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Research Article

Effect of Ethanol Extract of Green Tea Leaves (*Camellia Sinensis*) for Lowering Iron Level in Ferrous Sulfate Induced Male Rats

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Abstract

Green tea (*Camellia sinensis*) has developed potentially as a natural agent for iron overload in therapy of thalassemia. The purpose of this research was to observe the ability of ethanol extract of green tea as a natural iron chelating agent in animal iron overload model. Ethanol extract of green tea leave (GTLE) was prepared by maceration using 75% ethanol. Sprague-Dawley male rats were divided into three groups (5 rats each), a normal control group (group I) received daily p.o. of deionized water, the iron overload group received 100 g/Kg BW (group II) of GTLE that given two hours before ferroussulfate0.5 g/Kg BW administrations and the last group (group III) received only 0.5 g/Kg BW of ferrous sulfate. GTLE and ferrous sulfate were given orally every 24 hours for 30 days. At the end of the experimental period, rat blood serum samples were collected. Iron content and alanine aminotransferase (ALT) levels were measured using a spectrophotometer followed by observing the histologic preparation of rat liver organ. The results showed that administration of green tea leaves ethanolic extract of 100 g/Kg BW was able to keep down iron and ALT levels in the rat blood to a normal level.

Keywords: Green tea; histophatology; iron-overload; rat model; liver

INTRODUCTION

One of the therapy management for patients with thalassemia is performing blood transfusions to maintain haemoglobin levels within normal levels (Choudhry, 2017; de Dreuzy *et al.*, 2016). Excessive iron levels or comonly known as iron overload, are happened due to the influence of blood transfusion. This therapy which done repeatedly will lead to iron overload in the form of hemosiderosis; siderosis myocardium often plays a role in early death of patient with thalassemia (Berdoukas *et al.*, 2015; Saito, 2014; Saito and Hayashi, 2015). It can be reduced by providing an iron-chelating agent that does not settle into various organs and tissues and easy to be excreted. An iron overload condition can cause dysfunction of multiple organs of the body. One of them is the liver. Liver is the main organ that is disturbed due to iron overload because it is the primary storage organ of iron and a place of iron metabolism. Chronic accumulation of iron causes plasma transferrin to become saturated with iron which may later lead to damage to the liver (Kumfu *et al.*, 2016; Saito, 2014; Sikorska *et al.*, 2016; Verlhac *et al.*, 2015).

Tea is the most famous and favorite beverage in the worldwide (Yang *et al.*, 2014). Tea was made from young shoots of the tea plant. Green tea (*Camellia sinensis*) is known as a drink that has many benefits for health and widely consumed by the people in the world (Hirun and Roach, 2011; Wardani *et al.*, 2016). Daily consumption of more cups of tea could protect against some chronic diseases in individuals (Henning *et al.*, 2003). The main compounds in green tea are catechins and other polyphenol compounds. Catechins in the tea proven to minimize oxidation of the fatty tissue's ability in order to chelating of iron and copper, which catalyze the structure of free radicals (Chander *et al.*, 2005).

Flavonoid of catechin in the tea could be chelating the iron and it have anti-inflammatory activity to protect neuronal death in neurologycal disease research and animal cells (Mandel *et al.*, 2005). It inhibits the activity and neutrophil release of matrix metalloproteinase-9 that have been shown to prevent or treat chronic inflammatory diseases (Kim-Park *et al.*, 2016). The content of polyphenols in tea can be extracted and expected to as a chelating agent of iron in the body. The tea leaf extract is used as alternative medicine and more convenient to be used for lower levels of iron in patients with thalassemia. The previous study in iron-overloaded experimental rats showed that green tea reduced iron overloads and regulated serum hepcidin levels as well as improving liver fibrosis (Al-Basher, 2017). This research was conducted to develop a green tea (*Camellia sinensis*) as a natural agent to overcome iron overload by evaluating other parameters regarding iron level in blood serum, alanine aminotransferase levels, and histologic preparation of rat liver organ. Therefore, scientific findings can support the use of green tea in the future as supportive therapy to overcome iron overload.

METHODOLOGY

Animal model

The animals that used as an iron overload model were sixty days old *Sprague-Dawley* male rats (*Rattus norvegicus*) with the weight of 120-170 grams. The animals were obtained from the Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia. All procedures performed in this

studies involving animals were in accordance with the ethical standards of the practice at which the studies were conducted. Animals were treated with compliance and conditioned for one week before treatment. Animals were given standard pellet diet and water *ad libitum*.

Materials

Green tea leaves were harvested from a tea plantation in Bandung, Indonesia in the *dry season* that spans from April to September. The leaves then determined at Taxonomy Laboratory, Faculty of Biology, Jenderal Soedirman University, Purwokerto, Indonesia. Ethanolic extract of green tea leaves (GTLE) was prepared by the maceration procedure using 75% ethanol (Bratachem, Purwokerto). The macerate was concentrated using a rotary evaporator. Ferrous sulfate for iron overload induction was obtained from Merck, Germany. Iron FS Feren reagent diagnostic (DyaSys, German) and Fluitest GPT ALT diagnostic reagent (Analyticon, German) were used for quantification of iron content and alanine aminotransferase (ALT) in rat blood serum samples respectively and both of them measured using spectrophotometer (Mapada, China). Deionized water (Bratachem, Purwokerto) was used as the solvent of ferrous sulfate. Sodium Carboxymethyl Cellulose (CMC-Na) (E. Merck, Germany) was used as a suspending agent of GTLE.

Experimental design

Iron overload rat model was performed and adopted from Pari *et al.* with modified some parameters (Pari *et al.*, 2014). Male rats were divided into three groups, each group consisting of five animals. The group I was given deionized water as a normal control group. The iron overload group received 100 g/Kg BW (group II) of GTLE that given two hours before ferrous sulfate 0.5 g/Kg BW administrations, and the last group (group III) received only 0.5 g/Kg BW of ferrous sulfate. GTLE and ferrous sulfate were given orally every day for 30 days. At the end of the experimental period, rat blood serum samples were collected, and iron content was measured by spectrophotometer at a wavelength of 595 nm and deionized water as a blank. Alanine aminotransferase (ALT) levels as a marker of hemosiderosis in the liver were also measured using spectrophotometer at 340 nm. The rat liver organ for each group was also observed by making histologic preparations with hematoxylin and eosin (HE) staining. Data analysis of iron and ALT levels were done statistically by *ANOVA* ($p < 0.05$) and *Tukey HSD* test, while histopathological data of liver was done descriptively.

RESULT AND DISCUSSION

Iron is absorbed in the colon through the duodenum and ileum (Saito, 2014). When it absorbed, it will be channeled to the body and passed to the blood vessels. Measurements of serum iron levels in the blood of rats were performed using a reagent kit of Iron FS ferene. Iron bound to transferrin is released in an acidic medium as ferric iron than reduced to ferrous iron in the presence of ascorbic acid. The iron concentration can be measured by spectrophotometer based on ferrous iron forms a blue complex with ferene. The intensity of the blue color that formed will be proportional to the concentration of the iron so that by comparing with known standards iron levels, it will be known the iron levels in the serum sample. The intensity of the blue color formed was observed at a wavelength of 595 nm, which is the maximum wavelength of the system.

The mean of iron concentration levels in the blood serum of rats after treated for 30 days can be seen in Figure 1. Results of iron levels measurement after treatment in rats based on a statistical calculation of *Kolmogorov-Smirnov* showed that data were normally distributed and using *ANOVA* test ($p < 0.05$) showed that iron value from all of three groups has not a significant difference. Nevertheless, rats were fed a ferrous sulfate at a dose of 0.5g/kg BW have a higher iron content than the control group that was given deionized water during treatment, this result suggests that the administration of ferrous sulfate at a dose of 0.5g/kg BW for 30 days was able to increase the iron level in the blood. Rats on a diet of iron for 30 days with ferrous sulfate and also GTLE with a dose of 100 g/kg BW was able to keep down the iron levels to normal conditions.

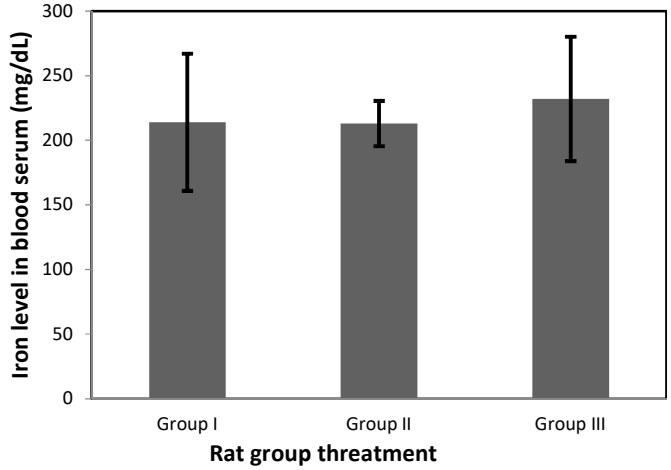

Figure 1. The iron level [mg/dL] in rat blood serum samples treated with iron overload and GTLE. Rats were divided into three groups, (Group I) deionized water as a normal control group; (Group II) 0.5 g/Kg BW ferrous sulfate + 100g/Kg BW GTLE; (Group III) 0.5 g/Kg BW ferrous sulfate.

The iron in the blood binds to transferrin then transferred and stored to the liver and spleen in the form of ferritin and hemosiderin. Excess iron in the body causes hemosiderin to accumulate in the liver, heart, and spleen tissue that can cause hemosiderosis that can damage the liver (Kumfu *et al.*, 2016; Saito, 2014). ALT levels are used as a marker of hemosiderosis and liver damage due to iron toxicity. Results of ALT levels measurement after treatment in rats are presented in Figure 2. Based on the statistical calculation of *Kolmogorov-Smirnov* showed that data were normal distributed and using *ANOVA* test ($p < 0.05$) showed that ALT value from all of three groups has a significant difference. *Tukey HSD* test results of ALT Group I and Group II levels were significantly different ($p <0.05$) with Group III. In addition, ALT levels of Group I and Group II showed no significant difference ($p<0.05$). These results demonstrated that the administration of ferrous sulfate could increase ALT levels in rat and GTLE prevent ALT levels to the normal group. These results in accordance with the previous research (Crespy and Williamson, 2004) which also proves that consuming green tea will decrease the concentration of enzyme marker of liver damage such as aspartate aminotransferase, alanine transfer, and ferritin.

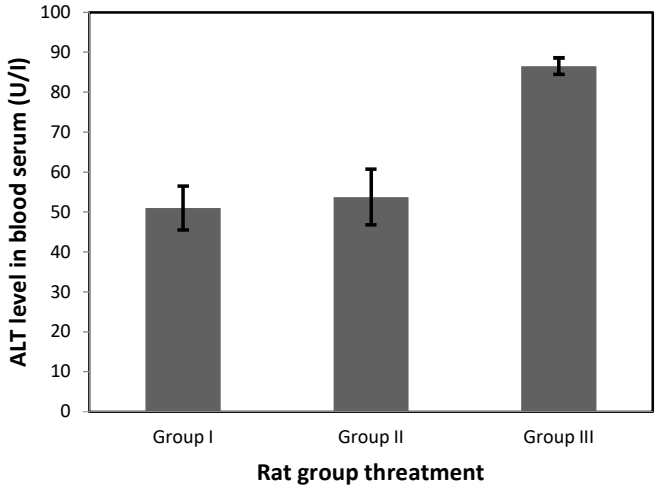

Figure 2. The ALT level [U/l] in rat blood serum samples treated with iron overload and GTLE. Rats were divided into three groups, (Group I) deionized water as a normal control group; (Group II) 0.5 g/Kg BW ferrous sulfate + 100g/Kg BW GTLE; (Group III) 0.5 g/Kg BW ferrous sulfate.

Observation of liver histopathology performed after 30 days of treatment. The results of histopathological examination of the liver in this study illustrated that each group was found no histopathological changes in rat liver (Figure 3). This outcome indicates the administrating of ferrous sulfate has not shown significant liver damage, but from observations of iron and ALT levels seems to be demonstrated iron overload condition on the rats.

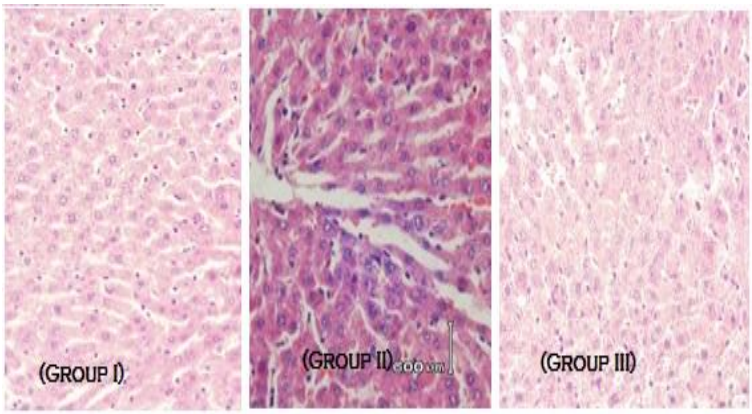

Figure 3. The liver histology observation using *HE* staining that treated with iron overload and GTLE. Rats were divided into three groups, (Group I) deionized water as a normal control group; (Group II) 0.5 g/Kg BW ferrous sulfate + 100g/Kg BW GTLE; (Group III) 0.5 g/Kg BW ferrous sulfate.

This study showed that administration of GTLE to the rats that fed a diet of excess iron in the form of ferrous sulfate with a dose of 0.5 g/kg BW for 30 days could make restoring the iron levels and the ALT concentration in the blood to normal value. Flavonoids in the form of catechin of green tea were

potentially chelate the iron in the blood and also have the anti-inflammatory activity to protect neuronal death in neurological disease and animal cells (Mandel *et al.*, 2005). Other research described that the administration of black tea to rats for 60 days could increase the activity of antioxidant and liver microsomes. Microsomal have a role in protecting fats from peroxidation stimulated by metal ions (Chander *et al.*, 2005). The tea diet in rats by the administration for ten days have better activity than on diet tannins in absorbing of iron. The content of polyphenols in tea can be extracted and is expected as a chelating agent of iron in the body (Kim *et al.*, 2004).

Other research reported that green tea improved iron overload-induced hepatotoxicity, oxidative stress and apoptosis in rat liver (Al-Basher, 2017), effectively inhibited the deposition of hepatic iron (Saewong *et al.*, 2010), induced hepcidin expression to inhibit iron absorption in duodenal (Upanan *et al.*, 2015), reduced plasma non-transferrin-bound iron concentration (Srichairatanakool *et al.*, 2006), and decreased nonheme-iron absorption (Samman *et al.*, 2001). This research findings support previous studies of green tea as a natural agent to overcame iron overload by evaluating other parameters regarding not only iron and ALT level in blood serum but also histological of the liver organ in the rat.

CONCLUSION

GTLE at a dose of 100 g/Kg BW was able to keep down iron and ALT to normal level although not in significant levels in the rat blood serum that intake of an iron excess of ferrous sulfate. Therefore, GTLE has potentially used as natural agent therapy to slightly lowering ferrous sulfate induced male rats.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHORS CONTRIBUTIONS

HW: Conceptualization, methodology, validation, investigation, resources, visualization, supervision. **HNB**: Methodology, formal analysis, supervision. **YRS**: Methodology, formal analysis, validation, investigation. All authors contribute to the data interpretation, writing and approving to the final version of the manuscript.