Xi'an Shiyou Daxue Xuebao (Ziran Kexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN: 1673-064X
E-Publication: Online Open Access
Vol: 65 Issue 07 | 2022
DOI 10.17605/OSF.IO/2UNHK

THE EFFECTIVENESS OF DEFERASIROX TO PREVENT FROM THE OCCURRENCE OF LIVER FIBROSIS IN BALB/C MICE WITH IRON OVERLOAD

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Abstract

Oxidation of hepatocyte mitochondria due to iron overload led to hepatocyte injury, elevated TGF β and fibrosis. Fibrosis starts with stellate cell fibro genesis activated by chronic hepatocyte injury. Liver fibrosis is the main cause of morbidity and mortality in iron overload. Deferasirox is an iron chelation drug serving to bind iron overload and having ant fibrotic effect. To examine the effect of deferasirox on liver fibrosis prevention due to iron overload. This experimental research used post-test only control group design on male Balb/c mice that were randomly divided into 3 groups. Group 1 (NaCl+S) was administered with 0.3 cc Na Cl 0.9% through intra peritoneal (I.P) injection and drug solvent (Aquabidest, CMC and Nipagin) per oral (P.O) intermittently. Group 2 (Fe+S) was administered with 0.3 cc 1.5 mg Fe+sucrose (Venofer®) through I.P injection and drug solvent (Aquabidest, CMC and Nipagin) P.O intermittently. Group 3 (Fe+Dfx) 0.3 cc 1.5 mg Fe+sucrose (Venofer®) I.P injection, and Deferasirox 20 mg/kgBW/day P.O intermittently. All of the treatment was given for 60 days. Fibrosis area fraction of liver was assessed using software ImageJ. The average fibrosis area fraction of group 1 was 0.00 ± 0.00%, group 2 was 9.17 ± 8.54% and group 3 was 1.38 ± 0.20%. The fibrosis area fractions between group 2 and 3 were significantly different with p value 0.000 ($p<0.05$). The body weight of group 2 was higher than that of groups 3 and 1. Iron overload causes liver fibrosis in male Balb/c mice. Deferasirox administration may lower the area of liver fibrosis in male Balb/c mice due to iron overload.

Keywords: Deferasirox, Iron chelation, Iron overload, TGF β, and Liver fibrosis.

INTRODUCTION

Iron is an important micronutrient for humans, but excessive iron content may increase oxidative stress and tissue damage due to lipid peroxidation [1][2]. Excessive iron content in plasma will accumulate in liver and other organs [3]. Iron overload is the main cause of chronic liver disease, besides fatty liver [4]. Accumulated iron in liver may cause chronic inflammation that later causes fibrosis, cirrhosis and even malignancy of the liver [5].

According to the World Health Organization (WHO) in 2019 hepatic cirrhosis was the 10th cause of death in low-revenue countries [6]. The Global Burden of Disease in 2019 reported that the number of death due to hepatic cirrhosis in Southeast Asia was over 442,000 making Southeast Asia the region with the biggest number of death due to hepatic cirrhosis in the world [7]. Hepatitis cases in Indonesia increased twofold from those in 2007 - 2013 [8]. Hepatitis has a risk of up to 50% to become chronic liver disease [9]. Besides due to chronic hepatitis virus and non-alcoholic fatty liver disease (NAFLD), over one third chronic liver disease cases are caused by iron accumulation.

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Administration of iron sucrose at a dose of 1.5 mg intra peritoneal injection intermittently initiates fibrosis on mouse's heart [10]. Iron overload also triggers inflammation and apoptosis on mouse's pancreatic cells administered with 180 mg/kg of iron [11].

Iron overload in the liver also affects oxidative stress with instigates excessive formation of Reactive Oxygen Species (ROS) which will cause cell death [12, 13]. Cell death or necrosis in the liver is commonly found in the area close to central vena or zone 3 [14]. Hepatocyte injury led to stellate cell activation that affects fibrosis on hepatic perisinusoidal and sinusoid [15]. Fibrosis in the tissue is easily observed in histopathology preparation with Masson's trichrome stain [16]. Deferasirox has tridentated molecule with BM 373, half-life 8 – 16 hours which will be easily absorbed in digestive tract. Metabolism and elimination of Deferasirox take place in the form of Fe-(Dfx) 2 through glucuronidase and hepatobiliary excretion to feces. Deferasirox is an iron chelation drugs with ant fibrotic effect [17]. According to Sobbe (2015), Deferasirox does not decrease fibrosis in mouse's liver [18]. The result of the study is different from that of the study conducted by Adel (2019) which reported that deferasirox decreased mouse's fibrotic band [17].

Based on the explanation, the effectiveness of deferasirox in preventing liver fibrosis in Balb/c mice due to iron overload need to be evaluated.

METHOD

Design

This study is post-test control group design experimental research. The transformation of hepatic histopathology was assessed by measuring of fibrosis area fraction using software image J. The research design was in accordance with The Declaration of Helsinki and was carried out upon approval of the Bioethical Commission of the Faculty of Medicine, Sultan Agung Islamic University (187/VII/2021/Komisi Bioetik).

Animal experiment

The subjects of the study were 8-week-old male Balb/c mice (n= 15) with body weight 25-40 g from the Experimental Animal Raising Unit (UPHP) LPPT Gadjah Mada University. The Balb/c mice were raised in the cage of the Biology Laboratory of Faculty of Medicine, Sultan Agung Islamic University, with 12:12 hours of light-dark conditions, humidity: 50-60%, temperature: 24°-26°. The male Balb/c mice were divided into 3 groups. Group 1 (NaCl+S) was administered with 0.3 cc Na Cl 0.9% intra peritoneal (I.P) injection and drug solvent (Aquabidest, CMC and Nipagin) 0.5 cc (P.O), group 2 (Fe+S) was administered with 1.5 mg (0.3 cc) Fe+ sucrose (Venofer®) through I.P. injection and drug solvent 0.5 cc per oral (P.O)., and group 3 (Fe+Dfx) was administered with 1.5 mg (0.3 cc) Fe+sucrose (Venofer®) through I.P. injection and deferasirox 20 mg/kg BW/day through P.O. Administration of the I.P. injection was carried out intermittently. The mice were given standard feed and water ad libitum and they were sacrificed after 60 days of treatment.

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Liver preparation

Before being sacrificed, the mice were anesthetized with pentobarbital 60 mg/kgBW through I.P. injection. After that, their abdomens were operated to take out the liver. The liver was fixated using PFA 4% in PBS for 24 hours and the tissue was stored in paraffin block.

Histological Analysis

Paraffin blocks were cut into 4 mm of thickness. They were deparaffined and stained using Masson's Trichrome stain. Fibrosis area fraction was examined using software ImageJ with 5 different fields of vision in zone 2 of the liver, with magnification of 400 X. Zone 2 livers is the zone between zone 1 which is close to portal triad and zone 3 which is close to central vein.

Statistical Analysis

The difference of fibrosis area fraction between group 2 and 3 were evaluated using Independent Sample T Test and the difference of Balb/c mice's body weight between pre-test and post-test were tested using Paired Sample T Test. The statistical analysis was carried out using software IBM SPSS Statistics 26.

RESULT

Balb/c mice's body weight was evaluated pre and post treatment. The highest average body weight of balb/c mice was of group 2 (Fe+S) compared to that of group 3 (Fe+Dfx) and group 1 (NaCl+S). The mice had significantly different body weight ($p<0.05$) pre and post treatment (see table 1). The balb/c mice's body weight was kept between 25-40gas per inclusion criteria.

Table 1: Balb/c mice's average body weight (gram) of various treatment groups

Mice	Average Body Weight (g)		p value
	Pre-Test	Post-Test	
Group 1 (NaCl+S)	27.9 ± 0.8	30.7 ± 3.6	0.00*
Group 2 (Fe+S)	24.8 ± 1.8	31.4 ± 3.7	0.00*
Group 3 (Fe+Dfx)	27.4 ± 1.3	31.8 ± 3.6	0.00*

* $p<0.05$ significantly different based on Paired Sample T-Test

The fibrosis area fraction in the liver was observed in zone 2 in avoidance of bias from damage commonly occurring in zone 3 because of acute hypoxia during sacrifice process. Fibrosis was not found in the mice's liver in group 1 (NaCl + S), the group without Fe sucrose administration (see figure 1).

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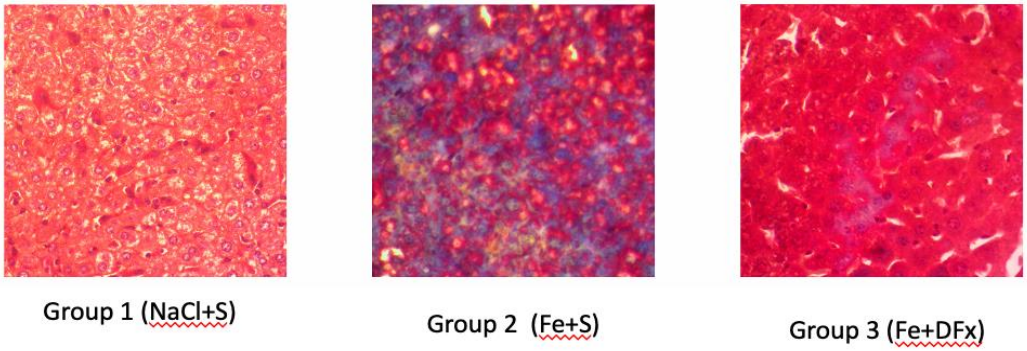

Figure 1 Representative figure of liver fibrosis area shown by Masson's Trichrome staining. Group 1 demonstrated normal liver without fibrosis area. Blue color confirmed deposition of thick collagen fiber in group 2 dan deposition of fine collagen fiber in group 3.

Deferasirox administration for mice with iron overload may reduce liver fibrosis area fraction. Fe sucrose administration 1.5 mg through I.P. injection intermittently may trigger formation of liver fibrosis. Fibrosis area fraction adequately occurred within group 2 (Fe + S) was higher than that of group 3 (Fe + Dfx). There is significant difference ($p<0.05$) of fibrosis area fraction between group 2 (Fe + S) and group 3 (Fe + Dfx) (see table 2).

Table 2: Percentage of Fibrosis Area Fraction in Various Groups of Balb/c Mice

Group	Mean+ Standard Deviation	p value
Group 2 (Fe + S)	9.17+8.54	0.000*
Group 3 (Fe + Dfx)	1.38+0.20	

* $p<0.05$ significantly different based on Independent Sample T-Test

DISCUSSION

Liver has per sinusoidal space as the place for substance exchange from blood in sinusoid to basolateral hepatocyte (Gartner & Hiatt, 2014). The stellate cell that is located in per sinusoidal space serves to help remodel hepatocyte and sinusoid epithelium (Cordero-Espinoza & Huch, 2018). Stellate cell produces hepatocyte growth factor (HGF) to initiate hepatocyte regeneration and secrete collagen that composes fibrosis in the liver. TGF β is cytokine that will induce fibrosis by synthesizing extracellular matrix components, namely collagen types I and III, α-SMA, laminin and fibronectin [15]. Other

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fibrotic collagens are II, IV, XI, XXIV and XXVII (Karsdal et al., 2020). This fibrosis is the scaffold for hepatocyte to proliferate [19].

In this study, fibrosis was not found in mice without iron administration since physiologically iron deposition (hemosiderin) [20] and fibrosis [21] does not occur in the liver. Iron in hepatocyte is stored in ferritin and free iron in cells (LCI) will partially be used by mitochondria [13]. Extra iron causes formation of hemosiderin iron [22]. Electron charge of Fe^{2+} and Fe^{3+} in hemosiderin may be stained blue using Prussian blue staining [23]. Fibrosis in the liver will be formed due to hepatocyte damage [19]. Liver damage occurs due to increased Reactive Oxygen Species (ROS) [24] caused by the increase of LCI [13]. LCI content in the liver is kept about 0.5 – 1.5 µM in normal condition [25].

Administration of iron sucrose 1.5 mg through I.P. injection intermittently causes liver fibrosis. Iron overload causes hepatocyte and kupffer cell to produce TGF β [26]. Transforming growth factor β (TGF β) causes lipid hepatocyte peroxidation and activates stellate cell to produce collagen [26] and fibrosis formation in perisinusoidal space [27]. The research conducted by Sangartit (2016) explained that iron sucrose through I.P.injection increased iron serum, ferritin, transferrin saturation (TfSat), and NTBI level [28]. Iron sucrose administration through I.P injection increased TGF β level and fibrosis in the heart [10]. Oral administration of iron supplement ($FeSO_4$) did not increase NTBI level [29]. It takes time of absorption of oral iron administration compared to parenteral administration since iron is absorbed in intestine only 1-2 mg/day equally to body's iron excretion [30]. Intraperitoneal administration increases iron level in the liver and heart [31] since it does not pass iron regulation in intestine [32].

Deferasirox (Dfx) administration inhibits fibrosis growth in Balb/c mice's liver in this research. Dfx has significant hepatoprotective and ant fibrotic roles through inhibition of inflammatory process in the liver [17]. Dfx administration in iron therapy can prevent proinflammatory activity by inhibiting TNFα [33]. Dfx is ant fibrotic since it can suppress signals from TGF β in mice with extra iron [34]. Dfx is also antioxidant [17] thus it can prevent apoptosis hepatocyte [35]. Decreased collage deposition in the liver of mice with extra iron occurs with the group of wistar rat administered with Dfx [36]. In clinical practice, Dfx is an important iron chelation management for patient with thalassemia and secondary iron overload[37]. Liver damage occurs with administration of ferumoxytol (FMX) and low molecular weight iron dextran(LMWID) through IV but the damage does not occur with administration of iron sucrose (IS) and ferric carboxymaltose(FCM) [38]. Meanwhile, according to Ito (2016), parenteral administration of iron sucrose may increase NTBI [39]. Sodium ferric gluconate (SFG), compared with IS, shows higher retention of iron [40]. Lower weight of SFG (37.5 kDa dalton) and IS (43 kDa) molecules may increase NTBI and LPI higher than FCM (150 kDa), iron dextran (103 kDa), and FMX (185 kDa) [41, 66, 69, 70]. Each type of iron therapy has different capability to increase iron serum in the body.

Iron sucrosecan be administered intraperitoneally for extra iron test in mice [10, 31]. Mice administered with IS 50 mg/kg intraperitoneally 2 times a week increased iron deposit in

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E-Publication: Online Open Access
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the liver, lien and bone marrow [42]. Mice administered with IS intraperitoneally for 4 weeks with low dose (5 mg/mL) did not show any pathological change in the liver's structure, damage started occurring and there was minimum iron deposit with medium dose (10 mg/mL). Meanwhile with high dose of iron sucrose (20 mg/mL) showed strong damage of liver structure with thick brown iron deposit in hepatocyte [43]. Therefore, it is necessary to consider using variety of doses of iron sucrose to trigger iron overload within the liver.

Liver damage is not only caused by the increase of NTBI/LPI [44]. Toxicity of tissue iron is directly proportional to tissue reactive species (NTBI/LPI), genetics, environment and duration of exposure [13]. Liver fibrosis can also be reduced with stellate cell apoptosis processed by NK cell [45,53-60]. NK cell produces INFγ with antifibrogenic effect through inhibiting TGF β signaling, thus stellate cell cannot be activated [45, 46-58]. Fibrosis in liver, lung and lien is marked with TGF β level increase [47]. Hepcidin administration for mice reduces TGF β level [48-55]. BMP6 and BMPR will increase hepcidin expression when iron count or load increases [49, 50-71]. Hepcidin prevents ferroportin releases iron from enterocyte to blood circulation [13]. Hepcidin can also directly protect liver from fibrosis by deactivating stellate cell in perisinusoidal space in the liver [50, 60-71]. Hepcidin level recovery may prevent fibrosis by preventing activation of stellate cell [51, 53-66].

This study is limited that the dose of deferasirox did not vary, thus the authors cannot explain the dose of deferasirox that inhibits liver fibrosis.

CONCLUSION

Intermittently intraperitoneal injection of iron sucrose may cause liver fibrosis in Balb/c mice. Deferasirox administration decreases the percentage of mice's fibrosis area fraction induced by iron sucrose.

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