

The Effect Of L-Citrulline on Doxorubicin-Induced Cardiotoxicity Using Serum Oxidative Stress Biomarkers

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Abstract

Doxorubicin (DOX) is a chemotherapeutic agent widely used against various cancers. However, its usage is linked to cardiotoxicity side effects via production of reactive oxygen species, causing oxidative stress and cardiac myocyte death. L-Citrulline (CIT) is a potent anti-oxidant agent with potential to prevent DOX-induced cardiotoxicity. This study analyzed the effect of CIT against DOX-induced cardiotoxicity using oxidative stress biomarkers. Administration of DOX (intraperitoneal injection) was performed to induce cardiotoxicity in 20 Wistar rats, divided into 4 groups. Two doses (300 and 600 mg/kg of body weight) of CIT were administered via gavage prior to, during, and after DOX injection. Serum levels of glutathione (GSH) and malondialdehyde (MDA) were measured using colorimetric analysis. Both doses of CIT increase slightly the concentration of GSH ($P=.063$ vs. control). The concentration of MDA was also increased in the low-dose ($P=0.103$), and high-dose groups ($P<0.001$). The observed elevations were dose-dependent.

Administration of CIT did not exert a beneficial effect against DOX-induced cardiotoxicity. The increased level of MDA in CIT group indicates increased oxidative stress and lipid peroxidation. Moreover, the elevation of the level of GSH is more likely to be caused by the apoptosis-related GSH efflux mechanism, explained in other literature.

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Introduction

Cancer is a major cause of both morbidity and mortality. In 2015, this disease was responsible for 8.8 million deaths worldwide.¹ There are numerous treatment options available for the treatment of cancer. Frequently, treating physicians administer powerful cytotoxic drugs to suppress the growth of cancerous cells. Consequently, this also affects healthy cells, resulting in the development of adverse effects. Doxorubicin (DOX) is a chemotherapeutic agent widely used against various types of cancer. Its function involves several mechanisms, such as inhibition of topoisomerase II, DNA intercalation, generation of semiquinone free radicals, and iron-dependent production of oxygen free radicals.²

However, the iron-dependent production of oxygen free radicals has been linked to the development of cardiotoxicity. This cumulative-dose dependent adverse effect greatly limits the usage and safety of DOX as a chemotherapeutic agent.³ Numerous studies have shown that superoxide produced through the metabolism of DOX reacts with iron to create hydroxyl radicals, leading to damage in various cell compartments and cardiac myocyte death (the iron-reactive oxygen species [ROS] hypothesis).⁴

Studies have extensively investigated options for the management or prevention of this adverse effect of DOX. Although various agents (i.e., PEGylated liposomal form, beta-adrenergic blocker carvedilol) and methods (altering the duration of DOX infusion) have been examined, the results have been inconsistent.^{5,6} Currently, the only approved drug used to counteract the adverse effects of DOX is the iron chelator dexrazoxane. However, it was revealed that dexrazoxane reduces the efficacy of DOX, induces various adverse effects, and is not cost-effective.⁷ Therefore, there is an unmet need for alternative cardioprotective agents to prevent the

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development of DOX-induced adverse effects.

L-Citrulline (CIT) is a widely available non-protein, non-essential amino acid. It has been shown that CIT possesses anti-oxidant properties and acts as a scavenger of superoxide.⁸ In the human body, CIT is metabolized into L-Arginine and, subsequently, to nitric oxide (NO) by nitric oxide synthase (NOS).⁹ The potent vasodilator NO plays an important role in vascular tonus regulation and heart contractility.¹⁰ Based on this fact, the purpose of this research was to study the effect of CIT on DOX-induced cardiotoxicity using serum oxidative stress biomarkers.

Materials and methods

This was an experimental, randomized, negative-controlled, and parallel-group trial model study. The study was conducted between February and June 2018 in the Pharmacokinetics Laboratory, Pharmacology and Therapeutics Department of Fakultas Kedokteran Universitas Indonesia, Salemba, Jakarta, Indonesia. Wistar rats were used to obtain primary data. The animals were male, weighing 250–350 g and maintained in the Pharmacology Laboratory under standardized conditions (i.e., 25°C temperature, 55% humidity, and a 12-hour light/dark cycle).

Two doses of CIT were tested in this study, namely the low-dose (300 mg/kg of body weight [BW]) and high-dose (600 mg/kgBW), which served as independent variables. The doses were determined based on a study by Liu et al.¹¹ The measurements of the concentration of GSH and MDA in the serum, reflecting oxidative stress in the rats, served as dependent variables.

Treatment and sample extraction

The weight of the animals was measured to determine the appropriate dosage for each rat. This was performed every day for the 6-day treatment period. Subsequently, the rats were randomly classified into 4 groups (5 rats per group). Group I (control) received aquades via gavage daily and NaCl 0.9% through intraperitoneal (IP) injection. Group II received DOX (10 mg/kgBW) through IP injection, instead of NaCl, to induce cardiotoxicity.¹² Group III received low-dose CIT (300 mg/kgBW) via

gavage daily and DOX (10 mg/kgBW) through IP injection. Group IV received high-dose CIT (600 mg/kgBW) via gavage daily and DOX (10 mg/kgBW) through IP injection. IP injection of NaCl and DOX were done on days 4 and 5.

The rats were sacrificed on the sixth day of the experiment under anesthesia through IP injection of a mixture of ketamine (80 mg/kgBW) and xylazine (8 mg/kgBW). Subsequently, a supracardiac puncture was performed to obtain blood from the rats. The blood was placed in plain blood tubes without addition of an anticoagulant. The tubes were centrifuged at 3,000 rpm for 10 minutes to isolate the serum. The supernatant (serum) was removed and stored (–80°C).

Measurement of the concentration of GSH in the serum

The concentration of GSH in the serum was measured using the Elabscience GSH assay kit E-BC-K030, purchased from PT Genetika Science. A standard curve was prepared using 1 mmol/L GSH standard solution. The standard solution was diluted to produce several solutions with known concentration. Subsequently, the samples were prepared by mixing the reagent 1 (precipitant) into the serum. The mixture was centrifuged to isolate the supernatant for measurement. The standard solutions and samples were prepared for the measurement of absorbance by adding reagent 2 (GSH assay buffer) and reagent 3 (chromogenic agent or dinitrothiocyanobenzene), which reacts with GSH and generates yellow-colored 2-nitro-5-thiobenzoic acid. The absorbance of the standard solutions and samples was measured using a Ultraviolet-Visible (UV-VIS) spectrophotometer at 405 nm. The standard curve and its equation were produced from the absorbances of the standard solutions. The concentration of GSH in each sample was calculated from the standard curve by adding the absorbance of the sample as x at the equation.

Measurement of the concentration of MDA in the serum

The concentration of MDA in the serum was measured through the thiobarbituric acid (TBA) reactive substances assay, with trichloroacetic acid (TCA) 20% and TBA 0.67%

dissolved separately using distilled water. The process was initiated by preparing standard solutions using a MDA standard solution. Similarly, the samples were prepared by first adding TCA 20% solution. After vortexing, the TBA 0.67% solution was added. Subsequently, the samples and standard solutions were mixed and heated using water bath for 10 minutes. This process was performed because TBA binds to MDA only in an acidic and high-temperature environment, forming pink-colored adducts. After heating, the samples and standard solutions were centrifuged and the supernatants were collected. The concentration of MDA was measured using an UV-VIS spectrophotometer at 530 nm.

Protein measurement

Protein concentration was also measured to standardize the result to 1 mg protein. The standard curve was produced using bovine serum albumin. All samples were diluted by 100-fold, to be measured using the standard curve. The Bradford assay was used to perform the measurement of protein. The Bradford reagent utilized in this assay binds to protein and produces a blue-colored solution. Absorbance was measured using an UV-VIS spectrophotometer at 595 nm.

Data analysis

SPSS 24 for Windows was used to perform the statistical analysis of the data. The Shapiro-Wilk test was used to check the normality of data distribution. In addition, Levene's test was used to check the homogeneity of data variances. One-way analysis of variance (ANOVA) using Tukey's multiple comparison as a post-hoc test was used if the criteria were fulfilled (normal data distribution and homogeneous variance). For non-normally distributed data, the Kruskal-Wallis non-parametric analysis using the Mann-Whitney U post-hoc test was performed.

Results

Concentration of GSH

The statistical analysis showed that the data was normally distributed (Shapiro-Wilk test; $P > 0.05$) with homogeneous variances (Levene's

test $P > 0.05$). Hence, the data are presented as mean \pm standard error of the mean (SEM) and in a bar chart with SEM. One-way ANOVA was performed to determine the statistical significance of the observed differences, showing $P = 0.063$.

As Table 1 shows, the concentration of GSH in the serum was decreased in the DOX group (Group II) compared with that measured in the control group. In contrast, the concentration of GSH increased in the group treated with CIT in a dose-dependent manner. This effect was greater in the higher dose. However, these differences between the groups were not statistically significant.

Group (n=5)	GSH Concentration \pm SEM (nmol/mg protein)	95% Confidence Interval (upper limit, lower limit)
Control	0.4 \pm 0.04	0.29–0.51
DOX	0.37 \pm 0.03	0.28–0.46
DOX+Low	0.43 \pm 0.04	0.31–0.54
CIT		
DOX+High	0.51 \pm 0.01	0.47–0.54
CIT		

Table 1. The concentration of GSH in the serum.

Concentration of MDA

The concentration of MDA in the serum was normally distributed with homogeneous variance. Therefore, the data are presented as mean \pm SEM and a bar chart with SEM. One-way ANOVA was performed to determine the significance of the observed differences. In contrast to the results obtained for GSH, the differences observed for MDA were statistically significant ($P < 0.001$). Hence, Tukey's multiple comparison test was performed to compare the differences between the groups.

As Table 2 shows, the level of MDA in the serum of rats belonging to the DOX group increased compared with that measured in the control group ($P > 0.05$). Moreover, the level of MDA increased in the group treated with CIT in a dose-dependent manner. The difference was slight in the low-dose CIT group ($P > 0.05$) and was more prominent in the high-dose CIT group ($P < 0.001$).

Group (n=5)	MDA Concentration±SEM (nmol/mg protein)	95% Confidence Interval (upper limit, lower limit)
Control	0.48±0.06	0.32–0.64
DOX	0.58±0.04	0.48–0.68
DOX+Low CIT	0.64±0.03	0.54–0.73
DOX+High CIT	0.97±0.04	0.86–1.08

Table 2. The concentration of MDA in the serum.

Discussion

GSH concentration

The observed decrease in the concentration of GSH in the DOX group compared with that measured in the control group may be the result of several mechanisms. First, GSH is a major intracellular anti-oxidant agent, possessing a free thiol group. Thiol reacts and neutralizes ROS, including superoxide. Following the reaction of GSH (i.e., reduction), a superoxide or other ROS oxidizes into oxidized glutathione (GSSG) through glutathione peroxidase.¹³

The generation of ROS by DOX is the most widely studied cause of cardiotoxicity. Moreover, it is thought that this process acts as a major trigger for several forms of cell death, including apoptosis, necrosis, and autophagy.¹⁴ As DOX produces more superoxide radical, more GSH is also oxidized into GSSG to neutralize the superoxide and prevent the formation of the hazardous hydroxyl radical. Furthermore, GSSG may be reduced back into reduced GSH through glutathione reductase, which requires NADPH as an electron donor. NADPH itself is produced by the G6PD enzyme. In apoptotic cells, the G6PD enzyme is inactivated, resulting in depletion and oxidation of NADPH. These events impair GSH recycling and contribute to GSH depletion.¹⁵

The dose-dependent elevation of GSH concentration in the serum after treatment with CIT may reflect the anti-oxidant properties of CIT. Studies have shown that CIT is a potent superoxide and hydroxyl radical scavenger. In addition, it is metabolized into L-Arginine – another superoxide radical scavenger – in the kidney. L-Arginine exerts protective effects from oxidative stress by decreasing the activity of xanthine oxidase and increasing that of GSH, superoxide dismutase, and catalase.⁹ Moreover, CIT has been shown to increase the activity of

eNOS and production of NO.¹⁶ Treatment with DOX reduces the level of NO, causing vascular endothelial dysfunction. However, the observed increase in NO shows that CIT may limit the cardiotoxicity of DOX.

MDA concentration

The level of MDA in the serum of rats belonging to the DOX group increased compared with that measured in the control group. This reflects the increased production of ROS due to the metabolism of DOX. In addition to the aforementioned production of superoxide in the semiquinone cycle, DOX is metabolized into doxorubicinol (an alcohol metabolite) via 2-electron reduction. Doxorubicinol may react and interrupt the work of the ACO1 gene, resulting in accumulation of iron within the cell.³ The produced superoxide reacts with doxorubicinol and undergoes the Fenton reaction, producing hydroxyl radical (the aforementioned iron-ROS hypothesis).⁴

Hydroxyl radical initiates the lipid peroxidation cycle – a process in which a hydrogen is abstracted from the bisallylic methylene of a lipid membrane PUFA, forming a lipid radical. The lipid radical reacts with oxygen to produce lipid peroxy radical. This process may continue into the propagation phase. The most widely studied end product of this termination process is MDA.¹⁷ Hence, high concentrations of hydroxyl radical initiate the lipid peroxidation cycle, resulting in increased concentration of MDA. This condition is worsened by depletion of reduced GSH, which results in decreased activity of glutathione peroxidase. This condition permits the accumulation of lipid peroxides and often cell death (due to alteration of proteins and nucleic acids).¹⁸

In the CIT group, the concentration of MDA in the serum was increased in a dose-dependent manner. Under normal conditions, eNOS produces NO and CIT from L-Arginine in a 1:1 conversion ratio. It utilizes an electron from NADPH, transported by flavins to the heme group in the oxygenase domain, where it also binds to L-Arginine. Subsequently, it transfers the electron to molecular oxygen, which becomes activated and cleaved. The production of NO from L-Arginine requires 2 cleaved oxygen molecules.⁹

Treatment with DOX induces NOS

uncoupling by decreasing the level of L-Arginine and BH4 through the oxidation process. The effect of uncoupled eNOS is the impairment of the reduction process of molecular oxygen by heme iron, favoring the production of superoxide.¹⁹

In addition, peroxynitrite is a highly toxic molecule produced when NO reacts with superoxide radicals. Moreover, it is produced after the induction of NOS uncoupling by DOX. Among its numerous hazardous effects, peroxynitrite is involved in the oxidation of zinc thiolate clusters. When a zinc thiolate cluster of eNOS is oxidized and forms disulfide bond, eNOS becomes unstable and ultimately cannot bind to BH4. This cycle continues, resulting in further NOS uncoupling.²⁰ As mentioned before, the administration of CIT has been shown to increase the activity of eNOS. The enhanced activity of uncoupled eNOS, which results in increased production of ROS, may explain the dose-dependent increase observed in the serum level of MDA in the CIT-treated group.

This finding contradicts the explanation provided for the GSH results. This pathway is a hallmark of the initiation of apoptosis.¹⁵ The increase in the level of serum GSH is caused by the GSH efflux mechanism, reflecting the initiation of apoptosis in cardiac myocytes. Cytotoxicity assay should be done to prove this possible mechanism.

Conclusions

Based on the present findings, administration of CIT does not exert a protective effect against DOX-induced cardiotoxicity, as demonstrated by the increased concentration of MDA in the serum. The increased of MDA in the serum indicates increased lipid peroxidation and cellular damage. Moreover, the increased concentration of GSH in the serum indicates the initiation of the mechanism of apoptosis. This effect is directly proportional to the administered CIT dosage (i.e., in a dose-dependent manner).

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Declaration of Interest

No conflict of interest.

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