Oral Glutamine Protects against Acute Doxorubicin-Induced Cardiotoxicity of Tumor-Bearing Rats\textsuperscript{1,2}

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Abstract

Doxorubicin (DOX), a widely used anticancer drug, has a dose-dependent cardiotoxicity, attributed mainly to free radical formation. The cardiomyocyte oxidative stress occurs rapidly after DOX treatment, resulting in harmful modifications to proteins, lipids, and DNA. Previous data showed that oral \textit{l}-glutamine (Gln) prevented cardiac lipid peroxidation and maintained normal cardiac glutathione (GSH) levels in DOX-treated rats. Our aim in this study was to examine the effect of Gln on DOX-induced cardiac oxidative stress in a tumor-bearing host. Female Fisher344 rats with implanted MatBIII mammary tumors were randomized into 2 groups: a Gln group that received \textit{l}-Gln (1 g kg\textsuperscript{-1} d\textsuperscript{-1}) (n = 10) via a Gln-enriched diet and/or gavage with 50% Gln suspension during the whole experiment and a control group that was fed the same diet formulation without Gln and/or were gavaged with water. All rats received a single injection of 12 mg/kg DOX and were killed 3 d later. GSH levels of hearts, livers, tumors, and blood, as well as cardiac histological alterations, lipid peroxidation, peroxinitrite levels, and caspase-3 activation were determined. Cardiac physiologic alterations were assessed by ultrasonic imaging before and 3 d after DOX administration. The Gln supplementation resulted in lower cardiac lipid peroxidation and peroxinitrite levels and elevated cardiac catalase enzyme activity and GSH compared with the controls, without affecting those of the tumors. DOX-induced alterations of the echocardiographic parameters were significantly reduced in the Gln-supplemented rats. These data indicate that Gln is able to reduce the oxidative damage of cardiomyocytes that occurs soon after DOX administration and thus protects the heart of a tumor-bearing host from DOX-induced cardiomyopathy.


Introduction

The anthracycline antibiotic doxorubicin (DOX;\textsuperscript{6} adriamycin) is an antineoplastic agent with high antitumor efficacy in solid malignancies. Although the cardiomyopathy of DOX is well documented (1), it remains a major anticancer agent. Anthracycline-induced cardiotoxicity was observed in 2–20% of the patients receiving anthracyclines and was dose dependent (2). The antitumor activity of DOX is likely to be distinct from the mechanism of its cardiotoxicity. DOX antitumor activity is thought to be due to DNA damage and inhibition of cell replication of highly proliferative tumors (3). Cardiomyocytes, however, are minimally replicative cells that should be resistant to such antimitotic mechanisms. Evidence indicates that free radical generation and oxidative stress of the cardiomyocytes that occur soon after exposure to DOX significantly contribute to the cardiotoxic effects of DOX (4,5). The oxidative stress plays an important role in the early injury of cardiomyocytes, causing harmful and incompletely repaired modifications of proteins (6), lipids (7), and DNA (8). Increased production of superoxide, OH\textsuperscript{-}, conjugated dienes, malonaldehyde, and enzymatic activity changes of glutathione (GSH) peroxidase and catalase following anthracycline treatment has been observed in several biological systems (9,10). Accordingly, the use of antioxidants in combination with chemotherapy prolonged the survival time of patients compared with the expected outcome without antioxidant supplements (11).

Pharmacokinetic studies have shown that the rapid tissue uptake of DOX within 5 min after administration was followed by a terminal half-life of 20–48 h (12). DOX is metabolized in the liver where the quinone nucleus of DOX undergoes redox cycling. The reduction of DOX to a semiquinone free radical is catalyzed by NADPH-dependent cytochrome P450 reductase and reconversion to the quinone is accompanied by reduction of molecular oxygen to superoxide anions (O\textsubscript{2}\textsuperscript{-}), forming peroxynitrite (ONOO\textsuperscript{-}) in a reaction with nitric oxide. Both hydroxyl radicals and peroxynitrite can initiate protein

\begin{itemize}
  \item \textsuperscript{1} Supported by Susan G. Komen for the Cure to V.K.T. (grant no. BCTR78206).
  \item \textsuperscript{2} Author disclosures: V. K. Todorova, Y. Kaufmann, L. J. Hennings, and V. S. Klimberg, no conflicts of interest.
  \item \textsuperscript{6} Abbreviations used: DOX, doxorubicin; GSH, glutathione; LV, left ventricle; LVEF, left ventricle ejection fraction; LVPW, left ventricle posterior wall; MDA, malondialdehyde; PARP, polyADP-ribose polymerase; SOD, superoxide dismutase.
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\end{itemize}
Glutamine (Gln) is the most abundant amino acid serving multiple metabolic functions that account for its extremely high turnover rate (14). As one of the precursors for the synthesis of the major natural antioxidant of body GSH, Gln plays an important role in maintaining the cellular redox state, especially during periods of stress (15). The safety of enteral and parenteral Gln and its benefits on improving amino acid metabolism, immune function, and outcome in normal volunteers and patients with catabolic diseases have been established in numerous studies (16), including at least 18 clinical trials [reviewed in (17)]. Our previous studies showed that dietary Gln maintained normal cardiac GSH levels in rats given DOX and prevented cardiac lipid peroxidation (18). We have also found that Gln did not affect tumor GSH (19). This is important, because GSH is a key regulator of the cellular redox state and the redox environment within the tumor cells determines the response of tumors (and protection of the normal cells) to chemotherapy and radiation (20). Moreover, we have shown that a Gln supplement reduced tumor growth in experimental 7,12-dimethylbenz[a]anthracene-induced breast cancer (19).

The present study investigated the effect of oral Gln on DOX-induced oxidative stress of cardiomyocytes in tumor-bearing rats. Based on the above-mentioned beneficial effects of Gln, we hypothesized that oral Gln will reduce DOX-induced cardiac oxidative damages in the presence of a growing tumor and thus it can intensify the chemotherapeutic regimens containing DOX by preventing its limiting toxicity.

**Methods**

**Animals, cell culture, and treatment.** A total of 20 female Fisher344 rats (NCI) 35–40 d old and weighing 130–150 g were used. All studies were approved by the Animal Care and Use Committee at the Central Arkansas Veterans Healthcare System. The rats were maintained 2 per cage in standard cages in the animal care facility. The rats were approved by the Animal Care and Use Committee at the Central Arkansas Veterans Healthcare System. The rats were maintained 2 per cage in standard cages in the animal care facility and were subjected to a 12-h dark/light cycle. One week before tumor cell implantation, the rats were randomized to receive either a purified ingredient AIN-93G diet (21) that did not contain Gln (Harlan Teklad) (n = 10) or its modification with 5% Gln (TD.07199, Harlan Teklad) (n = 10). The TD.07199 modification had reduced casein from 20 to 15% and added 5% L-Gln.

Both groups of rats were randomized to receive either a purified ingredient AIN-93G diet (21) that did not contain Gln (Harlan Teklad) (n = 10) or its modification with 5% Gln (TD.07199, Harlan Teklad) (n = 10). The TD.07199 modification had reduced casein from 20 to 15% and added 5% L-Gln. Preliminary data showed that these rats consumed 12–18 g/d. The amount needed per cage that would add Gln to the daily amount of Gln intake calculated. Based on these calculations, the rats in the cages were withdrawn by heart puncture with a syringe containing heparin, and the hearts were removed. Samples from the left ventricle (LV) were fixed in neutral buffered formaldehyde (4% wt/v) or snap-frozen in liquid nitrogen and stored at −80°C until used. Samples from the livers and tumors were also snap-frozen in liquid nitrogen and stored at −80°C until used.

**Histopathological analysis.** Tissue samples from the LV of 6 rats from each group were processed and embedded in paraffin and sectioned at 4 μm. Sections were stained with hematoxylin and eosin for routine histological microscopic analysis and myocardial necrosis was evaluated using a scale of 0 (no change) to 3 (severe lesion).

**Lipid peroxidation.** Cardiac and liver lipid peroxidation was measured by the malondialdehyde (MDA)-thiobarbituric acid reactive assay. Briefly, tissue homogenates in 5 mmol/L butylated hydroxytoluene in 20 mmol/L phosphate buffer, pH 7.4, were centrifuged at 3000 × g for 10 min and 200 μL of the supernatant was mixed with 400 μL 30% trichloroacetic acid + 0.67% thiobarbituric acid in the presence of 200 mmol/L butylated hydroxytoluene. The mixture was incubated in a boiling water bath for 20 min and centrifuged (3000 × g; 10 min). The OD of the supernatant was measured at 532 nm. Commercially available 1,1,3,3-tetraethoxypropane (Sigma) was used as a standard for MDA. The results were expressed as nmol/mg protein.

**Peroxynitrite production.** Cardiac peroxynitrite production was measured using dihydrodihydrodiamine (22). Briefly, 0.05 g tissue was incubated in dihydrodihydrodiamine solution (1 g/L in dimethylsulfoxide) in 24-well plates at 37°C for 90 min. Samples were washed with PBS and homogenized in PBS with 5% SDS and 15% Tween. After centrifugation, the fluorescence of the supernatant was measured using Spectramax M5 microplate reader (Molecular Devices) at excitation 488 nm/emission 515 nm. The results were expressed as fluorescence/mg protein.

**GSH determination.** GSH concentrations of blood, heart, liver, and tumors were determined by a standard enzymatic recycling procedure (22). Briefly, 500 μL heparinized blood or 0.5 g tissue was mixed and homogenized with equal volumes of 10% or 5% 5-sulfosalicylic acid and centrifuged at 5°C at 3000 × g for 15 min. Ten microliters of the supernatant was added to 1 mL of reaction mix (0.2 mmol/L NADP, 0.6 mmol/L 5,5-dithio-bis-2-nitrobenzoic acid, and 1.33 U GSH reductase) and the absorbance was measured at 412 nm. The data were expressed as nmol/g tissue and μmol/L (for the blood).

**Catalase.** Cardiac catalase was measured using assay based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H$_2$O$_2$ (Cayman Chem). The formaldehyde produced was measured spectrophotometrically at 540 nm with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which upon oxidation changes from colorless to a purple color. The results were expressed as U/mg protein.

**Superoxide dismutases.** Cardiac superoxide dismutase (SOD) activity was assessed using an assay that measures the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine (Cayman Chem). The results were expressed as U/mg protein.

**Caspase-3-like enzymatic activity.** Cardiac caspase-3 enzymatic activity was measured using Caspase-3 Fluorimetric assay (Biovision), which is based on detection of cleavage of substrate Asp-Glu-Val-Asp (DEVD)-7-amino-4-trifluoromethyl coumarine. Heart tissue homogenates were incubated with DEVD-7-amino-4-trifluoromethyl coumarine for 2 h at 37°C and fluorescence read in a Spectramax-M5 microplate reader (Molecular Devices). The results were expressed as U/mg protein.

**Western blotting analysis.** The cardiac protein levels and activation of poly(ADP-ribose polymerase) (PARP), a target of caspase-3, were analyzed using a standard Western blot procedure (20) and anti-rat PARP antibody (Santa Cruz Biotechnology). Equal protein amounts (40 μg) of each sample loaded on 10% polyacrylamide gels were

Glutamine and doxorubicin cardiotoxicity 45
analyzed by Western blotting using specific antibodies. The equal loadings were controlled by staining with Ponso S and reprobing the membranes with tubulin.

**Echocardiographic assessment of cardiac physiological alterations.** Two-dimensional B-mode and anatomical M-mode imaging were performed using ultrasound imaging system Vevo 770 High-Resolution In Vivo Imaging system (VisualSonics) designed for small animals. M-mode images were acquired and used for the calculation of the LV function using procedures provided by the manufacturer. Ten rats of each group were examined before DOX administration (baseline) and 3 d after DOX administration. The rats were anesthetized with 2% isoflurane/oxygen by a facemask during the entire procedure. The procedure was as follows: the rat was laid supine on the platform with all legs taped to echocardiographic electrodes for heart rate monitoring. All hair was removed from the chest using a chemical hair remover (Nair; Carter-Horner). With the scanhead 716 and on B-mode, the short-axis imaging was taken to mainly visualize LV. M-mode was used to obtain anatomically correct LV measurements, including LV posterior wall (LVPW) and LV ejection fraction (LVEF). Data analysis was performed offline using a customized version of Vevo 770 Analytic Software.

**Statistical analysis.** Data are presented as mean ± SE. Data analysis was carried out using StarView software. A paired t test was used to test the differences between the values of LVEF and LVPW before DOX administration and 3 d after DOX administration within each group. Unpaired t test was used to determine the differences of MDA, peroxinitrite, GSH, catalase, and caspase between the Gln and control groups. P < 0.05 was considered significant.

**Results**

**Food intake and body weight.** DOX treatment resulted in reduced food intake. The mean food intake at baseline for each cage was 24–36 g/d compared with 3–5 g/d at 3 d after DOX administration. Therefore, all rats were gavaged daily with 0.1–0.2 mL 50% Gln suspension or water depending on the food consumption as described in Methods. Between the start of the Gln supplement and DOX administration (2 wk), all rats gained an mean of 35 g and the 2 groups did not differ. DOX administration resulted in body weight loss, which was 10 ± 1.5 g for the Gln group and 13 ± 2.1 g for the control group, but the differences did not reach significance (P = 0.4).

**Histopathological examination.** The histopathological examination showed that 50% of the rats in the Gln group did not have cardiac necrosis compared with 12.5% in the control group. Necrosis 3+ was found in 12.5% of Gln-fed rats compared with 37.5% in the controls.

**Lipid peroxidation and peroxinitrite production.** The results (Table 1) showed that Gln supplementation significantly lowered heart and liver lipid peroxidation by 16 and 32%, respectively, in comparison with the controls and had no significant effect on tumors (not shown). Gln significantly lowered the cardiac peroxinitrite compared with the controls.

**GSH concentrations.** The content of the reduced GSH in the hearts, livers, and blood of Gln group was significantly elevated by 17, 20, and 32%, respectively, compared with the control group (Table 1). Gln did not affect tumor GSH levels (P = 0.7).

**Cardiac catalase-, SOD-, and caspase-3 enzyme activities.** Gln significantly elevated heart catalase activity by 18% compared with the controls and did not significantly affect tumor catalase (Table 1). Cardiac SOD activity did not differ significantly between the groups (P = 0.3). The cardiac caspase-3 enzyme activity in the Gln group was 23.2% lower than in the control group.

**PARP activation.** The reduction of apoptosis by Gln was further confirmed by the lower activation of cardiac PARP, a target of caspase-3, as determined by Western blotting (Fig. 1). During apoptosis, caspase-3 cleaves the 116-kDa PARP molecule into 2 fragments of 89 kDa (catalytic domain) and 24 kDa (DNA-binding domain).

**Echocardiographic assessment of cardiac physiological alterations.** Heart rate was similar between the groups and was not significantly affected by tumor presence or DOX treatment (260–325 beats/min). Before DOX administration (baseline), the groups did not differ in LVPWd or LVEF. At 3 d after DOX administration, the mean LVPW end-diastolic thickness of the control group was 0.97 mm greater than at baseline (P = 0.043), whereas in the Gln group, the LVPWd was 0.3 mm greater than at baseline (P < 0.048) (Table 2). At d 3, reduction of LVEF in the Gln group was less than in the control group (P = 0.03).

**Discussion**

This study was designed to examine the effect of oral Gln on the DOX-induced cardiac oxidative stress of a tumor-bearing host. DOX-induced cardiototoxicity of rats was found to recapitulate

**TABLE 1** Effect of Gln supplementation on tissue MDA, peroxinitrite, GSH, and enzyme activities of catalase and caspase-3 of tumor-bearing rats treated with DOX

<table>
<thead>
<tr>
<th>Variable</th>
<th>Gln group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA, μmol/mg protein</td>
<td>1.47 ± 0.09*</td>
<td>1.76 ± 0.07</td>
</tr>
<tr>
<td>Liver</td>
<td>0.314 ± 0.05*</td>
<td>0.45 ± 0.11</td>
</tr>
<tr>
<td>Heart peroxinitrite, fluorescence/mg protein</td>
<td>944.3 ± 263*</td>
<td>1076.1 ± 341</td>
</tr>
<tr>
<td>GSH, mmol/g tissue, μmol/L blood</td>
<td>0.571 ± 0.01*</td>
<td>0.475 ± 0.02</td>
</tr>
<tr>
<td>Heart</td>
<td>1.23 ± 0.01*</td>
<td>0.997 ± 0.02</td>
</tr>
<tr>
<td>Liver</td>
<td>0.638 ± 0.03</td>
<td>0.623 ± 0.04</td>
</tr>
<tr>
<td>Tumor</td>
<td>748.0 ± 43.8*</td>
<td>510.0 ± 24.4</td>
</tr>
<tr>
<td>Heart catalase enzyme activity, U/mg protein</td>
<td>821.59 ± 76.9*</td>
<td>677.81 ± 123</td>
</tr>
<tr>
<td>Heart SOD, U/mg protein</td>
<td>5.85 ± 1.9</td>
<td>5.72 ± 1.7</td>
</tr>
</tbody>
</table>

*Values are means ± SEM, n = 8. *Different from control, P < 0.05.

![FIGURE 1](https://academic.oup.com/jn/article-abstract/140/1/44/4600427/8.4600427)
the physiological and histological findings in patients, making this model particularly suitable for experimental studies (23).

The results showed that Gln protected the heart from DOX-induced oxidative stress in the presence of a malignant disease through stimulation of cardiac antioxidant properties. Gln-supplemented rats had enhanced cardiac catalase activity and higher levels of cardiac GSH. At the same time, Gln supplementation resulted in lower cardiac lipid peroxidation, lower cardiac peroxinitrite production, and inhibition of myocyte necrosis/apoptosis compared with the controls. A decline of LVEF by >10% was suggested as a criterion for suspending the treatment (24). In the present study, the echocardiographic examination showed a 12% reduction of LVEF in tumor-bearing rats treated with DOX alone compared with only 2% in the rats treated with DOX+Gln.

DOX cardiotoxicity in patients has been subdivided into acute and chronic effects, depending on their occurrence following administration of the drug. The most frequently encountered and investigated form of cardiac toxicity is a cumulative, dose-related myocardial dysfunction occurring 1–6 mo after chemotherapy (25). However, the presence of pathological alteration in human heart samples, such as contraction and nuclei ring formation, were found as early as 24 h after DOX administration (26). It has been generally accepted that the oxidative stress of cardiomyocytes plays a major role in the early injury of cardiomyocytes by DOX (27). The heart is particularly susceptible to DOX toxicity due to its low antioxidant enzyme activities, including catalase, SOD, and GSH peroxidase (28). As such, the heart is more vulnerable to DOX-generated reactive oxygen species insults than other organs in the body (28). The level of DOX-induced oxidative stress was found to be up to 10 times greater in the heart than in the liver, kidney, and spleen (29). A number of reports using DOX acute toxicity have shown a marked cardiac protection by antioxidant supplements. Antioxidants such as coenzyme Q (30) and N-acetylcysteine (31) reduced DOX cardiotoxicity through prevention of the early oxidative effects of DOX. The present work demonstrates that the oxidative stress is a significant component of DOX cardiotoxicity in a tumor-bearing host and is reduced by Gln supplementation.

The importance of Gln in maintaining the body’s metabolic homeostasis becomes evident during periods of stress, when it becomes a conditionally essential amino acid. The excessive needs of Gln during catabolic states such as an advanced malignant disease are supplied from muscle stores and this might lead to a massive depletion of skeletal muscle Gln (32). Gln starvation results in energy depletion, decreased immune defense, stimulated apoptosis, and reduced the levels of GSH (33). Experimental data suggest that Gln may protect the cardiac metabolism during periods of stress. For example, Khogali et al. (34) found that in the absence of Gln, ischemia-reperfusion caused immediate decreases in myocardial glutamate and myocardial ATP and an accumulation of myocardial lactate, whereas the presence of Gln prevented these deleterious changes in myocardial metabolites. Postischemic treatment with Gln completely restored the myocardial metabolites to normal and significantly enhanced the myocardial ATP:ADP and GSH:oxidized GSH ratios.

Our previous studies found that oral Gln supplementation stimulated GSH production in heart, blood, normal mammary tissue, and gut while significantly reducing tumor GSH levels in implantable sarcoma and 7,12-dimethylbenz[a]anthracene-induced breast cancer models (19). This is important, because GSH is a key regulator of the cellular redox state and as such it also determines the response of tumors (and protection of the normal cells) to chemotherapy and radiation (20). The paradoxical effect of Gln on GSH metabolism in tumor and host tissues may be a result of the relatively more acidic intracellular environment of tumors compared with normal structures, thus inactivating the pH-sensitive enzyme 5-oxoprolinase, which catalyzes the formation of γ-glutamyl-Gln dipeptide, an immediate precursor of GSH in the γ-glutamyl cycle to glutamate (35). Our data showed that Gln downregulated several enzymes of the γ-glutamyl cycle, including 5-oxoprolinase and glutaminase in tumor, but upregulated them in normal tissues (36). The differential effect of Gln on GSH recycling of normal tissues and tumors was associated with a reduction of tumor growth in breast cancer model (19). We have also found that dietary Gln elevated blood and cardiac Gln and GSH in rats treated with DOX (18), cyclophosphamide (37), or methotrexate (38) and this inversely correlated with cardiac tissue lipid peroxidation.

In a phase I trial, 9 patients diagnosed with inflammatory breast cancer received Gln (0.5 g kg⁻¹ d⁻¹) during methotrexate neoadjuvant therapy, followed by a DOX-based regimen (39). No patient showed any sign of chemotherapy-related toxicity. Recently, Wischmeyer (40) reviewed the clinical and mechanistic data examining Gln’s ability to reduce morbidity and mortality in critical illness. The author analyzed the clinical data over the past 15–20 y showing that Gln supplementation improved infectious morbidity and mortality, reduced hyperglycemia, reduced inflammation, and improved liver dysfunction in critically ill patients. Given the enormous amount of data from small clinical trials, we conclude that there is need for a large, multicenter, multinational clinical trial.

The results from this study indicate that oral Gln has a significant protective effect against acute DOX cardiotoxicity in a tumor-bearing host. Gln was able to reduce the cardiotoxicity of DOX mainly through the stimulation of the antioxidant capacity of cardiomyocytes. Further clinical studies will determine whether Gln supplementation could prevent cardiac oxidative damages and therefore enhance the therapeutic index for DOX in cancer patients.

Acknowledgments
V.K.T. and V.S.K. designed research; V.K.T., Y.K., and L.J.H. conducted research and analyzed data; V.K.T. wrote the paper; and V.K.T. had primary responsibility for final content. All authors read and approved the final manuscript.

Literature Cited


