Novel liver fibrosis model in Macaca fascicularis induced by thioacetamide

（チオアセトアミドによるカニクイザル肝線維化モデルの確立）

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Abstract

Although transplantation is the only definitive treatment of liver cirrhosis, there remains a shortage of donors, necessitating that novel treatments be developed. We aimed to establish a liver fibrosis model in *Macaca fascicularis* that can help accelerate preclinical research. Liver fibrosis was induced by administering thioacetamide (TAA) and carbon tetrachloride (CCl_4) respectively. Analysis of residual liver function and fibrosis progression was based on clinical indices such as the Child–Pugh score or fibrotic markers besides histology. TAA-induced marked fibrosis, whereas CCl_4 did not induce fibrosis. Concerning residual liver function, both of TAA and CCl_4 worsened the indices of the Child–Pugh score, but only the TAA model increased retention ratio of indocyanine green. The TAA-induced fibrosis model in *Macaca fascicularis* worsens fibrosis and residual liver function, mimicking Child–Pugh grade B. Given that our model was evaluated by clinical indices, it could be applicable to preclinical research.

Introduction

Liver cirrhosis represents end-stage liver injury in alcohol abuse and chronic hepatitis, ultimately leads to hepatocellular carcinoma, and is associated with high morbidity and mortality [1, 2]. Cirrhosis is characterized by widespread disruption of the normal liver architecture due to fibrosis and decreasing residual liver function, with liver transplantation being the only definitive treatment for end-stage liver injury. However, donor shortages mean that mortality rates remain high among patients on waiting lists [3], meaning that there is an urgent need to develop alternatives.

A barrier to developing alternative treatments for end-stage liver injury is the lack of a reliable model of liver fibrosis in non-human primates (NHPs) that can facilitate preclinical research. Although many fibrosis models have been reported in rodents [4,5], it is unclear how accurately rodent data predict outcomes in humans because of the many biological and anatomical differences [6]. By contrast, old-world monkeys like *Macaca fascicularis* and rhesus monkeys have many anatomical and immunological similarities to humans [6, 7]. Therefore, experiments in NHPs have been widely employed when developing vaccines or novel procedures for clinical application [8,9]. To date, however, there have only been a few reports of liver fibrosis models in NHPs [10–12]. These existing reports have also failed to cover all relevant clinical indices, such as the Child–Pugh score [13], the Liver damage grade which was defined by

Liver Cancer Study Group of Japan [14], and the Model for End-Stage Liver Disease (MELD) score [13,15] necessary to evaluate residual liver function or prognosis when determining the clinical indication for liver transplantation.

An article previously reported that administering carbon tetrachloride (CCl_4) with a high fat diet and alcohol for 16 weeks induced liver fibrosis in *Macaca fascicularis* [10]. Fibrotic progression was shown both histologically and serologically by the presence of hyaluronic acid (HA), collage type IV (Col-IV), and type III procollagen-N-peptide (P-III-P). However, the report lacked data about total bilirubin levels or ascites, which are both included in the Child–Pugh score. More recently, a liver fibrosis model was reported in another species of new world monkey, common marmosets, that was induced by thioacetamide (TAA) [12]. They reported four protocols with the appearance of decreasing serum albumin levels and increasing fibrosis in some monkeys. However, they also failed to cover all clinical indices, and the total bilirubin level increased transiently, which deviates from the clinical features of chronic liver diseases. To the best of our knowledge, there are no reports of liver fibrosis models in old world monkeys induced by TAA.

In this study, we evaluate liver fibrosis models induced by CCl_4 and TAA in *Macaca fascicularis*, using clinical indices of both residual liver function and fibrotic progression to establish a model that mimicked Child–Pugh grade B to C.

RESULTS

General appearance and Macroscopic appearance

Figure 1 outlines the study protocol and shows the group allocation and Table 1 summarized general information of allocated monkeys in this study. Monkeys treated by TAA lost appetite and some vomited just after administration during the first week, but their appetites and activities recovered after 2–3 weeks of administration. By contrast, monkeys treated with CCl_4 did not show any change during the first week and lost their activity and appetite gradually over 3–4 weeks after the initial administration. In addition, subcutaneous administration of CCl_4 caused skin erosion.

Monkeys treated by TAA lost body weight over time (Figure 2a). Macroscopically, monkeys treated by TAA showed coarse granulation formation on their liver surfaces while those treated by CCl_4 showed fatty injury like change (Figure 2b). Abdominal ultrasound revealed that ascites did not develop in any monkeys and any change of biliary tract indicating biliary obstruction or cholecystitis did not appear in any monkeys (Figure 2b). Whereas, splenomegaly appeared in monkeys receiving TAA and CCl_4 from 2 weeks after initial administration (Figure 2c).

Residual liver function

Albumin levels decreased over time in monkeys treated with both TAA and CCl_4 from 2

weeks after initial administration. The prothrombin time increased persistently in monkeys receiving TAA, whereas the total bilirubin level increased significantly in monkeys receiving CCl_4. Only TAA increased ICGR15. Serum NH_3 levels increased in both the TAA and CCl_4 groups, while AST and ALT increased transiently 2–4 weeks after initial administration and recovered over time. Another feature of chronic liver disease, decreasing platelet levels, was also induced by both TAA and CCl_4. However, neither model affected creatinine levels. Interestingly, white blood cell (WBC) and C-reactive protein (CRP) significantly increased in monkeys receiving CCl_4 (Figure.2d).

Histological analysis and progression of liver fibrosis

Histology in control monkeys revealed normal cellular architecture (Figure 3a). Fibrosis developed significantly in monkeys receiving TAA, but was less marked in monkeys receiving CCl_4 based on the analyzes of tissue samples and fibrotic markers. Histology of the biopsy specimens from monkeys receiving TAA at 4 and 8 weeks showed bridging fibrosis, a typical feature of F3 fibrosis, and massive inflammatory infiltration of lymphocytes around sinusoids (arrowhead of Figure 3a). The histology of monkeys receiving CCl_4 showed centrilobular congestion but not fibrosis formation at 4 weeks. We conducted 2nd liver biopsy at 7 weeks because general condition of monkeys receiving CCl_4 became poor and we considered they would not survive until 8th week. The histology of biopsy specimen at 7 weeks showed

ballooning that are characteristic of fatty liver injury (arrow, Figure 3a), but did not show significant fibrosis formation.

Quantitative analysis of fibrosis based on the area positive for Sirius red showed fibrotic progression in monkeys receiving TAA but not in those receiving CCl_4. The hydroxyproline level tended to increase in monkeys receiving TAA, but this increase was not over time as with the other fibrosis assessments (Figure 3b). HA levels increased 4 weeks after initial dosing with both TAA and CCl_4; however, it only increased persistently to week 8 in monkeys receiving TAA. The levels of P-III-P and Col-IV increased 2 weeks after initial administration and persisted throughout the administration of both TAA and CCl_4. Finally, levels of HA and Col-IV increased markedly in monkeys receiving TAA (Figure 3c).

DISCUSSION

Liver cirrhosis is characterized by devastation of residual liver function and replacement of the normal parenchyma by fibrosis. Transplantation is the only definitive, albeit radical, treatment for liver cirrhosis, but donor shortages remain an issue. Several novel treatments have been developed, such as cellular transplantation [16] and molecular targeted drugs [17, 18]; however, none of these has proven suitable to replace liver transplantation. Therefore, the development of viable alternates continues to be an unmet public health need. To accelerate the development

and clinical application of novel treatments or procedures through effective preclinical research, we must first establish reliable models of liver fibrosis in NHPs (e.g., *Macaca fascicularis*), which are more closely related to humans[6].

Clinical applicability requires that any usable model of liver fibrosis model in NHPs be formulated based on clinically relevant indices. Otherwise, the models may not truly simulate liver cirrhosis in clinical settings. Quantitative analysis of histological data is not always used clinically, but it can be used to establish liver fibrosis in animal models objectively. To date, no model of fibrosis in NHPs has been established based on clinical indices and quantitative analyses of fibrosis. In this study, based on these relevant clinical indices, we showed that TAA and CCl_4 induced quantitative fibrosis progression in *Macaca fascicularis*.

Liver damage induced by TAA more closely reflected the clinical features of liver cirrhosis than that induced by CCl_4 in terms of both residual liver function and fibrotic progression in our protocol. Fibrosis progression was much more significant in monkeys receiving TAA than in monkeys receiving CCl_4. Although histological analysis by biopsy is the most accurate assessment of fibrosis, it is invasive. Therefore, fibrotic markers such as HA, P-III-P, and Col-IV are generally used as supplemental markers of fibrosis progression in clinical settings. In this study, each of these markers predicted fibrogenesis and correlated well with the histological findings. Changes in the fibrotic markers showed that TAA administration for 4 weeks induced

liver fibrosis and that administration for an additional 4 weeks worsened that fibrosis. Hydroxyproline assays are widely used as a quantitative analysis of fibrosis in animal models, but we did not show a correlation with either the histological results or the fibrotic markers. Given that fibrogenesis is not homogeneous in the liver, hydroxyproline assays of small specimens may not always reflect overall fibrosis formation.

The blood chemistry results indicate that liver damage began 2 weeks after the reagents were initially administered. We considered that these changes indicated the onset of acute liver damage because AST and ALT increased transiently 2–4 weeks after initial administration and recovered over time. Because of this acute injury, a monkey (TAA-2) died just 5 days after administration. To prevent acute death by TAA administration, we should start lower dose and increase up to effective dose gradually. Additional administration 2–4 weeks after initial administration induced chronic liver damage and resulted in persistent exacerbation of clinical indices of Child–Pugh and the Liver damage grade. According to the indices of Child–Pugh score, such as albumin, total bilirubin, NH_3, and prothrombin time, both reagents induced liver damage that mimicked grade B severity by the eighth week. However, only TAA administration worsened ICG clearance function. The ICG clearance test is widely used clinically for dynamic assessments of the capacity of the liver to metabolize and eliminate drugs and it indicated whether liver damage is present. Our study is the first we know of in the published literature to

show ICG clearance data in NHPs.

TAA was superior to CCl_4 in our study because it both induced liver fibrosis progression and worsened residual liver function. Notably, the protocol took only 8 weeks to establish a fibrosis model, which was the shortest time requirement among the existing models in NHPs, which took more than 16 weeks at least. This could contribute to accomplishment of the three R's principle[19]; Replacement, Reduction and Refinement. Furthermore, research with this model could predict effects in humans with greater accuracy because the results were based on clinical indices and objective data specific to fibrosis.

Our study has several limitations. First, we found individual differences in the effects of the reagents. One monkey that received TAA showed neither significant fibrosis formation nor a reduction in liver function, as was apparent in the other monkeys treated by TAA; it may be that the response to reagents is much less predictable in NHPs than in rodents. Individual modifications to reagent administration may be necessary to establish usable models of fibrosis in all NHPs, as previously reported [12]. Second, we could not assess what changes might have occurred after stopping the reagents. Previous reports indicate that fibrosis can reverse after stopping the reagents in rodents[20] and NHPs [11, 12]. It will be essential to determine the effects of stopping TAA and CCl_4 in future research. Because we strictly complied the three R's principle, we designed this study with minimum required monkeys. Additional study is necessary to verify

our result to establish the preclinical model.

In this study, we showed that TAA administration for 8 weeks in *Macaca fascicularis* induces the clinical changes of chronic liver damage consistent with Child–Pugh grade B and class F3 fibrosis (Inuyama classification or Metavir scoring system). This model could prove to be of great benefit in preclinical research for the development of novel treatments for liver cirrhosis.

METHODS

Animals

All animal studies were approved and performed according to the Tsukuba Primate Research Center. All of the experiments were performed under the guidelines of the Tsukuba Primate Research Center, as well as the guiding principles for animal experiments using NHPs set out by the Primate Society of Japan. Tsukuba Primate Research center provided 11 healthy *Macaca fascicularis* (6–18 years old, 3.38±0.86 kg). All monkeys were caged individually in a room with ventilation and the 12 h cycle of artificial light from 7 AM to 7 PM. Temperature and humidity were maintained at 23℃–27℃ and at 50%–70%, respectively.

Reagent administration

Figure 1 outlines the study protocol and shows the group allocation. TAA (Sigma-

Aldrich, St. Louis, MO, USA) was dissolved in normal saline (Otsuka Pharmaceutical, Tokyo, Japan) and administrated three times a week at a dose of 100 mg/kg (n = 5). CCl_4 (WAKO) was diluted with corn oil (WAKO) and administrated twice a week at a dose of 1000 mg /kg (n = 3). The control group received normal saline at a dose of 1 ml/kg three times a week (n = 3). All reagents were administrated subcutaneously for 8 weeks.

Assessment of residual liver function

To assess the Child–Pugh score, the MELD score, and the Liver damage grade, blood tests were taken for albumin, total bilirubin, prothrombin time, NH_3, indocyanine green (ICG) clearance, and creatinine to evaluate residual liver function. ICG clearance test was measured by retention of ICG at 15 minutes (ICGR15). In addition, aspartate transaminase (AST) and alanine aminotransferase (ALT) were measured as general indices of liver damage. Blood was drawn from femoral veins every 2 weeks. We also performed abdominal ultrasonography (AUS) every 2 weeks to assess ascites and splenomegaly. Spleen index was calculated with short diameter by long diameter of spleen on AUS.

Assessment of fibrosis progression

Open liver biopsy was conducted before reagent administration and at 4 and 8 weeks after initial administration under sedation with xylazine. Tissue samples were then fixed with 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin, Sirius red and

Masson trichrome. We evaluated fibrosis progression according to the new Inuyama classification[21] or the Metavir scoring system[22], as follows: F0, no portal fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with rare septa: F3, numerous septa or lobular distortion without cirrhosis; and F4, cirrhosis. The area stained by Sirius red was calculated by ImageJ software (National Institute of Health, Bethesda, MD, USA).

We also conducted a hydroxyproline assay for additional quantitative analysis of fibrosis, as previously described[23]. Briefly, homogenous samples of N_4HCl from solid material were hydrolyzed for 12 hours in N_6HCl at 97℃. The hydrolyzed hydroxyproline was then converted into pyrrole reaction products with a Chloramine T solution (Sigma-aldrich). After adding Ehrlich's reagent, absorbance was measured by spectrophotometer at a wavelength of 550–565 nm. Enzyme-linked immunosorbent assay of fibrotic markers, such as HA, Col-IV, and P-III-P[24], was also performed by a commercial laboratory (SRL Tokyo Laboratories, Tokyo Japan).

Statistical analysis

Statistical data were analyzed with JMP 13 (SAS Institute Japan, Tokyo, Japan). Mann–Whitney *U* tests were used to compare parameters. Statistical significance was defined by a p value of <0.05.

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Author Contributions

M.M., S.M. and H.T. designed the study, convinced the experiments and analyzed the data. M.M., S.M., S.H. and Y.H. performed the experiments and analyzed the data. M.M and S.M. wrote the manuscript. M.O. and H.T. supervised the study.

Disclosure/ conflict of interest

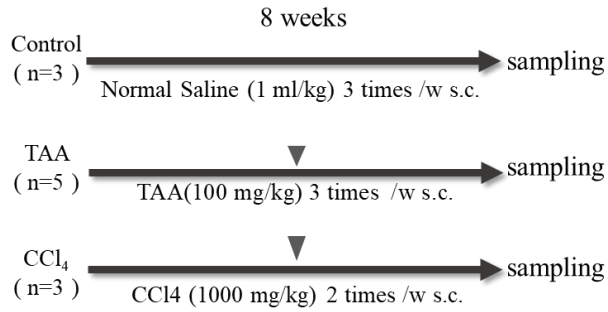
The authors declare no conflicts of interest.

Table 1. Summary of general information of each monkey

	Sex	Age	Initial BW (kg)	Last BW (kg)	Survival (day)	Adverse effect
control-1	F	8	2.74	2.72	56	none
control-2	F	8	2.73	2.65	56	none
control-3	F	12	3.38	3.42	56	none
TAA-1	F	7	3.26	3.04	56	vomiting after administration
TAA-2	F	18	3.17	2.75	5	vomiting after administration
TAA-3	M	6	3.94	2.90	42	vomiting after administration
TAA-4	F	17	3.45	3.03	56	vomiting after administration
TAA-5	M	8	5.00	3.40	56	none
CCl_4-1	M	10	3.46	3.47	51	skin erosion
CCl_4-2	F	13	2.98	2.76	49	skin erosion
CCl_4-3	M	12	5.53	4.08	42	none

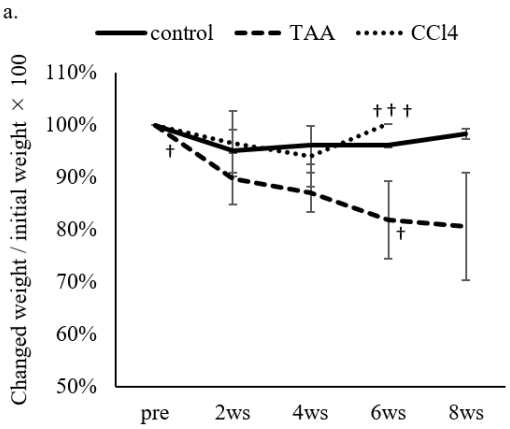
11 healthy Cynomolgus monkey (6–17 years old, 3.38±0.86 kg) allocated into control, TAA treatment, and CCl_4 treatment group respectively. Mean ± SD.

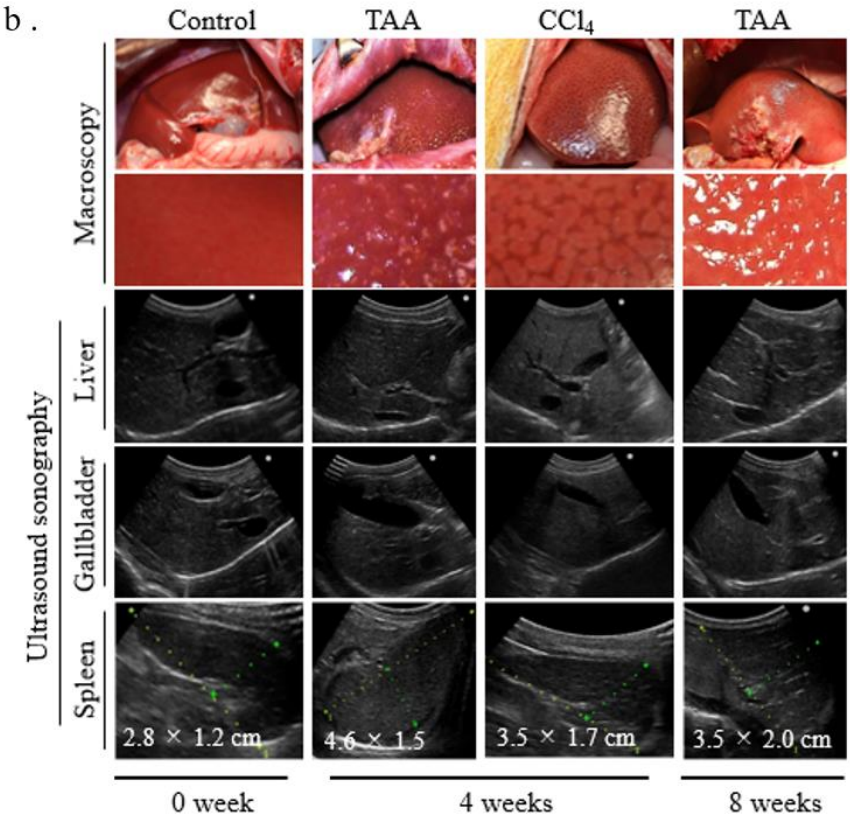
Figure 1. Fibrosis model protocol

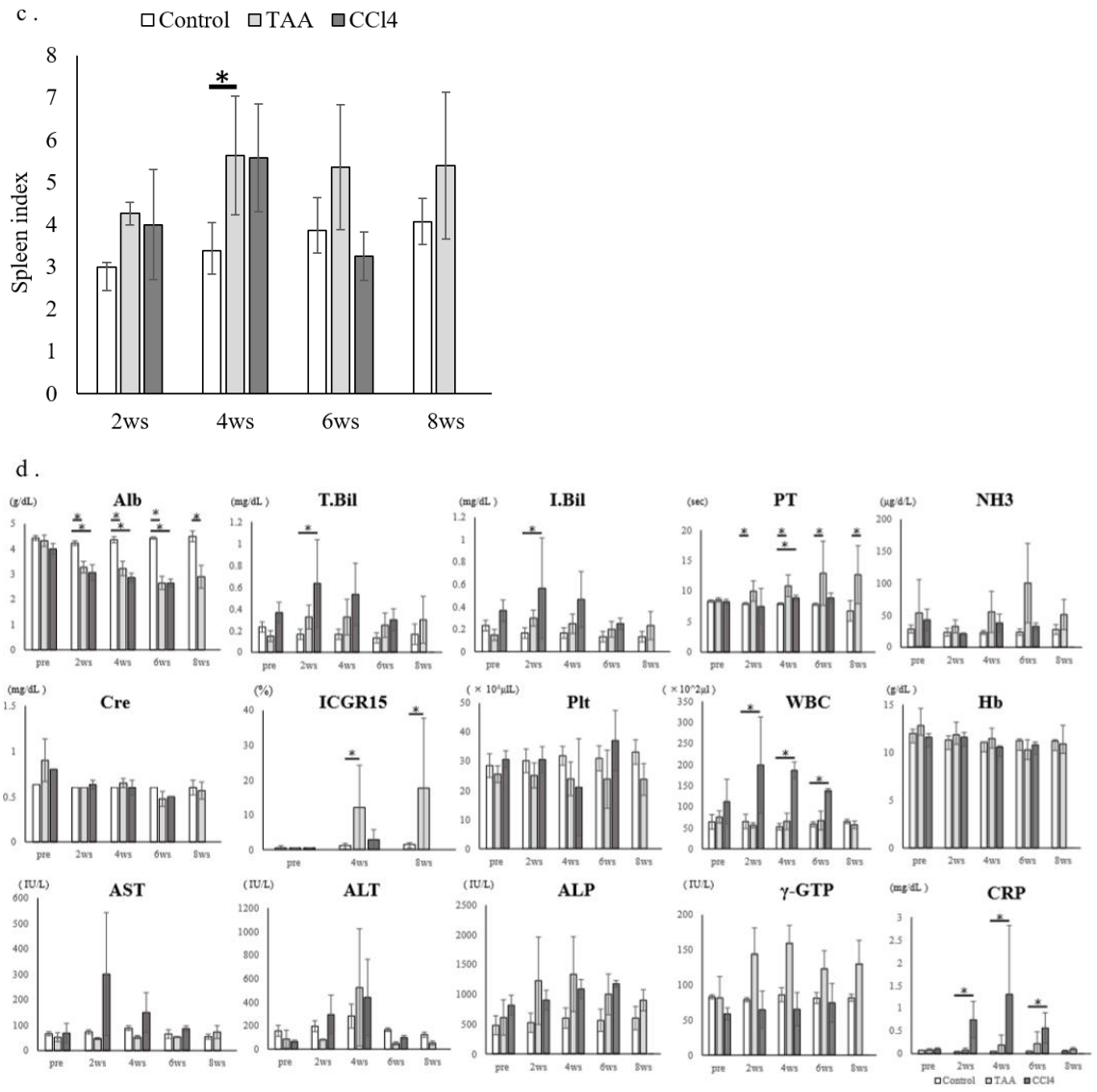


Eleven monkeys were allocated to three groups and received either control, thioacetamide (TAA), or carbon tetrachloride (CCl_4) for 8 weeks. Inverted triangle = liver biopsy at week 4.

Figure 2. Changes in body weight, ultrasound sonography findings, spleen index and Change in blood chemistry in each group



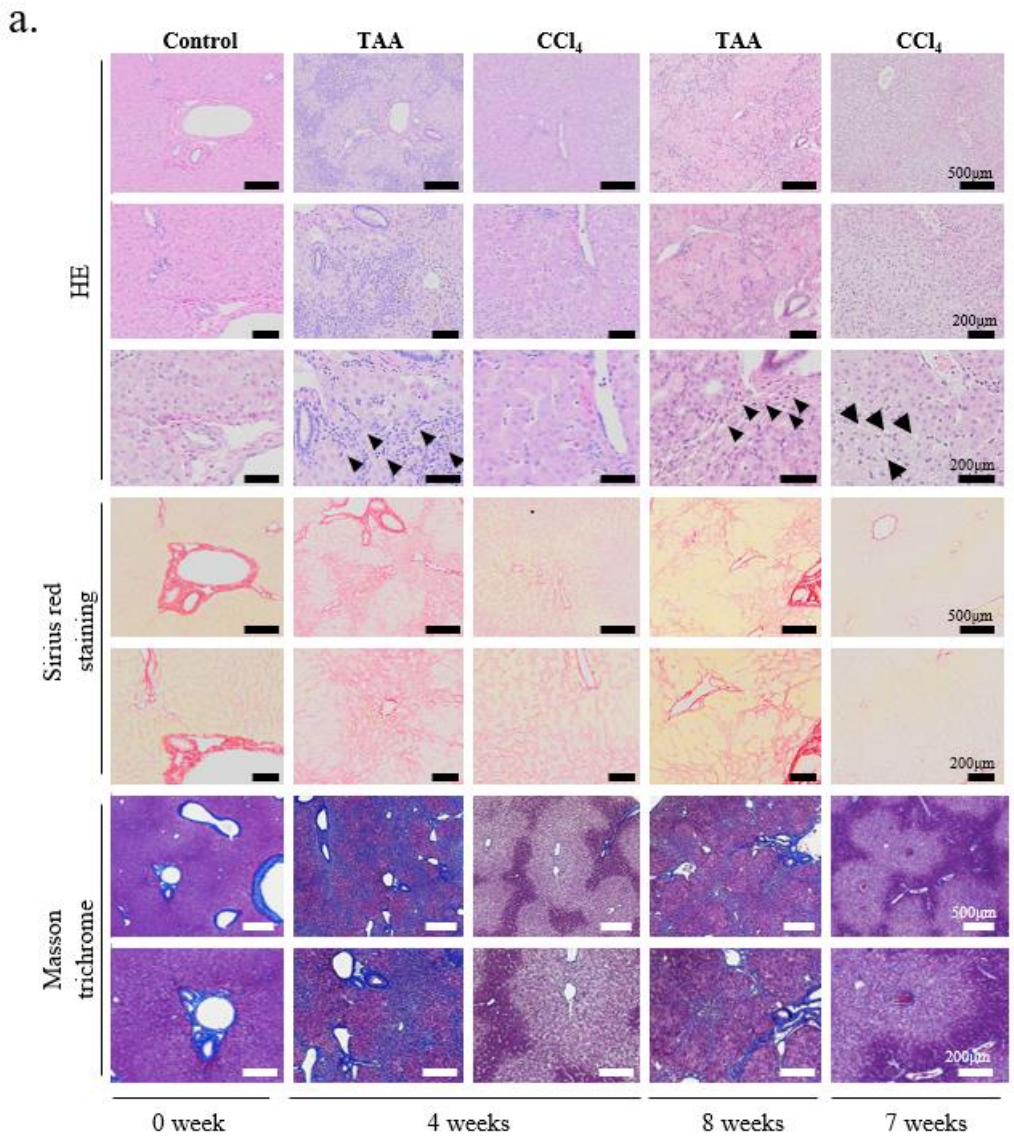




(a) Body weight changes per group (mean ± SD): straight line, control group; dashed line, thioacetamide (TAA) treatment; and dotted line, carbon tetrachloride (CCl_4) treatment. (b) Macroscopic and ultrasound changes. Macroscopically, there were changes to the liver surfaces by both reagents. Ultrasound sonography (US) did not show ascites in any group, but did show splenomegaly in the monkeys receiving TAA and CCl_4 (c) Spleen index was calculated by long diameter (cm) × short diameter (cm) of spleen on US. The index increased in monkeys receiving

TAA significantly (d) Blood chemistry was analyzed every 2 weeks except ICGR15 analyzed every 4 weeks: white bar, control; light gray bar, TAA treatment; gray bar, CCl_4 treatment. * $p < 0.05$ vs control by Mann–Whitney U test. Mean ± SD.

Figure 3. Histological analysis and progression of liver fibrosis



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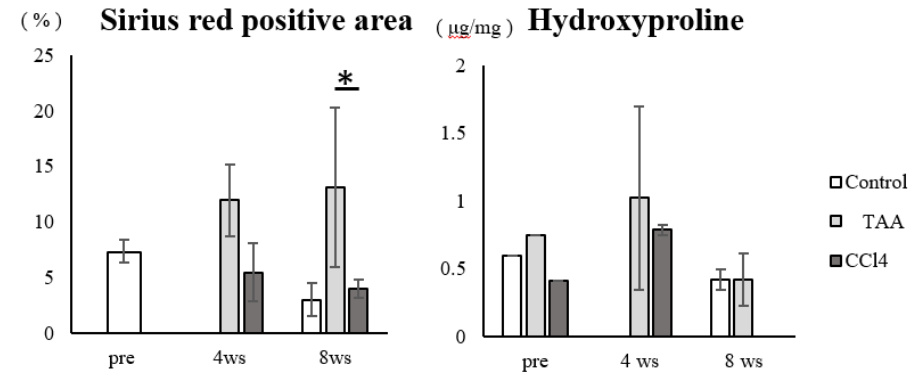

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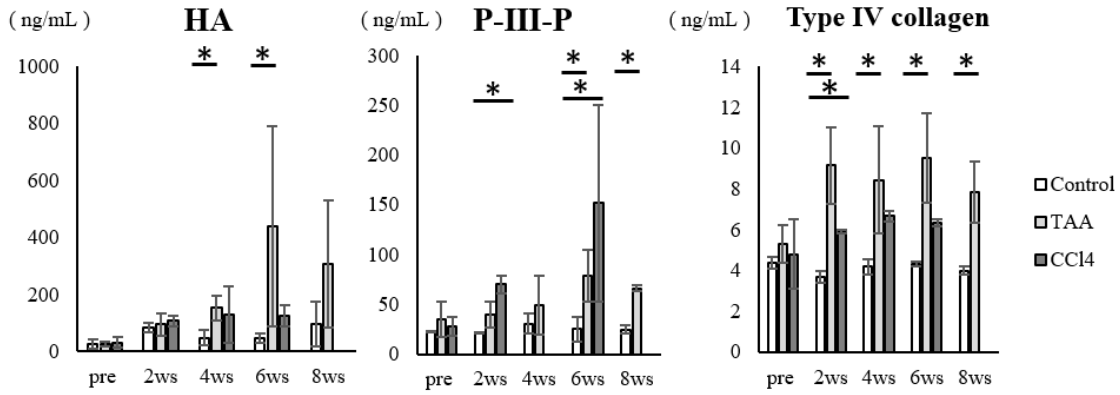

(a) Hematoxylin/eosin, Sirius red staining, and Masson trichrome staining: arrow heads in specimens treated by thioacetamide (TAA) show inflammatory infiltration by lymphocytes around the sinusoids at weeks 4 and 8, whereas the arrows in specimens treated by carbon tetrachloride (CCl_4) show hepatocyte ballooning at week 7. (b) Quantitative analysis of the Sirius red positive area by ImageJ and hydroxyproline assay. (c) Enzyme-linked immunosorbent assay of hyaluronic acid (HA), collage type IV (Col-IV), and type III procollagen-N-peptide (P-III-P): White bars = control group; light gray bars = TAA treatment;

and gray bars = CCl_4 treatment. *$p < 0.05$ vs control by Mann–Whitney U test. Mean ± SD.

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