Research Article

Comparison of Two Methods of 2AAF/CCL_4 Exposure to Induce Rat Animal Model of Chronic Liver Injury

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Abstract

Since chronic liver injury has becoming a global public health problem, many research to elucidate the pathological and regeneration mechanism, that lead to the need of creating an animal model of chronic liver injury. The aim of the study was to develop and compare two method of 2AAF/CCl_4 exposures in male wistar rats and to analyze the subject survival, weight gain, liver anatomy and histopathology. This research was experimental study, conducted in Animal Research Facilities, IMERI Faculty of Medicine Universitas Indonesia, from October 2018-October 2019. We compared two methods of 2AAF/CCl_4 exposures to 8 weeks male Wistar rats (n=18), i.e. repetitive 12 weeks (twice a week) subcutaneous CCl_4 (2 ml/kg) administration, combined with administration of 2 weeks intragastric 2AAF (10 mg/kg) once a week (group II, n=6 and daily (group III, n=6). The control group (group I, n=6) received equal quantities of subcutaneous olive oil (CCl_4 diluent) and intragastric polyethylene glycol (2AAF solvent). Group II showed lowest (p=0.000; one way ANOVA, followed by Tukey's multiple comparisons test) weight gain (23.4±7.46 g) and highest liver/body weight ratio (4.4±0.20), and loss of liver surface slipperiness. Histopathological examination of treatment group using hematoxylin eosin and Masson's trichrome stainings showed liver cells damage (fat degeneration, cell swelling, necrosis and inflammation) and fibrosis. Severe pathology was seen in group III than group II, however both have high survival rate (>83%). Daily administration of 2 weeks high dose 2AAF (10mg/kg) in combination with 12 weeks repetitive CCl_4 (2 ml/kg) could induce severe rat liver cells damage and extensive liver fibrosis with an equivalent safety figure compared to that given once a week.
Keywords: *2AAF, CCl_4, 2AAF/CCl_4, liver injury, animal model.*

Perbandingan Dua Metode Pajanan 2AAF/CCL_4 dalam Pembuatan Model Hewan Tikus Cedera Hati Kronik

Abstrak

Cedera hati kronik merupakan masalah kesehatan masyarakat global sehingga banyak penelitian dilakukan untuk mencari mekanisme patologi atau regenerasinya, sehingga dibutuhkan suatu model hewan coba cedera hati kronik. Penelitian ini bertujuan untuk mengembangkan dan membandingkan dua metode pajanan 2AAF/CCl_4 pada tikus jantan dan menganalisis tingkat keberhasilan hidup, peningkatan berat badan, anatomi hati dan histopatologisnya. Penelitian ini merupakan studi experimental yang dilakukan di Animal Research Facilities-IMERI Fakultas Kedokteran Universitas Indonesia. Kami membandingkan dua metode pajanan 2AAF/CCl_4 pada tikus wistar jantan usia 8 minggu (n=18), yaitu pemberian CCl_4 (2 ml/kg) subkutan berulang selama 12 minggu (2 kali/minggu), dikombinasikan dengan pemberian 2AAF (10 mg/kg) intragastrik 1 kali/minggu (kelompok II, n=6) dan setiap hari (kelompok III, n=6). Kelompok kontrol (kelompok I, n=6) menerima cairan pelarut, yaitu minyak zaitun (pelarut CCl_4) subkutan dan polietilen glikol (pelarut 2AAF) intragastrik dalam jumlah setara. Kelompok III menunjukkan peningkatan berat badan terendah (23,4±7.46 g) dan rasio berat hati/berat badan (4,4±0.20) terbesar (p=0,000; one way ANOVA dilanjutkan dengan Turkey's multiple comparison test), serta kehilangan kelicinan permukaan hati. Pemeriksaan histopatologi kelompok perlakuan dengan pewarnaan hematoxylin eosin dan Masson's trichrome memperlihatkan kerusakan sel hati berupa degenerasi lemak, pembengkakan sel, nekrosis dan peradangan. Gambaran patologi lebih berat pada kelompok III dibandingkan kelompok II, namun keduanya mempunyai angka ketahanan hidup yang tinggi (>83%). Disimpulkan, pemberian setiap hari selama 2 minggu dosis tinggi 2AAF (10 mg/kg) dikombinasi dengan CCl4 (2 ml/kg) berulang selama 12 minggu dapat memicu kerusakan sel hati berat dan fibrosis luas dengan tingkat ketahanan hidup setara dibandingkan pemberian 1 kali/minggu.
Kata kunci*: 2AAF, CCl_4, 2AAF/CCl_4, cedera hati, model hewan.*

Introduction

Chronic liver injury has become a global public health problem that is responsible for the death of around one million people per year or 2% of all deaths in the world.[1] Deaths of people with cirrhosis of the liver in Indonesia increased from 16.925 to 49.224 in 1980 to 2010.[2] Based on the data of Indonesian Ministry of Health, 7.1% of Indonesian population suffered from hepatitis B and it has potential to become liver cirrhosis.[3] Liver is a main organ that responsible for metabolizing drugs, toxic chemicals and endogenous by products for the body.[4] Consequently, liver is vulnerable to damage. Yet, the liver has an extraordinary regenerating ability that is mediated by hepatocytes and facultative stem cells.

In rat, facultative stem cells or hepatic progenitor cells (HPC) are also known as oval cells which are characterized by small sized cells (about 10 µm) with an oval-shaped nucleus and a high ratio of nuclei and cytoplasm.[5] The emergence of oval cells in several studies is associated with a second line regeneration process involving liver progenitor cells when the ability of hepatocyte proliferation is inhibited.[6] These cells are located in the canals of Hering, have the ability to infiltrate along the liver plate and to differentiate into hepatocytes and cholangiocytes (bipotent ability).[7] Nevertheless, other studies have been linked the emergence of oval cells with fibrosis and cirrhosis of the liver although the mechanism has not been clearly defined.[8]

Animal models have been used to emulate many of human diseases, so does in chronic or severe liver injury. One of the animal models used to induce chronic/severe liver injury and induced HPC responses is a combination exposure of 2-acetylaminofluorene (2AAF) and carbon tetrachloride (CCl_4) in adult mice or rats.[9-13] CCl_4 is one of the most widely used liver toxin that induces liver fibrosis and cirrhosis in experimental animal models. The main features of liver histopathology after injection CCl_4 are infiltration of inflammatory cells, hepatocyte regeneration, stellate cell proliferation and connective tissue deposition.[4] CCl_4 induction will cause hepatic cells necrosis, mainly in pericentral area because of cells toxicity due to the high expression of cytochrome P450 2E1 (Cyp2E1). Robust hepatocyte necrosis occurs within 36 hours after CCl_4 exposure seen by a significant increase of plasma alanine aminotransferase (ALT). Repeated and prolonged exposures should be performed to induce severe liver injury and regeneration involving HPC as the ALT levels are reduced to almost normal after 72 hours; however, this does not seem sufficient.[14] To produce severe liver injury and activate the HPC, there should be a liver damage and suppression of hepatocyte proliferation; 2AAF can suppress hepatocyte proliferation and stimulate adult stem cells (oval cells) proliferation.[9]

Developing an animal model for chronic or severe liver injury, method of exposure (method of application, administration dose, and duration of exposure) is crucial. It aims to induce both liver injury and inhibition of hepatocyte proliferation, so that it can induce HPC response to regenerate the liver; meanwhile, it has to keep the animal models alive. Until now, there has not been a standard procedure in developing 2AAF/CCl_4 rat as an animal model for severe liver injury. The aim of the study was to develop and compare two method of 2AAF/CCl_4 exposures in male wistar rats and to analyze the subject survival, weight gain, liver anatomy and histopathology.

Methods

Study Subject and Design

This research was experimental study, conducted in Animal Research Facilities (ARF), IMERI Universitas Indonesia, from October 2018-October 2019. Eighteen male wistar rats, aged 8 weeks with a body weight of 160-200 g, were obtained from Animal Research Division of Research and Development Agency, Ministry of Health of the Republic Indonesia. Animals were housed in a constant temperature, under a 12-hour dark/light cycle and supplied with laboratory chow and water ad libitum. We used 2AAF and CCL_4 to induce severe liver injury. CCl_4 was purchased from Merck® (Munchen, Germany) and 2AAF was purchased from Sigma-Aldrich® (Darmstadt, Germany). All animal experiments were approved by ethics committee of Faculty of Medicine Universitas Indonesia (No. 1277/UN2.F1/ETIK/2018).

2AAF/CCl_4 Exposure to Induce Severe Liver Injury

A total of 18 rats were divided into three groups of six rats. Group I is rats as a control group and received equal quantities of subcutaneous olive oil and intragastric polyethylene glycol (PEG), Group II is experimental rats that were induced by subcutaneous injection of 1:1 solution of CCl_4 in olive oil (2 ml/kg), administered twice a week for 8 weeks. At 9^{th}-12^{th} weeks, the dose was changed into 3:7 solution of CCl_4 in olive oil (2 ml/kg) plus intragastric administration 2AAF in PEG (10 mg/kg) once a week. Grup III is experimental rats that were induced with CCl_4 in olive oil in the same dose

and administration as group II with difference in the administration of 2AAF in PEG which was given every day (at 9th-12th weeks).

Livers were collected after sacrificing animals at the end of 12th week, 24 hours after the last induction. Gross anatomy of the liver was observed, the weight was measured (in gram) and the liver weight/body weight was counted. For histopathological analysis, liver specimens were fixed for 12-24 hours in 10% neutral formalin solution, embedded in paraffin, sectinoned as thick as 3 µm, and were stained with two kinds of staining, i.e. hematoxylin eosin (HE) to evaluate the liver morphology and pathological process, and Masson's trichrome staining (MT) to evaluate the degree of fibrosis. Histopathological evaluation was performed with standard light microscopy (Leica type DM750).

Severity of the liver damage was determined according to the French at al[15] as folows: score 0: no visible cell damage, score 1: focal hepatocyte damage on less than 25% of the tissue, score 2: focal hepatocyte damage on 25–50% of the tissue, score 3: extensive, but focal hepatocyte lesions, and score 4: global hepatocyte necrosis. The degree of fibrosis was based on the characteristic pattern and progression of fibrosis according to Onyekwere et al[16] as follows: stage 0: none of fibrosis, stage 1a: mild fibrosis in the perisinusoidal zone 3, stage 1b: moderate fibrosis in perisinusoidal zone 3, stage 1c: fibrosis in the portal of periportal, stage 2: fibrosis in the perisinusoidal zone 3 and portal/periportal, stage 3: bridging fibrosis, and stage 4: cirrhosis.

Statistical Analysis

The statistical analysis was carried out using SPSS 20.0 for windows statistical package. Data (body weight, body weight gain, liver weight, and liver weight/body weight ratio) were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. The criterion for statistical significance was $p < 0.05$. All values were expressed as mean values ± SE.

Results

The two method of 2AAF/CCl_4 exposures have high survival rate, i.e. 83-100%. One of 18 rats, rat from group II, died during CCl4 induction. The causal death was not detected.

Body Weight and Liver Weight

After 12 weeks 2AFF/CCl_4 exposures, there were changes in the body weight and liver weight (Table 1). All parameters were analyzed by one way ANOVA, followed by Tukey's multiple comparisons test. Initial body weight was rats body weight at the beginning of research (before exposures). Each group had no significant different in their initial body weight (p=0.173). Final body weight was rats body weight after 12 weeks 2AAF/CCl4 exposures. Weight gain is the difference between final body weight and initial body weight. Group III showed significant lower of body weight compared to the group I and group II (both p = 0.000), whereas there was no different between group II and group I (p=0.894). The same condition was also seen in their weight gain. Group III had the lowest weight gain compared to the group I and II (both p=0.000); whereas, group II was not significantly different from group I (p=0.888). The rat liver weight of experimental groups (group II and group III) were significantly higher compare to the control group (group I; p=0.001 and p=0.045 respectively). However, there was no significant difference between experimental group (group II and group III; p=0.076). The liver index (liver/body weight ratio) showed significant differences all groups (p=0.000).

Tabel 1. Rats Body Weight, Weight Gain, Liver Weight, and Liver/Body Weight Ratio Between Groups

Groups	Initial body weight (g)	Final body weight (g)	Weight gain (g)	Liver weight (g)	Liver weight/ body weight x100
I	187.0 ± 1.95	282.6 ± 12.35	95.6 ± 12.69	6.5 ± 0.38	2.3 ± 0.08
II	186.8 ± 3.28	288.6 ± 9.68#	101.8 ± 6.88#	10.7 ± 0.72*	3.7 ± 0.18*#
III	176.8 ± 5.95	200.2 ± 4.19*#	23.4 ± 7.46*#	8.7 ± 0.56*	4.4 ± 0.20*#

*significant different between group II and group I or group III and group I
#significant different between group II and group III
The mean difference is significant at the 0.05 level

Anatomy of the Liver

Gross anatomy observation of the liver showed a different appearance of group III compared to the group I. Normaly, liver has a reddish-brown colour, with a fresh (shiny) and smooth (slippery) surface. There was a gradual changes from the normal morphology in group I, slight changes in group II (n=5; one rat died during the experimental), and significant changes in group III. In the experimental group, stand out in the group III, the surface became rough and lost its freshness. The size of the group III liver also bigger than the other groups (Figure 1.).

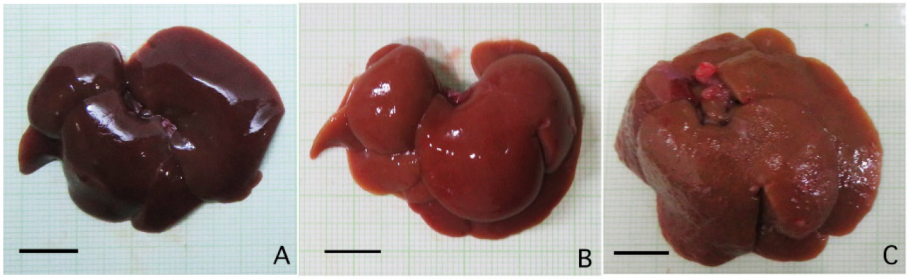

Figure 1. Gross Anatomical Features of the Livers. A. Group I (control). B. Group II. C. Group III. The livers were laydown on the milimeter paper, in the same magnification. Scale bar: 10 mm.

Histopathology of the Liver

Observation of the HE staining of liver tissues shown that the experimental groups experienced changes in their morphology that imply tissue damage compared to the control group. Group I had a classic lobular appearance with radially arranged hepatocytes against the central venous, separated by the sinusoids (figure 2, B). Radial arrangement of the hepatocytes was maintained in the group II, yet there was appearance of liver damage, such as fat degeneration, cell sweeling, necrosis and inflamation. There was also seen ductular reaction in the portal area. Group III had an irregular lobules, the picture of the liver lobules appeared irregular, the damage area was wider, and the ductular reaction has invaded the liver parenchyma. Moreover, group III showed nodular foci that were not found in other groups (Figure 2).

Semi-quantitative analysis of the liver (Figure 4) includes the coverage area of the liver damage and liver fibrosis (percentage of damage area counted from microscopic five large field of view in each samples), scores of the liver damage (addopted from French et al)[15] and stage of the liver fibrosis (adopted from Onyekwere et al)[16] in the coverage area were also done to get more objective picture about the pathological phenomena between experimental groups.[15,16] All samples of the control group did not show any pathological process, whereas the experimental groups showed liver damage. Both group II and group III showed fat degeneration, necrosis, cells swelling (ballooning), inflamation and fibrosis (Figure 2 and 3). Group III had wider coverage area of liver cells damages and fibrosis compared to the group II, except the fat degeneration process. The severity of the liver cells damage and fibrosis in the coverage area were also higher in the group III compared to the group II (Figure 4). Most of the fibrosis of the group II was limited in the perisinusoidal zone 3 (stage 1; 80% of the coverage area; detail data are not shown). There was only slightly fibrosis that extended and affected the portal/periportal area (stage 2; 16% of the coverage area). In group III, the fibrosis has extended to the portal/periportal (stage 2; 30% of the coverage area) and even bridge the central vein and portal triad (stage 3; 40% of the coverage area). The remaining 30% of the fibrosis coverage area was limitted to the perisinusoidal zone 3 or portal/periportal (stage 1).

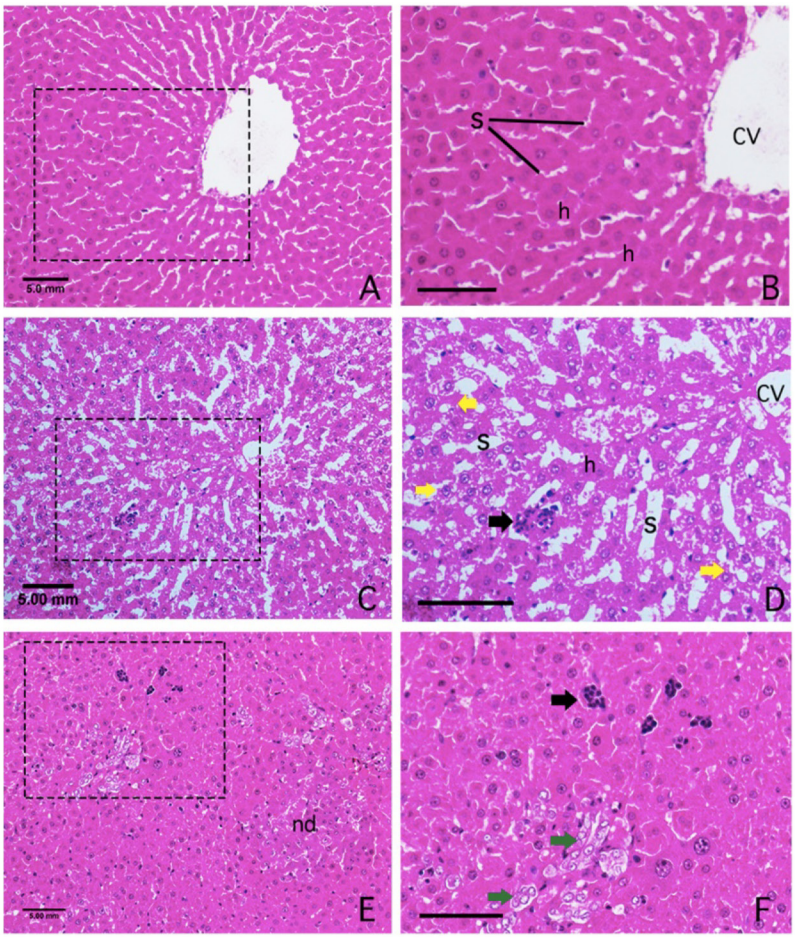

Figure 2.
Photomicrograph of Liver Stained with HE. A, B. Normal morphology of liver tissue (group I/control). B is a magnification of dashed lines box in A. C, D. Group II. D is a magnification of dashed lines box in C. Liver damage was clearly seen: cells swelling (yellow arrows), inflammation (black arrow) and necrotic cells. E, F. Group III. F is a magnification of dashed lines box in E. The swelling cells, inflamation (black arrow) and necrotic cells are seen. In this group, ductular reaction (green arrows) and nodular foci (nd) were prominent. CV: central vein, s: sinusoid, h: hepatocyte, black bar: 500µm.

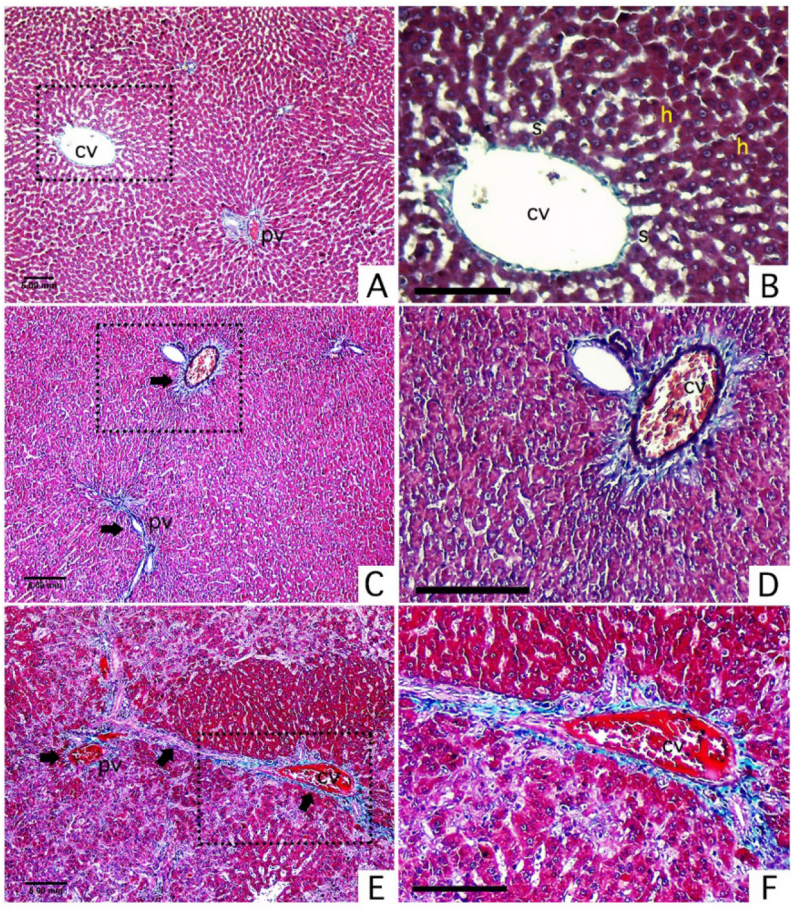

Figure 3.
Photomicrograph of liver stained with Masson's Trichrome. A, B. Group I (control). B is a magnification of dashed lines box in A. This shows normal distribution of collagen fibers in the portal area (pv) and central vein (cv). C, D. Group II. D is a magnification of dashed lines box in C. This shows increased distribution of collagen fibers (black arrows) in perisinusoid in zone 3 and portal/periportal. E, F. Group III. F is a magnification of dashed lines box in E. This shows distribution of collagen fibers in perisinusinoid in zone 3 that connect to the portal/periportal area (bridging fibrosis). h: hepatocyte, black bar: 500µm.

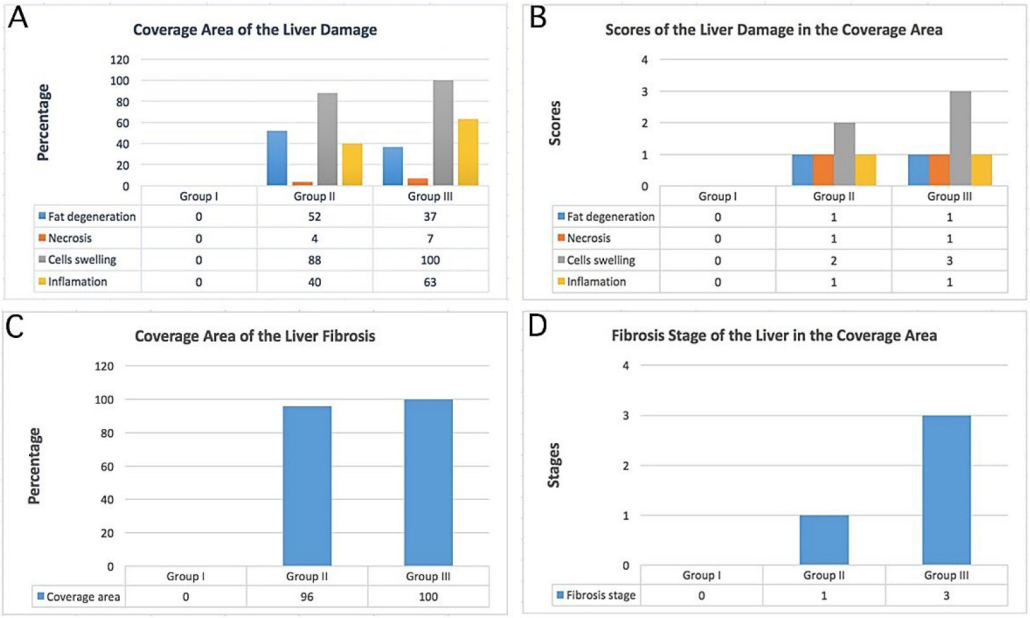

Figure 4. Semi-quantitative Analysis on The Coverage Area of The Liver Damage and Fibrosis, Severity of The Damage and Fibrosis in The Coverage Area. A. Coverage area of the liver damage (%). B. Severity of the liver damage was determined according to the French at al.15 C. Coverage area of the liver fibrosis (%). D. Summary of the severity of the fibrosis. The degree of fibrosis was based on the characteristic pattern and progression of fibrosis according to Onyekwere et al.16

Discussion

Developing an animal model for severe liver injury has critical role to reveal the mechanism of chronic/severe liver injury and liver regeneration. Repeated injection of CCl_4 in combination with the exposures of 2AAF to the rats was expected to be able to induce liver damage that can mimics human severe or chronic liver damage. Our study showed that combination exposures of 2AAF/CCl_4 could induce rat's liver damage with high survival rate. Degree of the liver damage and the histopathological features was influenced by the administration dose of the inducing chemicals. This result was in accordance with the report of Paku et al[17] and Constandinou et al.[18] Paku et al[17] found that the rate of oval cell differentiation into hepatocytes is contact dependent. Low dose of AAF exposure (2.5mg/kg daily) resulted in mild liver cells damage and induce rapid and synchronous differentiation of oval cells. High dose 2AAF exposure (5 mg/kg daily) resulted in extensive liver cells damage that delayed oval cells differentiation and was proceeded an accumulation of hepatocytes into focal nodules. Constandinou et al[18] found that repetitive CCl_4 exposures can induce different level of liver fibrosis, starting on bridging fibrosis (4 weeks of twice-weekly dosing), cirrhosis (8 weeks of twice-weekly dosing) until advanced micronodular cirrhosis (12 weeks of twice-weekly dosing). Figure 4 showed that combination of repetitive CCl_4 and low dose 2AAF exposures (group II) resulted in focal liver damage (score 1-2) and limited liver fibrosis (stage 1); whereas, combination of repetitive CCl_4 and high dose 2AAF exposures (group III) resulted in focal until extensive liver cells damage (score 1-3) and bridging liver fibrosis (stage 3).

In this study, 12 weeks repetitive subcutaneous CCl_4 exposure (2 ml/kg) combine with low dose 2AAF exposure (10 mg/kg, intragastric, once a week, for 2 weeks) or high dose 2AFF (10mg/kg, intragastric, daily, for 2 weeks) could not induce hepatic cirrhosis, eventhough previous study has been showed that 6-10 weeks oral or intraperitoneal administration of CCl_4 could induce hepatic cirrhosis. The possible explanation is that subcutaneous CCl_4 administration might takes longer time to give and effect than oral or intraperitoneal administration. Previous study reported that subcutaneous injection in mice results in a low mortality (<5%), however it took longer time for cirrhosis to be

occurred, i.e. after 20 weeks exposure or more.[19] Nevetheless, combination repetitive exposure of CCl_4 and high dose of 2AAF exposure (group III) could induce more severe liver cells damage and liver fibrosis compare to the combination with low dose of 2AAF exposure (group II). This result implied that high dose 2AAF that was carried out every day for 4 weeks can accelerate the progression of fibrosis formation induced by CCl_4. This phenomenon is undestandable because rat liver has extraordinary regeneration capacity. It will regenerate as soon as CCl4 exposure is stopped; therefore, in combination with 2AAF, the capacity of hepatocyte to proliferate is inhibited by 2AAF, that results in progression of liver damage and decreased hepatocyte regeneration capacity.[9,14]

Every day intragastric administration of high dose 2AAF could induce ductular reaction and formation of nodular foci compare to the once a week administration. This phenomenon can be explained that every day administration of 2AAF will continuesly inhibit the proliferation of hepatocyte that resulted in the progression of liver damage, delayed the hepatocyte differentiation, and cause the accumulation of small hepatocyte into nodular focus. Whereas, once a week administation of 2AAF give a time to hepatocyte to be recovered and proliferate, in the same time induce the oval cells rapidly and synchronously differentiate into small hepatocytes. That is why, in this study ductular reaction could be seen both in the group II and III, but the group II was limitted in the portal/periportal. In the group III ductular reaction expanded to the liver parenchyma and nodular foci were formed.

The histopathological data supported the gross anatomy description of the liver and the rat's physical condition. Liver damage and inflammation cause changes in the liver morphology, including colour (much lighter in the experimental group) liver dimension (bigger and increase weight in the experimental group, especialy the group III), and liver surface (lost of slipperiness). The liver damage cause the decline of rat's health as reflected by the lower weight gain, especially in the group III.

Conclusion

Daily administration of 2 weeks high dose 2AAF (10 mg/kg) in combination with 12 weeks repetitive CCl_4 (2 ml/kg) can induce severe rat liver cells damage and extensive liver fibrosis compared to that given once a week with comparable survival rate. Combination of CCl_4 and 2AAF exposures in rat as an animal model for chronic/severe liver injury can be used to study the role of HPC during liver regeneration.

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