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Research Article

HEPATOPROTECTIVE EFFECTS OF L-CITRULLINE AGAINST DOXORUBICIN-INDUCED LIVER DAMAGE IN RATS: AN ANALYSIS OF SERUM BIOMARKERS

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ABSTRACT

Objective: The antineoplastic agent doxorubicin (DOX) is known for causing liver toxicity. Its metabolism in hepatocytes causes oxidative stress, which, in turn, induces DNA damage, lipid peroxidation, ATP depletion, and apoptosis. L-citrulline (CIT), a commonly found agent in fruits like watermelon, has piqued interest due to its antioxidant properties. In the body, CIT is converted to nitric oxide, which has been shown to mitigate hepatic injury by scavenging free radicals, improving hepatic sinusoidal microcirculation, and inhibiting neutrophilic infiltration. This study aims to investigate CIT ability to prevent DOX-induced hepatotoxicity.

Methods: A total of 20 Wistar rats were randomized to receive either DOX (10 mg/kg BW) or NaCl 0.9%. DOX-intoxicated group was further randomized to either received low-dose CIT (300 mg/kg BW), high-dose CIT (600 mg/kg BW), or aquadest. CIT was given orally for 6 days and DOX through intraperitoneal injection on days 4 and 5. Serum was obtained and hepatotoxicity was assessed with serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (GGT). Statistical analysis was done with one-way ANOVA and Tukey's test.

Results: Serum ALT, AST, and GGT were increased significantly compared to that of normal group. CIT administration in both the doses could decrease the serum levels of ALT and AST significantly compared to that of DOX group. In this study, CIT in both the doses could reduce the serum levels of GGT compared to that of DOX group though not statistically significant.

Conclusions: This study suggests that CIT exerts hepatoprotective effect, as evident by the attenuation of serum biomarkers.

Keywords: Hepatotoxicity, Doxorubicin, L-citrulline.

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INTRODUCTION

Doxorubicin (DOX), an anthracycline antibiotic, is a widely used antineoplastic agent with a well-established efficacy against many types of cancer, including breast, lung, gastric, ovarian, and thyroid cancers as well as lymphoma. However, its clinical use is limited due to its toxicity to healthy cells, affecting the heart, liver, brain, and kidneys [1,2].

Hepatotoxicity commonly results from DOX therapy. Overall, 30%–40% of patients receiving DOX injection had elevated serum levels of liver function biomarkers [3,4]. Moreover, DOX use increased the risk of developing liver injury, for example, acute/subacute liver necrosis, toxic hepatitis, and hepatic coma, by 1.29× across all types of cancer [5,6]. Metabolism of DOX in hepatocytes generates an abundance of free radicals, and the resulting oxidative stress induces DNA damage, lipid peroxidation, disturbance of membrane integrity, and ATP depletion, all of which culminate in apoptosis [1,7]. Currently, toxicity prevention only comprises dosage optimization and antioxidant supplementation. Further, no proposed hepatoprotective agent has been approved as a prophylactic drug against DOX-induced liver damage [3,8].

In this regard, L-citrulline (CIT), found in common fruits such as watermelon, has generated interest. A non-protein, non-essential amino acid, CIT, was previously thought to be a mere intermediate product of ureagenesis. However, in specific cells of the body, for example, periportal hepatocytes, renal cells, macrophages, and endothelial cells, CIT is recycled into L-arginine (ARG), from which nitric oxide (NO) is synthesized [9,10]. NO is shown to mitigate liver injury by improving hepatic sinusoidal microcirculation, inhibiting neutrophilic infiltration and cytokine production, and scavenging free radicals [11,12]. Remarkably, CIT is reportedly better than ARG in increasing NO bioavailability in the body [13,14]; this may be attributable to CIT-specific metabolism. Unlike ARG, CIT bypasses intestinal first-pass metabolism, thus reaching the systemic circulation in copious amounts [9,10].

An important role of CIT in NO metabolism demonstrates its potential to become a hepatoprotective agent. Only a few studies have evaluated CIT ability to maintain liver function [15,16]. Thus, the present study aimed to evaluate the hepatoprotective effects of CIT administration toward DOX-induced hepatotoxicity in animal models. We believe that this research will help in establishing CIT efficacy in ameliorating organ toxicity caused by DOX and other antineoplastic drugs, thus providing an inexpensive, natural, and widely available prophylactic hepatoprotective agent.

METHODS

Animals

In total, 20 male Wistar rats (250–350 g) were used in this study. The rats were maintained under standardized conditions: Temperature, 23°C; humidity, 55%; and light/dark cycle, 12/12 h. They were allowed to acclimatize 1 week before the initiation of the experiment. All rats had access to food and water *ad libitum*. This study was approved by the ethics committee in Indonesia (ethical clearance #0928/UN2.F1/ETIK/2018).

Experimental design

This was an experimental laboratory study. The rats were randomly divided into four groups (n=4 rats/group) and received treatment throughout the 6-day experiment accordingly (Table 1). Determination of dosage was based on a study by Hayward and Hydock (for DOX) and

Yi *et al*. (for CIT) [17,18]. At the end of the experiment, the rats were euthanized with an intraperitoneal injection of ketamine (80 mg/kg BW) and xylazine (8 mg/kg BW). Supracardiac puncture was performed to euthanize the rats and collect blood samples.

The blood samples were stored in a plain tube without an anticoagulant, allowed to clot at room temperature (±28°C), and centrifuged at 3000 rpm for 10 min. The separated supernatant, i.e. serum was collected in microtubes and stored at −80°C until needed.

Assessment of hepatotoxicity

Hepatotoxicity was assessed on the basis of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (GGT) serum levels. Assays were performed using commercial diagnostic kits from DiaSys®, following the provided protocol. Serum biomarker activity is expressed in units/liter.

Statistical analysis

Data were analyzed using IBM SPSS Statistics version 22 and expressed as mean ± standard deviation. The Shapiro–Wilk test was used to assess data normality and Levene test to assess data variance. One-way ANOVA test and Tukey's multiple comparison test were used to determine differences between the treatment groups. Kruskal–Wallis test and Mann–Whitney U-test were performed in case of heterogeneity of variance. $p<0.05$ was considered to be statistically significant.

RESULTS

Effects of CIT administration on serum ALT activity of DOX-intoxicated rats

DOX intoxication resulted in ×2.61 elevation of mean serum ALT activity, which was significantly higher compared with that in the normal group ($p<0.05$; Table 2, Fig. 1a). Concomitant administration of CIT with DOX resulted in significantly lower serum ALT activity compared with DOX alone ($p<0.05$), and this was observed for both the doses of CIT. High-dose CIT administration decreased ALT activity significantly more than low-dose CIT ($p<0.05$), suggesting a dose-dependent effect.

Effects of CIT administration on serum AST activity of DOX-intoxicated rats

DOX treatment caused a ×2.26 increase in serum AST levels, which was significantly higher than that in the normal group ($p<0.05$). Low-dose CIT administration with DOX only caused an insignificant reduction in AST levels in comparison with DOX administration alone ($p>0.05$). However, high-dose CIT administration with DOX resulted in significantly lower serum AST activity compared with administration of DOX alone ($p<0.05$). Further, there was a significant difference ($p<0.05$) between the low- and high-dose groups; hence, a dose-dependent effect of CIT may also exist (Table 2 and Fig. 1b).

Effects of CIT administration on serum GGT activity of DOX-intoxicated rats

DOX treatment significantly elevated the levels of GGT in serum, with a mean activity that was ×7.93 higher than that observed in the normal group ($p<0.05$). Concomitant CIT administration, regardless of whether it was low or high dose, resulted in an insignificant decrease of mean serum GGT activity when compared with administration of DOX alone ($p>0.05$). While high-dose CIT administration lowered GGT level more than its low-dose counterpart, no statistically significant difference existed between the two ($p>0.05$; Table 2 and Fig. 1c).

DISCUSSION

In the present study, DOX intoxication of rats significantly elevated all serum biomarkers of liver function; this finding was expected and is consistent with those of previous studies. Dewanjee *et al*. reported that DOX-treated rats had increased serum AST, ALT, total cholesterol, and triglyceride levels accompanied with enhanced lipid peroxidation and increased apoptotic protein expression [19]. Damodar *et al*. reported that breast cancer patients undergoing DOX therapy had significantly increased AST, ALT, direct bilirubin, and total bilirubin levels from pre-chemotherapy to the fourth cycle of chemotherapy [3].

The underlying mechanism for DOX-induced elevation of serum biomarkers is well documented. An abundance of reactive oxygen species (ROS) is produced when DOX is metabolized in hepatocytes, and the resulting oxidative stress causes DNA damage, lipid peroxidation, and disruption of membrane integrity. Further, ROS attacks inorganic phosphates, thus depleting hepatocytes of ATP and consequently triggering apoptosis. These events permit the escape of cytosolic enzymes, for example, ALT, AST, and GGT, from hepatocytes into the circulation, thereby increasing their serum levels [1,2,7,20].

Elevations of serum ALT and AST were significantly attenuated with concomitant administration of CIT, an effect better achieved with the high-dose administration (600 mg/kgBW). This finding is in agreement with those of other studies evaluating CIT hepatoprotective ability. Moussaoui *et al.* reported on rats with modeled ischemia/reperfusion injury and found that CIT treatment significantly reduced elevations of ALT and AST ($p<0.05$), along with lactate dehydrogenase, arginase, and intrahepatic myeloperoxidase activity [15]. In addition, Jegatheesan *et al*. identified a significant decrease of plasma ALT levels due to CIT in rats with fructose-induced liver steatosis. They also reported a decreased, although statistically insignificant, elevation of AST level ($p>0.05$) [16].

Table 1: Treatment groups and their respective regimen

Group I (normal)	Days 1–6: Aquadest through gavage, ×1/day *Days 4–5: As above+IP injection of 0.9% NaCl
Group II (DOX)	Days 1–6: Aquadest through gavage, ×1/day *Days 4–5: As above+IP injection of DOX (10 mg/kg BW)
Group III (CIT low dose)	Days 1–6: Oral low-dose CIT (300 mg/kg BW), ×1/day *Days 4–5: As above+IP injection of DOX (10 mg/kg BW)
Group IV (CIT high dose)	Days 1–6: Oral high-dose CIT (600 mg/kg BW), ×1/day *Days 4–5: As above+IP injection of DOX (10 mg/kg BW)

CIT: L-citrulline, DOX: Doxorubicin, IP: Intraperitoneal

Table 2: Level of serum biomarkers of liver function across treatment groups[1]

Serum levels	I Normal	II DOX	III low-dose CIT	IV high-dose CIT	p-value
Serum ALT[2] (U/L)	23.96±6.96[a]	62.67±11.96[b]	41.99±5.89[c]	35.02±3.14[d]	<0.01
Serum AST (U/L)	39.68±5.22[a]	89.82±6.97[b]	83.81±9.87[b]	60.54±19.47[a]	<0.01
Serum GGT[2] (U/L)	1.09±0.45[a]	8.65±6.39[b]	4.31±4.27[a]	2.21±0.75[a]	0.026

ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, CIT: L-citrulline, DOX; Doxorubicin, GGT: Gamma-glutamyltransferase, U/L: Units/liter. [1]Data are presented as mean±standard deviation. In a row, data labeled with different letters differ significantly ($p<0.05$). [2]Heterogeneity of variance: Kruskal–Wallis test, followed by Mann–Whitney U-test, was performed

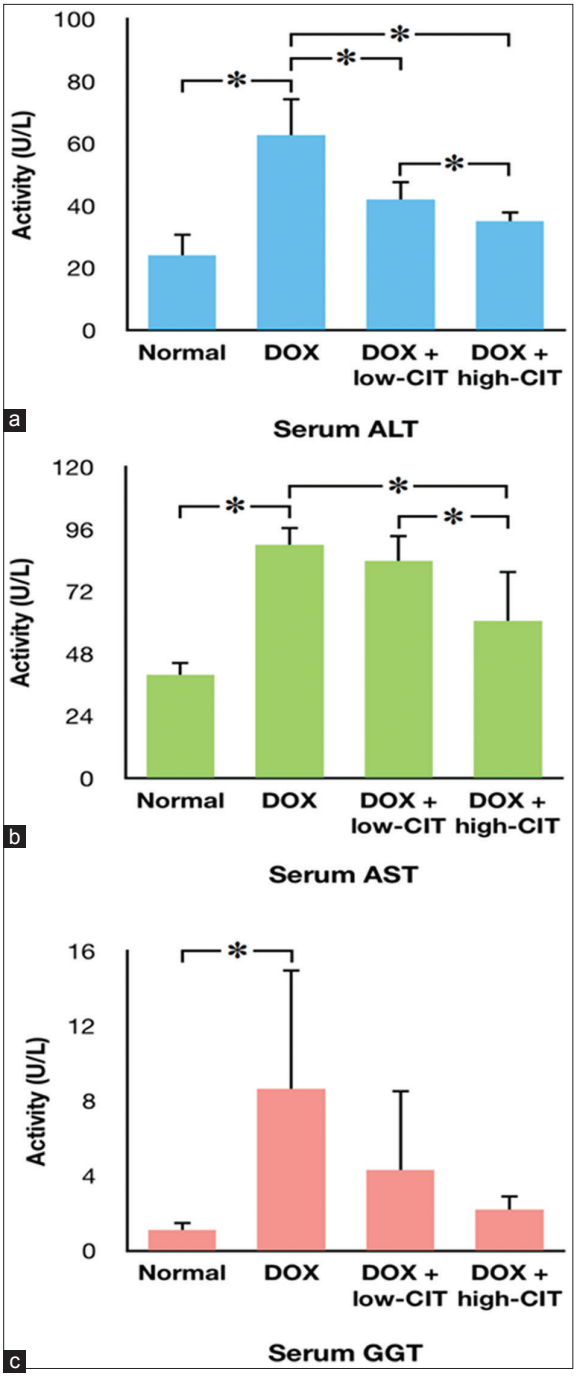

Fig. 1: Histogram representing the mean serum activity of alanine aminotransferase (ALT) (a), aspartate aminotransferase (AST) (b), and gamma-glutamyl transferase (GGT) (c) across treatment groups. (a) Doxorubicin (DOX) treatment significantly elevated mean serum ALT activity and citrulline (CIT) treatment significantly attenuated DOX-induced ALT elevation in a dose-dependent manner; (b) DOX treatment also significantly elevated mean serum AST activity and CIT only in a high dose was able to significantly attenuate DOX-induced AST elevation; (c) DOX treatment significantly elevated mean serum GGT activity and CIT treatment attenuate DOX-induced GGT elevation, although insignificantly. *p<0.05; ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, CIT: L-citrulline, DOX: Doxorubicin, GGT: Gamma-glutamyl transferase

A liver function assessment using serum ALT and AST levels is frequently performed in the clinical setting. Elevated ALT level is highly indicative of liver damage as ALT is found almost exclusively in hepatocytes [21]. Thus, its activity is considered a specific and sensitive indicator of hepatocellular damage. In addition, ALT is considered superior to AST in predicting hepatic injury among pre-clinical species such as rats and dogs [22,23]. AST, although found in hepatocytes, is also present in the heart, skeletal muscle, and kidneys, and thus, AST elevation is less specific for liver injury [21,23]. ALT elevation is usually more marked than AST elevation when the cause of elevation is hepatic due to longer half-life of ALT and higher proportion of AST bound to the mitochondria. Conversely, extrahepatic origin should be suspected when AST is more elevated than ALT [21-23]. The present study found greater ALT than AST elevation (×2.61 vs. ×2.26), confirming that DOX causes hepatocellular damage.

Furthermore, CIT capacity to significantly lower DOX-induced AST and ALT elevations in the present study demonstrates its hepatoprotective ability, which may be attributable to CIT antioxidative properties. Several studies have shown that CIT, as the precursor of ARG, potently increases NO levels in the body [13,14]. While we did not measure the NO levels following CIT administration in this study, we believe that CIT boosted NO production because NO is shown to scavenge superoxides and other free radicals, thereby mitigating oxidative stress caused by DOX metabolism in hepatocytes. Moreover, NO induces sinusoidal vasodilation, inhibits platelet aggregation, reduces neutrophilic infiltration, and inhibits cytokine release, all of which contribute to the mitigation of liver injury [11,12].

Introduction of CIT to DOX-intoxicated rats lowered the elevations of serum GGT, although insignificantly ($p<0.05$). Moussaoui *et al.* did not utilize GGT as part of their liver integrity assessment. However, they found that CIT significantly reduced elevations of all serum biomarkers, in which the present study failed to demonstrate [15]. Jegatheesan *et al.* used alkaline phosphatase (ALP), a marker of hepatobiliary injury similar to GGT; however, they also found that CIT only caused insignificant attenuation of ALP elevation [16].

Concentrated in the epithelial lining of bile ducts, GGT is an indicator of hepatobiliary injury. The findings of GGT support those of ALP, which is a more sensitive but less specific marker. GGT elevation also helps to confirm that elevations of AST and ALT are of hepatic origin [21,23]. Thus, decreased levels of GGT following the introduction of CIT as seen in this study also demonstrate CIT hepatoprotective ability.

CONCLUSIONS

The results of this study suggest that CIT applies a hepatoprotective effect, as evidenced by the attenuation of elevated serum biomarkers induced by DOX. The precise mechanism behind this must be further evaluated, especially in regard to the bioavailability of NO following the introduction of CIT, as previous studies have demonstrated NO ability in maintaining liver integrity. Nevertheless, using CIT, commonly found from food constituent, compensates organ toxicities induced by chemotherapeutic agents such as DOX may be a feasible clinical strategy.

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CONFLICTS OF INTEREST

The authors declare no competing interests.

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