1-2x elevated than the upper limit of normal compared to the control which is 2-3x elevated than the upper limit of normal. All other liver parameters revealed no significant difference between experimental and control groups, (Table 2).

Table 2. Serological parameters of the liver function between experimental and control groups

LIVER PARAMETERS	**NORMAL LIMITS** *range*	**EXPERIMENTAL** *mean +/- SD*	**CONTROL** *mean +/- SD*	**P-VALUE**
ALT, *U/L*	17.5 – 30.2	55.28 +/- 6.89	69.18 +/- 7.90	***<0.001***
AST, *U/L*	45.7 – 80.8	195.53 +/- 30.94	248.53 +/- 28.31	***<0.001***
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, $10^9/L$	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 in improving liver fibrosis compared to control. It can be noted that majority (94%) of rats exposed to HBOT revealed mild hepatic fibrosis, and on the other hand 76% of the control group revealed moderate fibrosis, with 24% revealing severe fibrosis.

Table 2. Description of Experimental and Control Groups on Histopathology

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%)	

IV. DISCUSSION AND CONCLUSION

Rats with hepatic injury induced by carbon tetrachloride that were exposed to Hyperbaric Oxygen Therapy (HBOT) revealed a very strong evidence (p-value, less than 0.001) in improving ALT, AST and degree of hepatic fibrosis compared to the control.

The above findings were compatible to the reported mechanism of liver injury induced by carbon tetrachloride, which is via lipid peroxidation by trichloromethyl radicals, which then leads to hepatocellular membrane damage and further liver damage occurs from exposure to reactive oxygen radicals released from activated Kupffer cells. The significant reduction in ALT, AST and degree of hepatic fibrosis among HBOT treated group, might mainly be attributed to the anti-oxidation and anti-inflammation properties of HBOT.

In conclusion, Hyperbaric Oxygen Therapy (HBOT) revealed a very strong beneficial evidence in improving ALT, AST and degree of hepatic fibrosis among rats with carbon tetrachloride induced liver injury.

V. RECOMMENDATIONS

Animal models are useful in advancing human clinical outcomes. However, as limitation of all animal studies: animals do not dependably predict human outcomes because of interspecies differences: altered susceptibility to and progression of diseases, loss of biological variability and lack of comorbidities or other human risk factors. In this study, the beneficial effect of Hyperbaric Oxygen Therapy on liver function and fibrosis was exposed using a rat model of CCl_4-induced liver injury. A future well-designed human study can be performed to clinically establish HBOT as an important adjunctive therapy to treat patients with acute and chronic liver diseases.

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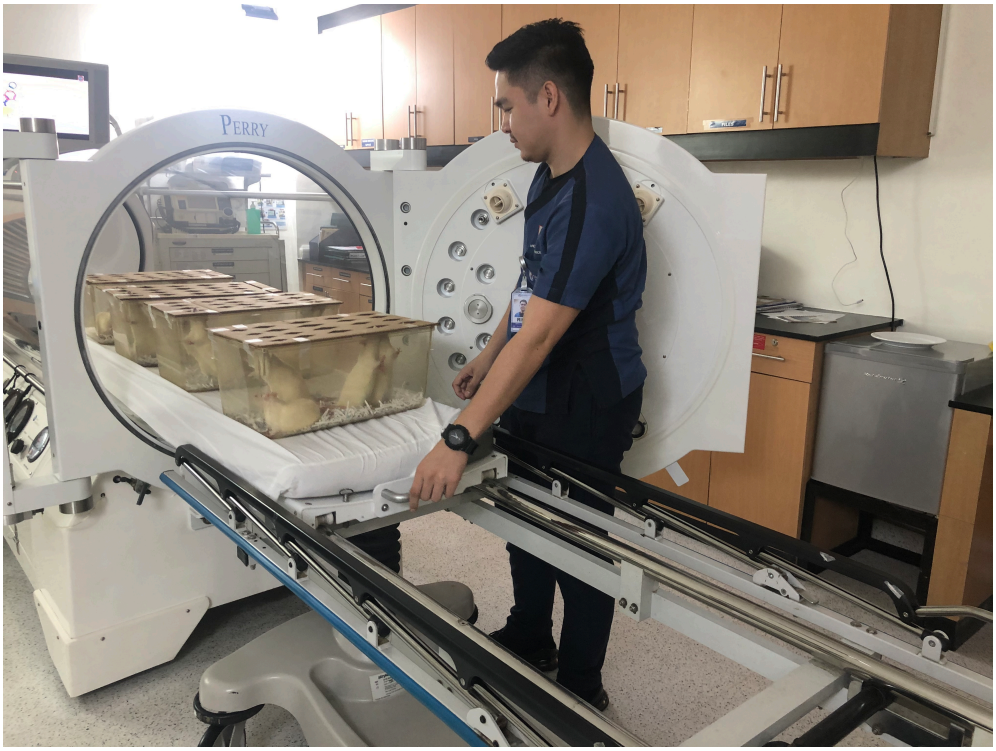
APPENDIX A: HYPERBARIC OXYGEN THERAPY (HBOT)

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APPENDIX B: BLOOD COLLECTION, HEPATECTOMY AND ANIMAL EUTHANASIA


Zoletil 50