Dose Dependent Results in an Induced Model of Chronic Hepatitis with CCl₄ in Mice

Ioana BEDECEAN, Raluca VIDRIGHINESCU, Orsolya SARPATAKI, Andras NAGY, Bogdan SEVASTRE*, Ioan MARCUS

University of Agricultural Science and Veterinary Medicine, Cluj-Napoca, Romania.

Corresponding author: bogdan.sevastre@usamvcluj.ro

Bulletin UASVM Veterinary Medicine 72(1) / 2015,
Print ISSN 1843-5270; Electronic ISSN 1843-5378
DOI:10.15835/buasvmcn-vm: 10574

Abstract

Animal models have been used for several decades to study fibrogenesis and to validate anti-fibrotic effects of potential therapeutic approaches. Until now, mainly rat models were used extensively, while the mice models were less investigated. The present study investigated the dose of carbon tetrachloride, and the time which was suitable for a model of experimental toxic chronic hepatitis in mice. The study was conducted on four groups, consisting of 16 Swiss female mice each. The animals were orally treated with carbon tetrachloride, in different doses; three groups received carbon tetrachloride, diluted in sunflower oil, in dose of 0.5 ml/kg, 1ml/kg, and respectively 1.5ml/kg, while the control group received sunflower oil only. The administrations were made on a weekly basis, three times a week, in the days 1, 3 and 5, until the end of the study. Four animals from each group were euthanized at 2, 4, 6 and respectively 8 weeks. Blood samples were collected and subjected to hematological analyses, then after euthanasia the animals were subject to gross examination and histopathology. The clinical and hematological investigations showed no variations among experimental groups. The gross and microscopic examination of liver revealed a complex pattern. The dose had no major influence on the microscopic lesions pattern; on the other hand, the lesions had a time-related evolution. Thus, microvesicular steatosis was found starting with the fourth week and the sparsely small necrotic areas were visible from the sixth week. The necrotic areas were surrounded by inflammatory cells infiltrate, and an increased number of Kupffer cells was found constantly within all liver tissue. In the eight week, the fibrotic lesions were clearly visible. Fibrosis was accomplished by alteration of liver parenchyma and, at the highest dose; fibrotic lesions were associated with sparsely distributed mineralization areas. The mice model of chronic hepatitis induced with carbon tetrachloride was a viable method, the lesions were similar to those described in rats, and comparable to the lesions found in human patients. Fibrotic lesions became visible after eight weeks; doses above 1ml /kg, are not recommended, as they were associated with mineralization areas.

Keywords: *carbon tetrachloride, chronic hepatitis, mice*

INTRODUCTION

The liver is a major target in toxicity and oncogenicity studies, therefore hepatic pathology is central to many toxicological pathology studies (Thoolen *et al.*, 2010). Liver functions are complex and diverse including endocrine and exocrine activity, metabolism, conjugation, detoxification, and hematopoiesis in early embryonic and fetal development (Harada *et al.* 1999). Intrinsic and induced enzymes responsible for hepatic function may be unevenly distributed throughout the hepatic lobule and between the different lobes (Geaves *et al.* 2007). There are differences of liver

function among species: adipopectin, a bioactive substance, and its ARNm is expressed exclusively in humans, monkeys and mice (Kamada *et al.*, 2003).

Repetition of a toxic insult to the liver is a classical way to induce liver fibrosis. Carbon tetrachloride (CCl₄), is transformed into a toxic CCl₃[·], radical by CYP2E1, an enzyme expressed in perovenous hepatocytes. It induces an acute centrilobular necrosis which triggers a wound healing response which would reconstitute liver integrity. When the insult is repeated, successive rounds of wound healing occur prior

to resolution of the previous one resulting in fibrosis accumulation. Microscopic lesions develop progressively with significant fibrosis after 2-4 weeks, severe bridging fibrosis after 5-7 weeks, and cirrhosis from eight to nine weeks of administration and after 10-12 weeks, micronodular cirrhosis, portal hypertension and ascites progressively appear. Timing for those lesions will vary depending on species, strain, dose, route and frequency of administration. Timing for the euthanasia in relation to the last exposure to CCl₄ is of importance; if done too early, necrosis and inflammation might be prominent, if done too late remodeling processes may prevail. The best time for being in the pro-fibrogenic phase of the response is to prelevate the livers 48h after the last exposure to CCl₄ (Starkel *et al.*, 2011).

The aim of the present study is to investigate the efficiency of oral route of administration of carbon tetrachloride, as well as the dose and the required time for a reliable model of experimental toxic chronic hepatitis in mice.

MATERIALS AND METHODS

The animals were caged in groups of 12 per cage, at a controlled temperature of 21-22°C, humidity of 40-60% and reversed 12/12h light/dark cycle (light off at 10 a.m.). Standard lab chow, provided by National Institute for Research and Development "Cantacuzino" Bucharest (Batch no. 2 / 26.03.2010), and water were freely available. The animal tests and experiments were approved by the Bioethical Board of the Faculty of Veterinary Medicine Cluj-Napoca.

Three groups of 16 mice were orally treated with different doses of carbon tetrachloride (Sigma), while the control group received saline solution only. The groups received three doses of carbon tetrachloride, respectively 0.5 ml/kg, 1ml/kg, 1.5ml/kg diluted in vegetable oil. The carbon tetrachloride was administrated orally three times a week, in the days 1, 3 and 5, using a lubricated catheter, then, subgroups of four randomly selected animals from each group were subject to euthanasia, 48h after the last administration of carbon tetrachloride, in the weeks 2, 4, 6 and 8. One examiner monitored the clinical state of the animals. The animals were weighed constantly starting with the first day of the experiment until the end.

Blood hematology was investigated with Abacus Junior Vet, automatic analyzer Diatron Messtechnik, Budapest, Hungary.

In the necropsy, the liver was weighed, after which individual liver lobes were examined carefully for gross, macroscopic lesions. Specimens of the liver were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned (5 µm thickness) and then subjected to hematoxylin – eosin stain.

The variation of hepatic cells number was conducted by selecting five 400x fields following the areas of hepatocytes, avoiding inflammatory infiltrate, which were photographed. Each photo was transferred to a computer and processed in Quick Photo Micro 2.3 software. For an easy counting to each image was applied a gridline of 0.05mm² at a magnification of 400x and afterwards the counting of hepatic cells was made with "object" tool.

The ratio between hepatocytes and other liver cells was obtained following the steps for the variation of hepatic cell number, with the exception that there were two counts made on the same field, one for the hepatocytes and one for the other cells.

The size variations of the hepatocytes nuclei was carried out using the same method as the previous counting, but using 400x fields and 1 000x fields for more accurate results.

Statistics – All data was expressed as the mean and standard error of the mean (SEM). The Gaussian distribution was checked by the Shapiro-Wilk normality test. Statistical analyses were performed by two-way ANOVA followed by Bonferroni post test for the variation of the body weight and the histological parameters.

Statistical significance was at $p < 0.05$ (95% confidence interval). Statistical values and figures were obtained using GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego California USA.

RESULTS AND DISCUSSIONS

In the first two weeks, the group treated with the highest dose showed apathy and loss of coat luster, and after that the animals have had no clinical signs. Additionally, no significant difference in the complete blood count was found. The weight of the body and liver had small and insignificant variations among the groups in study (Tab. 1).

Tab. 1. Variation of body weight and liver (g)

	T0	T1	T2	T3	T4	Liver
Control	34.95±0.33	35.82±0.68	34.40±0.68	35.53±0.41	36.49±0.58	1.99±0.13
Group 1	35.00±0.42	35.05±0.44	36.87±0.18	36.33±0.52	35.32±0.43	2.08±0.09
Group 2	35.19±0.68	34.73±1.22	35.90±0.47	35.41±0.43	35.76±0.36	2.41±0.15
Group 3	34.91±0.38	35.82±0.60	34.40±0.93	35.53±0.46	35.21±0.32	1.95±0.11

Group 1- group treated with 0.5ml/kg CCl₄, Group 2- group treated with 1ml/ kg CCl₄, Group 3- group treated with 2ml/ kg CCl₄, T0- the weights of animals at the beginning of the experiment, T1- values at two weeks, T2- values at four weeks, T3- values at six weeks, T4- values at eight weeks.

Data are expressed as mean ± S.E.M.

Gross examination was unable to find significant differences among various study groups. Expectedly, control animals showed no microscopical changes; however, into the animals subjected to carbon tetrachloride poisoning, histological examination revealed the presence of diffusely steatotic or steatotic areas in the liver; the lesions were not equally distributed in all animals in study. During the necropsy of the animals, the stomach was opened to observe modifications due to the route of administration, but there were no lesions, the gastrointestinal mucosa being normal and without macroscopic changes.

The severity of the lesions seems to be more related to time, than to dose of carbon tetrachloride, as long as, at similar time span, no obvious changes were found among animals intoxicated with different doses of carbon tetrachloride. After two weeks, the main lesion

was the loss of hepatic architectonics. The hepatocytes were no longer arranged in cords, this aspect was consistent during the all experiment, being found at four, six and respectively eight-time span. Additionally, after four weeks, carbon tetrachloride induced steatosis was observed, located mainly in the perilobular region; the hepatocytes were filled with a number ranging from two to eight small, clear, fatty vacuoles, and had small, hyper chromic, picnotic nuclei (Fig. 1). Rapidly, the whole lobule was modified and after another two weeks of administering the toxic agent, an inflammatory infiltrate was found, consisting of neutrophils and mononuclear cells (Fig. 2). The pathological changes present after eight weeks were dominated by the growing number of Kupffer cells and necrosis (Fig. 3). The regenerating mechanisms of the liver (present at the fourth histological examination) included

Fig. 1. Liver. Group 1 (0.5ml/CCl₄), two weeks, showed areas of steatosis located mainly in the perilobular region; the hepatocytes were filled with a number ranging from two to eight small, clearly, fatty vacuoles, and had small, hyper chromic, picnotic nuclei Bars 20mm

Fig. 2. Liver. Group 3 (2ml/CCl₄), four weeks, an inflammatory infiltrate was found, consisting of neutrophils and mononuclear cells. Bars 20mm the presence of collagen fibers, a high number of

cells in a mitotic stage and the presence of newly formed biliary ducts (Fig. 4).

The number of hepatocytes per high power field (HPF), represents a quantification marker of degeneration and fibrosis; when fibrosis develops the hepatic cells become more sparsely distributed within the liver tissue. However, no significant variations were found between control and experimental groups at different moments (two, four, six and respectively eight weeks) (Fig. 5). The size variation of the hepatocytes nuclei is considered a marker of degeneration, while the number of Kupffer cells per HPF an indicator of chronic inflammatory reaction (Aktas *et al.*, 2003). Unexpectedly, the size variations of the

Fig. 3. Liver. Group 2 (1ml/CCl₄), six weeks, showed necrotic areas and the growing number of Kupffer cells. Bars 20mm

Fig. 4. Liver. Group 2 (1ml/CCl₄), eight weeks, showed the presence of collagen fibers, a high number of cells in a mitotic stage and newly formed biliary ducts. Bars 100mm

hepatocytes nuclei and the Kupffer cells no / HPF show no relevant variations (Fig. 6 and 7).

Liver fibrosis is found in various liver diseases regardless of the etiology. It results from chronic injuries that over stimulate the wound healing response. Animal models have been used extensively to assess the hepatoprotective effect of various therapeutic agents. Models for fibrosis were developed in rats and then adapted in mice. The advantages of rat models are the

Fig. 5. The variation of hepatocytes number / HPF (average $\pm$ SEM)

Fig. 6. The size variations in diameter of the hepatocytes nuclei (μ m)

Fig. 7. The variation of Kupffer cells number / HPF

size of animals, especially for hemodynamic and imaging techniques (Starkel *et al.*, 2011). Rats were considered more suitable because they are bigger, providing a higher blood volume, required for plasma biochemistry and they are easier to handle for oral administration. However, the rats have higher mortality on oral administration than i.p. route, and the fibrosis less consistent. Reproducibility can be high only if all animals receive a similar dose of the inducing agent. This might, however, be difficult to control when the agent is provided in diet or in drinking water, or when absorption or metabolism of the toxic are affected in peritoneal reaction with CCl_4 i.p. (Starkel *et al.*, 2011).

This is why we chose to administer CCl_4 orally, the most likely natural route for a toxic substance, which prevents the local peritoneal reaction that might interfere with the lesional pattern of CCl_4 toxicity. The administration performed by using a lubricated catheter was easily tolerated by animals and no accidents were recorded, additionally allowing a precise dosage of the toxic. Our results proved that mice are a suitable model for experimental fibrosis, especially for oral administration. Moreover, mice require smaller dosages of therapeutic agents which is important when expensive newly synthesized molecules are checked for hepatoprotective effect. In the present study, the mortality of mice was absent. The pattern of lesions is the same as in the rat model of liver fibrosis by oral gavage, with alterations in parenchyma starting with the second week and resulting in significant fibrosis at eight weeks.

CONCLUSION

Mouse model is a reliable model of experimental liver fibrosis. The oral route is safe, well tolerated by animals, with no mortalities being reported. The histological pattern of carbon tetrachloride toxicity in mice resembles those described in rat which is the widely accepted model. The earliest lesions were found at a two weeks interval and consisted in perilobular steatosis, followed at four and six weeks by the presence of necrotic areas accomplished by inflammatory infiltrate of neutrophils and mononuclear cells and the growing number of Kupffer cells. The fibrotic lesions were visible only after eight weeks. The doses have little influence on the extensivity of the

lesions; morphometric evaluation was not able to find significant differences.

Acknowledgments. This paper was published under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/136893.

REFERENCES

1. Aktas S, Dinitz G, Ortac R, (2003). Quantitative analysis of ductus proliferation, proliferative activity, Kupffer cell proliferation and angiogenesis in differential diagnosis of biliary atresia and neonatal hepatitis. *Hepato-gastroenterology* 50(54):811-1813.
2. Amin A, Hamza AA (2005). Hepatoprotective effects of hibiscus, rosmarinus and salvia on azathioprine-induced toxicity in rats. *Life Sciences* 77:266-278.
3. Azeem AK, Mathew M, Nair C, Dilip C (2010). Hepatoprotective effect of Averrhoa carambola fruit extract on carbon tetrachloride induced hepatotoxicity in mice. *Asian Pacific Journal of Tropical Medicine*:610-613.
4. Gutiérrez R, Alvarado JL, Presno M, Pérez-Veyna O, Serrano CJ, Patricia Yahuaca P (2010). Oxidative Stress Modulation by Rosmarinus officinalis in CCl_4 -induced Liver Cirrhosis. *Phytotherapy Research* 24: 595-601.
5. Harada T, Enomoto A, Boorman GA, Maronpot RR (1999). Liver and gallbladder, in pathology of the mouse. Cache River Press, Vienna, IL, 119-83.
6. Rašković A, Milanović I, Pavlović N, Čebović T, Vukmirović S, Mikov M (2014). Antioxidant activity of rosemary (*Rosmarinus officinalis* L.) essential oil and its hepatoprotective potential. *BMC Complementary and Alternative Medicine* 14:225.
7. Starkel P, Leclercq IA (2011). Animal models for the study of hepatic fibrosis. *Best Practice & Research Clinical Gastroenterology* 25:319-333.
8. Thoolen B, Maronpot R, Harada T, Nyska A, Rousseaux C, Nolte T, Malarkey D, Kaufmann W, Ku"ttler K, Deschl U, Nakae D, Gregson R, Vinlove M, Brix A, Singh B, Belpoggi F, Ward J (2010). Proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system. *Toxicologic Pathology* 38: 5s-81s.
9. Yoshihiro K, Shinji T, Shinichi K, Hitoshi M, Yukiko S, Yuichi Y, Koji F, Norikazu M, Hitoshi N, Hiroyuki N, Yoshihisa O, Shinji K, Jun-Ichiro M, Yasuhisa S, Tohru F, Matsuzawa Y (2003). Enhanced carbon tetrachloride-induced liver fibrosis in mice lacking adiponectin. *Gastroenterology* 125:1796-1807.
10. Usha K, Mary Kasturi GM, Hemalatha P (2007). Hepatoprotective effect of *hygrophila spinosa* and *cassia occidentalis* on carbon tetrachloride induced liver damage in experimental rats. *Indian Journal Of Clinical Biochemistry* 22(2):132-135.