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RESEARCH ARTICLE

The Stages of Development of Liver And Renal Injuries in Rats Induced by Fixed Dose Combination of Antituberculosis Regimen

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SUMMARY

The use of fixed dose combination of four antituberculosis drugs (4FDC-AT) is recommended to eradicate tuberculosis infection. However, it may induce liver and renal dysfunctions. This study aimed to evaluate time-dependent development of liver and renal injury in rats induced by subchronic use of 4FDC-AT regimen. Male Wistar rats (150-250 g) were divided into two groups of six. Group 1 was given placebo while group 2 was treated with 4FDC-AT (89 mg/200 g) for 28 days. Blood samples were withdrawn to measure serum alanine aminotransferase (ALT) and creatinine levels on day 0 (baseline), 7, 14 and 28 of treatment. Following the last treatment, rat liver and kidney were harvested for histological analysis. Elevation of ALT and creatinine levels were considered noteworthy if the levels are >50% from upper baseline. None of the placebo rats experienced a significant increase in ALT and creatinine levels from day 7 to 28. In contrast, ALT escalated in 50% of rats in 4FDC-AT group starting at day 14; however, the 4FDC-AT treatment only increased creatinine levels in 17% of rats after 28 days. Histopathological analysis of liver and kidney in the placebo group showed normal histology structures, while in 4FDC-AT treated rats showed profound hydropic and lipid degeneration of hepatocytes. In renal tubules, infiltration of inflammatory cells and vacuolization were only evident in one rat. It is concluded 4FDC-AT administration rapidly induces liver dysfunction and structural damage in rats while development of renal injury was slower and limited.

Key Words: *Fixed dose combination, antituberculosis, experimental, organ toxicities, liver dysfunction, renal injury*

Sıçanlarda Antitüberküloz Rejiminin Sabit Doz Kombinasyonu Tarafından İndüklenen Karaciğer ve Böbrek Hasarı Gelişim Aşamaları

ÖZ

Tüberküloz enfeksiyonunu ortadan kaldırmak için dört antitüberküloz ilacın (4FDC-AT) sabit doz kombinasyonunun kullanılması önerilir. Ancak bunlar, karaciğer ve böbrek fonksiyon bozukluğuna neden olabilir. Bu çalışma, sıçanlarda 4FDC-AT rejiminin subkronik kullanımı ile indüklenen, zamana bağlı karaciğer ve böbrek hasarı gelişimini değerlendirmeyi amaçlamaktadır. Erkek Wistar sıçanları (150-250 g) altı üyeli iki gruba ayrıldı. Grup 1'e plasebo verilirken, grup 2 28 gün boyunca 4FDC-AT (89 mg / 200 g) ile işleme tabi tutldu. Serum alanine aminotransferase (ALT) ve kreatinin seviyelerini tedavinin 0.(bazal), 7., 14. ve 28. günlerinde ölçmek için kan örnekleri alındı. Son işlemin ardından, sıçan karaciğer ve böbrekleri histolojik analiz için toplandı. ALT seviyelerinin ve kreatinin seviyelerinin yükselmesinde,söz konusu seviyelerin üst sınırdan % 50'den fazla yüksek olması durumu dikkate değer olarak kabul edildi. Plasebo sıçanlarının hiçbiri ALT ve kreatinin seviyelerinde 7. günden 28. güne kadar önemli bir artış yaşamadı Buna karşılık ALT, 14. günde başlayarak 4FDC-AT grubundaki sıçanların % 50'sinde yükseldi. Ancak, 4FDC-AT tedavisi, 28 gün sonra sıçanların % 17'sinde kreatinin seviyelerini arttırdı. Plasebo grubundaki karaciğer ve böbreğin histopatolojik analizi normal histoloji yapılarını gösterirken, 4FDC-AT verilmiş sıçanlardaki hepatositlerde derin hidropik ve lipid dejenerasyonu görüldü. Renal tübüllerde, enflamatuar hücrelerin infiltrasyonu ve vakuolizasyon sadece bir sıçanda belirgindi. Sıçanlarda 4FDC-AT uygulamasının, hızlı bir şekilde karaciğer fonksiyon bozukluğuna ve yapısal hasara yol açarken, böbrek hasarı gelişiminin daha yavaş ve sınırlı olduğu sonucuna varılmıştır.

Anahtar Kelimeler: *Sabit doz kombinasyonu, antitüberküloz, deneysel, organ toksisiteleri, karaciğer fonksiyon bozukluğu, böbrek hasarı*

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INTRODUCTION

Tuberculosis (TB) is a global burden and around ten million new cases are still identified in 2017 alone. At least 60% of these cases are originated from Asian countries including India, Indonesia and China (WHO, 2018). The current strategy to control TB infection emphasizes the use of four Fixed Dose Combination regimen of antituberculosis (4FDC-AT), comprises of isoniazid, rifampicin, pyrazinamide, and ethambutol for two months, followed by the 2FDC regimen for four months (Gilpin et al, 2018). This regimen is found beneficial to lessen TB infection; however, there is a concern that 4FDC-AT may be associated with serious side effects, including hepatotoxicity and nephrotoxicity.

Several studies in Asia have reported around 10-30% of antituberculosis (AT)-treated patients develop liver dysfunction and hepatotoxicity (Mushiroda et al., 2016; Saha et al., 2016; Wu et al, 2015). Hepatotoxicity of AT is associated with isoniazid, rifampicin or pyrazinamide use alone (Yew and Leung, 2006), but the risk may increase if taken together due to drug-drug interactions (Jeong et al., 2015). This may negatively affect the patient's compliance with medication (Maria, Radji, and Burhan, 2017). In some cases, AT-induced hepatotoxicity may require the patients to reduce the dose of AT used, or in more extreme cases, compel the patients to stop taking the medication (Abbara et al., 2017). This may negatively impact the success rate of the treatment and even increase the risk of AT resistance (Maria et al., 2017).

In addition to hepatotoxicity, 7% of TB patients are found to develop acute kidney injury (AKI) during the intensive phase of AT (Ghori, et al., 2015). Although the prevalence is low, several cases may not be recovered leading to permanent renal damage (Afroz et al, 2017; Kim, Kim, and Choi, 2018). Renal toxicity of AT is predominantly triggered by the use of rifampicin (Covic, et al., 1998), yet again, when combined with isoniazid, the risk could be amplified (Chogtu et al, 2016).

The hepato-renal toxicity of AT is idiosyncratic in nature and widely dependent upon the characteristics and sensitivity of the host (Chen et al, 2015). The clinical manifestations can vary from asymptomatic elevations of liver enzyme levels to profound liver failure (Tweed et al., 2018). AT-induced hepatic and renal toxicities have been implicated with some risk factors, including age, comorbidities, co-medication, and Asian race (Chen et al., 2015; Tweed et al., 2018). Nevertheless, it is still difficult to predict whether someone would experience liver toxicity or merely an asymptomatic rise of liver enzymes during AT treatment (Saha et al., 2016).

The use of animal model might be essential to reveal the possible outcomes of subchronic use of AT especially in 4FDC dosage form. The 4FDC-AT form is of particular importance as not much data has used the regimen in animal studies. Therefore, this research aimed to study the development of liver and renal damage in rat animal model induced by subchronic use of 4FDC-AT regimen. Liver and renal damages are indicated by elevation of serum biomarker levels (> 50% from upper baseline) with evident histopathological changes in liver and kidney tissue structures following 28 days of treatments.

MATERIALS AND METHODS

Chemical and drug preparation

Diagnostic kits for alanine aminotransaminase (GPT (ALAT) IFCC mod.liquiUV) and creatinine (creatinine liquicolor) analysis were supplied from Human Diagnostic Worldwide® (Germany). The 4FDC-AT tablets were purchased from local pharmacy (Indofarma, Makassar, Indonesia), which comprised of rifampicin (150 mg), isoniazid (75 mg), pyrazinamide (400 mg) and ethambutol hydrochloride (275 mg).

The dose of 4FDC-AT used was calculated from human effective dose (HED) converted to animal effective dose (AED) by multiplying HED in mg/kg by 6.2, which is the conversion factor for rats (Nair and Jacob, 2016). The use of dose conversion factor is necessary as an animal model may have different biochemical, pharmacokinetics as well as physiological time that may affect drug outcomes in different species (Nair and Jacob, 2016). As the HED of 4FDC-AT for 60-kg human is 4 tablets (72 mg tablet/kg), hence, the dose given to rats was 446.4 mg tablet/kg or 89 mg tablet/200g rat bodyweight. This regimen contained rifampicin 62 mg/kg, isoniazid 31 mg/kg, pyrazinamide 164 mg/kg and ethambutol 113 mg/kg. The drugs were suspended in 1% sodium carboxilmethyl cellulose (NaCMC) prior to administration.

Animal preparation

Male Wistar rats (150-200 g) were cared in animal house of Biopharmacy Laboratory, Hasanuddin University (Makassar, Indonesia). Rats were kept in plastic well-aerated cages with maximum 4 rats per cage at a room temperature. The room was set up with 12 h light/dark cycle. All animals were provided with free access to water and food *ad libitum.* Rats were acclimated at least 7 days prior to use in the

experiments. All procedures applied on the animals complied with institutional standard of care and have been approved by Institutional Animal Ethics Committee under Hasanuddin University No. UH 170121091.

Experimental protocols

Twelve rats were assigned into 2 groups (n=6): Group 1 received the placebo (1% NaCMC) while Group 2 was treated with 4FDC-AT 89 mg/200 g rat body weight via oral gavage. Rats were weighed everyday to adjust the amount of 4FDC-AT administered daily. All treatments were given via oral gavage for 28 days (four weeks) to observe the development of liver and renal injuries during sub-chronic administration. Blood samples were withdrawn prior to treatment (day 0), on day 7, day 14 and 28 via lateral vein. Rats' livers and kidneys were harvested after 24 hours following the final treatments. Three additional rats that did not receive any treatments (only water and food) were sacrificed to provide healthy liver and kidney histology.

Biochemical assays

Blood samples were collected in vacuum tubes and centrifuged at 2500 rpm for 20 minutes. The sera were then analysed to measure alanine aminotransferase (ALT) and creatinine levels using Humalyzer 3500 (Human®) based on the kit instruction.

Histopathological examination

Livers and kidneys were immediately removed and washed in cold phosphate buffer solution (PBS) before fixed in 10% formaldehyde for 48 hours. The specimens were then processed in a tissue processor (Thermo Scientific®), embedded in paraffin wax, and cut into 5-µm thick sections with a microtome (Sakura®). Tissue sections were stained with Haematoxylin and Eosin (H&E). The presence of histological changes was examined under a light microscope (Olympus®) connected to a computer screen and a camera (Nikon®) by two observers blinded to the treatment given.

Statistical analysis

Numerical data are presented in mean ± standard deviation (SD). Data was analysed using SPSS version 22 software (IBM) and all statistical differences between the groups were analysed using paired T-test. Statistical significance was achieved if p value was <0.05.

RESULTS

Effects of 4FDC-AT on liver and renal biomarkers

This study was conducted using rat animal models that received 4FDC-AT regimen converted from human effective dose to rat dose. One daily dose contained rifampicin 62 mg/kg, isoniazid 31 mg/kg, pyrazinamide 164 mg/kg and ethambutol 113 mg/kg.

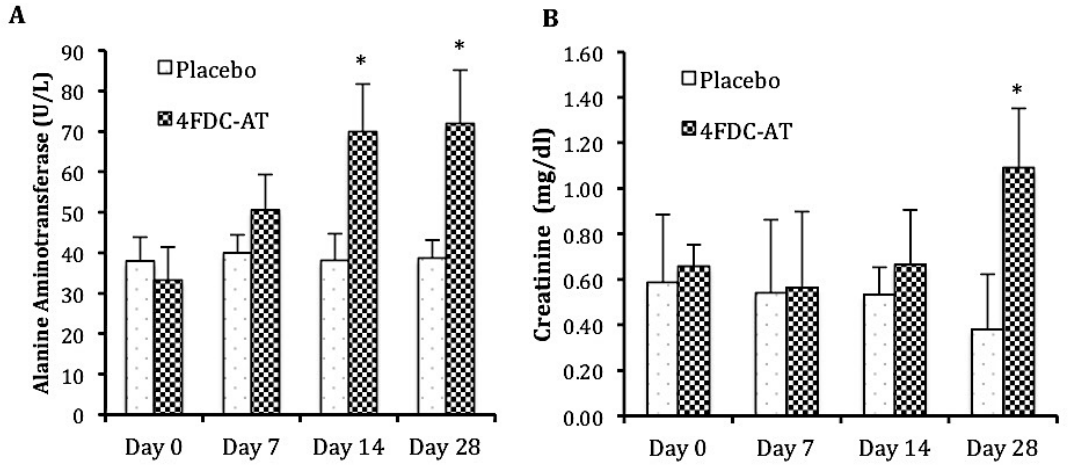

Figure 1. The serum level of alanine aminotransferase (A) and creatinine (B) before (day 0) and following treatments at day 7, 14 and 28 in placebo and 4FDC-AT groups

The baseline level of ALT was measured one day prior to treatment (day 0). It was found that the level of ALT in both groups was not significantly different with a range of 23-47 U/L. This range serves as the normal value in this study. Figure 1A illustrates the pattern of mean ALT level in placebo and 4FDC-AT treated rats. After 7 days of treatments, one rat experienced an increased ALT level, but it was not considered significant (less than 50% of upper normal value). At this stage, the overall increase in mean

ALT level of 4FDC-AT group was not significantly different from the placebo group. On day 14 of treatments, three out of six (50%) 4FDC-AT rats has shown an elevated ALT of >50% from normal range. Indeed, the mean ALT level of 4FDC-AT group was almost twice as high as that in the placebo group ($p<0.05$). On day 28 of treatments, the level of ALT in 4FDC-AT group did not further increase despite the continuous treatment. Yet, the mean of ALT in 4FDC-AT treated rats were still significantly higher than those treated with placebo ($p<0.05$).

The creatinine levels prior to treatments (day 0) ranged from 0.29 to 0.87 mg/dl and were not statistically different between treatment groups. Creatinine level was considered significant in this study if it reached 50% from upper normal limit of 0.87 mg/dl. Measurement of creatinine level on day 7 revealed no significant changes occurred in both placebo and 4FDC-AT groups (Figure 1B). Similar results also found after 14 days of treatments, where creatinine levels of rats from either group were still in the normal range. On that day, no significant difference was found between treatment groups. Following 28 days of treatments, one out of six (17%) rats in 4FDC-AT group have shown a significant increase in their creatinine levels. In overall, the mean creatinine level of 4FDC-AT group was significantly higher than that in placebo group ($p<0.05$).

Effects of 4FDC-AT on liver and renal histopathological changes

Hepatic tissue

Histopathological examination (Figure 2) showed that the liver tissue of healthy rats had normal appearance of hepatic tissue, the presence of red blood cells were depicted in some areas of sinusoid and several blood vessel were moderately congested (Figures 2A and 2B). Placebo groups did not show any lesions of pathological significance comparable to that of healthy rats (Figures 2C & 2D). In contrast, some animals in 4FDC-AT group experienced abnormal liver structure, characterized by scattered hepatic degeneration with extensive hemorrhagic area (Figures 2E, 2F & 2H). Most hepatocytes showed hydropatic degeneration characterized by cytoplasm swelling which was lighter in colour (Figure 2F). Vacuolization was also found in some hepatocytes (Figure 2F) with some necrotic area (Figure 2G). The sinusoids were markedly dilated and mostly congested with blood, with increased infiltration of inflammatory cells.

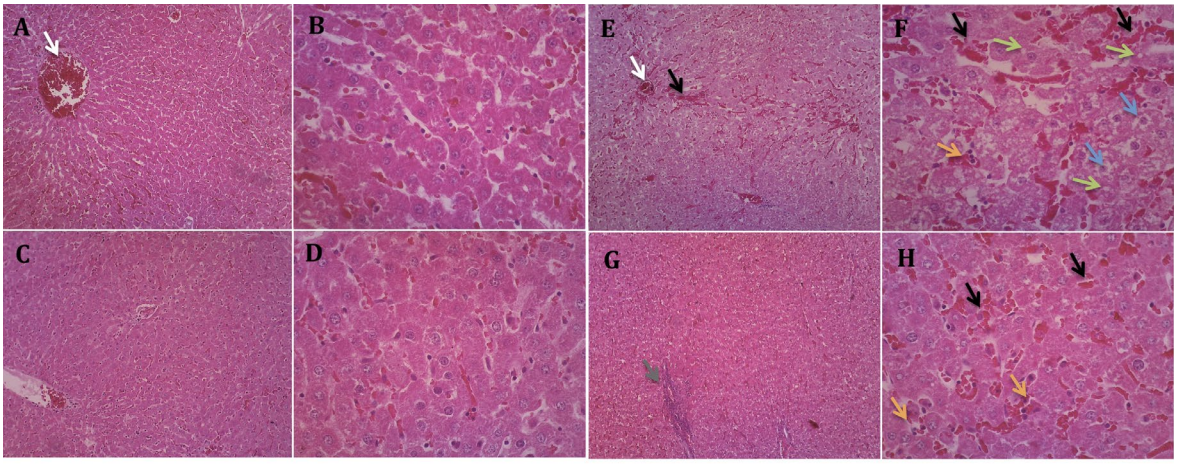

Figure 2. The liver histology in healthy rats (A, B), placebo (C, D) and 4FDC-AT-treated rats (E, F, G, H) following 28 days of treatments using H & E stain. Magnification 100x and 400x. Healthy rats and placebo group did not show significant histopathological changes. Liver structural damage found in 4FDC-AT-treated rats include extensive hemorrhage (black arrow), hepatocyte hydropic degeneration (blue arrow), vacuolization (green arrow), infiltration of inflammatory cells (yellow arrow), congestion (white arrow), and necrotic area (grey arrow)

Renal tissue

Based on examination of renal tissue (see Figure 3), the structural damage was less intense compared to that seen in liver following 28 days of 4FDC-AT treatment. However, signs of histological changes were shown, especially in renal tubules, where some haemorrhage and mild loss of tubule

lining were found (Figure 3H). One rat experienced significant degeneration of tubules characterized by almost complete loss of tubule lining and profound infiltration of inflammatory cells. With 4FDC-AT dose used in this study, the integrity of glomerulus was not significantly altered; although mild histological changes such as dilated Bowman's capsule was found.

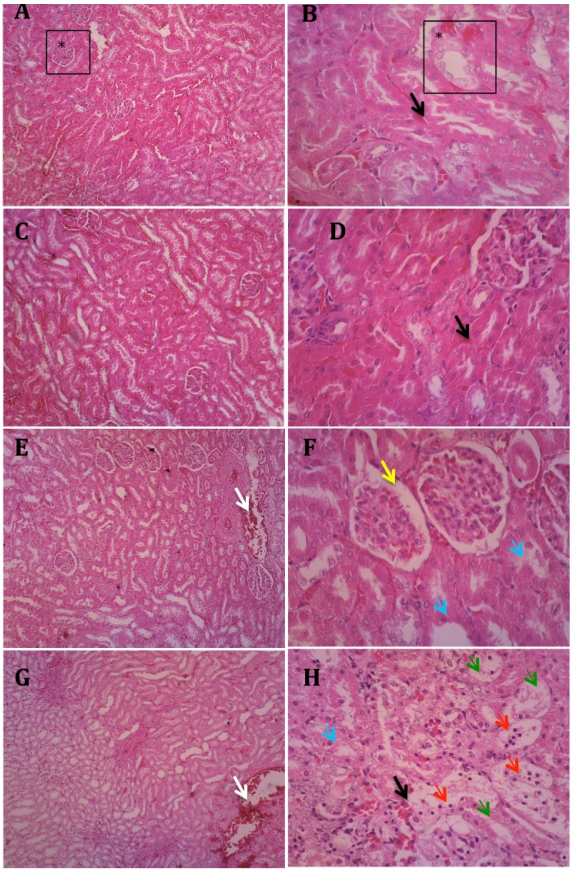

Figure 3. The renal histology in healthy controls (A, B), placebo (C, D) and 4FDC-AT-treated rats (E, F, G, H) following 28 days of treatments using H & E stain. Magnification of 100x and 400x. Healthy rats and placebo group did not show significant histopathological changes. Histopathological changes found in 4FDC-AT-treated rats include dilated Bowman capsule (yellow arrow), congestion (white arrow), mild loss of tubule lining (blue arrow), tubule degeneration (green arrow), hemorrhage (black arrow) and profound infiltration of inflammatory cells (red arrow).

DISCUSSION

The potential risk of hepatotoxicity and nephrotoxicity in TB patients with AT treatments has been recognized. However, it is difficult to study the pattern of hepatotoxicity and nephrotoxicity in humans. Although many studies have confirmed the presence of hepatotoxicity or nephrotoxicity induced by AT in animal models, but most studies only used combination of isoniazid and rifampicin (50 mg/kg to 150 mg/kg) (Aliosmanoglu et al., 2018; Amir et al., 2016; Hussain, Subaiea, and Firdous, 2018). Another study has induced liver toxicity using 4FDC-AT but the dose regimen is higher than those utilized in clinical settings (Mishra et al, 2018). This study was conducted using rat animal models that received 4FDC-AT dose calculated from AED conversion from HED (rifampicin 62 mg/kg, isoniazid 31 mg/kg, pyrazinamide 164 mg/kg and ethambutol 113 mg/kg). This allows a direct observation of the pattern of liver and renal dysfunction during treatments, as well as their histological structures following 28 days of 4FDC-AT treatments in rats.

The results showed that 4FDC-AT increased ALT levels starting from day 7, in which one rat experienced a 30% increase in ALT level. After 14 days of treatment, 50% of rats in 4FDC-AT group experienced >50% ALT elevation, but no further raise in ALT level after 28 days of treatment. The fact that only 50% of rats experienced ALT elevation at the end of experiment suggests not all rats are susceptible to 4FDC-AT hepatotoxicity at the particular dose given. In contrast, elevated creatinine level only appeared in 17% of 4FDC-AT rats during treatment, indicating that 4FDC-AT detrimental effects on renal function were not as severe as those in liver. This is also true in clinical setting. Among adverse effects associated with AT use, hepatotoxicity was more frequently reported than other unwanted effects (e Castro et al, 2015; Gaude, Chaudhury, and Hattiholi, 2015; Singh et al, 2015); whereas, the incidence of AKI due to the first-line AT was not mentioned in those studies. Apparently, the incidence of AT-induced AKI is more noticeable in geriatric patients (43 out of 61 patients), which may or may not recover after treatment discontinuation (Ghori et al., 2017).

The elevation of both ALT and creatinine levels showed a positive moderate correlation, implying that rat susceptibility to liver and renal toxicities might be influenced by similar factors. Both liver and renal toxicities of AT could be initiated by rifampicin toxic metabolites (Chogtu et al., 2016). The risk is shown to increase along with reduced antioxidant activities and increased oxidant exposure in individuals (Yew, Chang, and Chan, 2018).

The presence of hepatotoxicity is not adequately indicated by an elevation of ALT only. The histopathological evaluation would add important

information about liver tissue integrity and provide more evidence of hepatotoxicity. The liver sections of rats that received placebo showed normal liver tissue histology, quite similar with those of healthy rats (Figure 2). In contrast, 28 days of 4FDC-AT treatments had led to scattered hepatic degeneration and the centrilobular region showed a patchy appearance. Most sinusoid area was dilated and haemorrhagic, and often infiltrated by inflammatory cells (Figure 2). This finding has confirmed the presence of hepatotoxicity in rats treated with 4FDC-AT for 28 days. In fact, the rats with ALT elevation had more severe damage in their liver structures compared to those with insignificant increase of ALT. In TB patients, increased in ALT level could be asymptomatic, but may also prolonged and lead to hepatotoxicity in some cases (Saha et al., 2016). But in rats, the raise in ALT level >50% of upper limit value was followed by a significant damage in liver histological structure.

Unlike hepatic damage, the renal histopathological changes found in this study were considered mild to moderate. Only one rat had shown extensive damage in the tubular area with profound infiltration of inflammatory cells. Previously, the presence of histological renal damage with AT drugs have been studied in Wistar rats, but the result could vary from mild or moderate to significant alteration of rat renal histology (Hussein, Germoush, and Mahmoud, 2016; Muzika et al., 2016). Significant renal damage in Hussein's study (2016) was developed after 45 days of AT treatment. It is believed that the limited kidney injury found in this study was due to the short duration of the treatment (28 days). The result of this study was more similar to Muzika et al. (2016), which also observed a mild to moderate injury in kidney histology after 21 days of isoniazid and rifampicin treatments in rats.

This present study has some limitations. First, the presence of renal injury was not confirmed using urine biomarkers or blood urea nitrogen and was only based on elevation of serum creatinine. Serum creatinine was chosen since it is the closest to the ideal predictor of renal function than the other biomarkers. Secondly, additional measurement of liver function markers, such as aspartate aminotransferase (AST) and alkaline phosphatase (ALP), could also be useful to describe the range and intensity of liver injuries in the rats. Nevertheless, ALT was chosen to indicate rat liver injury in this study because it is the most specific marker for hepatocellular injury and elevated ALT has become the main feature of AT-induced hepatotoxicity in patients (Abbara, et al., 2017).

CONCLUSION

In conclusion, liver toxicity of 4FDC-AT was more evident and rapidly developed than renal toxicity in rats. The elevation of ALT appeared as soon as 14 days of treatments in 50% of 4FDC-AT rats and liver histopathological damage was evident after 28 days in those rats. In contrast, renal dysfunction, indicated by a significant increase in creatinine level, only appeared after day 28 of 4FDC-AT treatments in 1 out of 6 rats. The 4FDC-AT treatment mostly led to mild histopathological changes in rat kidney.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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