

# Nutrition Modulation of Cardiotoxicity and Anticancer Efficacy Related to Doxorubicin Chemotherapy by Glutamine and $\omega$ -3 Polyunsaturated Fatty Acids

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## Abstract

**Background:** Doxorubicin (DOX) has been one of the most effective antitumor agents against a broad spectrum of malignancies. However, DOX-induced cardiotoxicity forms the major cumulative dose-limiting factor. Glutamine and  $\omega$ -3 polyunsaturated fatty acids (PUFAs) are putatively cardioprotective during various stresses and/or have potential chemosensitizing effects during cancer chemotherapy. **Methods:** Antitumor activity and cardiotoxicity of DOX treatment were evaluated simultaneously in a MatBIII mammary adenocarcinoma tumor-bearing rat model treated with DOX (cumulative dose 12 mg/kg). Single or combined treatment of parenteral glutamine (0.35 g/kg) and  $\omega$ -3 PUFAs (0.19 g/kg eicosapentaenoic acid and 0.18 g/kg docosahexaenoic acid) was administered every other day, starting 6 days before chemotherapy initiation until the end of study (day 50). **Results:** Glutamine alone significantly prevented DOX-related deterioration of cardiac function, reduced serum cardiac troponin I levels, and diminished cardiac lipid peroxidation while not affecting tumor inhibition kinetics. Single  $\omega$ -3 PUFA treatment significantly enhanced antitumor activity of DOX associated with intensified tumoral oxidative stress and enhanced tumoral DOX concentration while not potentiating cardiac dysfunction or increasing cardiac oxidative stress. Intriguingly, providing glutamine and  $\omega$ -3 PUFAs together did not consistently confer a greater benefit; conversely, individual benefits on cardiotoxicity and chemosensitization were mostly attenuated or completely lost when combined. **Conclusions:** Our data demonstrate an interesting differentiality or even dichotomy in the response of tumor and host to single parenteral glutamine and  $\omega$ -3 PUFA treatments. The intriguing glutamine  $\times$   $\omega$ -3 PUFA interaction observed draws into question the common assumption that there are additive benefits of combinations of nutrients that are beneficial on an individual basis. (*JPEN J Parenter Enteral Nutr.* 2016;40:52-66)

## Keywords

fatty acids; research and diseases; amino acids; oncology; breast cancer; glutamine; doxorubicin; cardiotoxicity

## Clinical Relevancy Statement

The key for using a modulator for cancer chemotherapy is that the agent has to exhibit substantial selectivity in its effects on tumor vs host. Our data demonstrate that both single glutamine and  $\omega$ -3 polyunsaturated fatty acid (PUFA) treatments exhibit substantial selectivity in their effects on the host and tumor, and these 2 individual nutrients show therapeutic promise to be used as an adjunct to doxorubicin chemotherapy as a cardiotoxicity antagonist or chemosensitizer. Furthermore, one of the most controversial aspects of immunonutrition is that a number of distinct nutrients are usually combined at the same time based on a prevalent assumption that additivity or even synergism would be achieved by providing these nutrients known to be beneficial when given individually. Our data demonstrate that glutamine and  $\omega$ -3 PUFAs seem to be rather antagonistic on most end points examined when combined. This entails the need to understand potential nutrient-nutrient interactions, between but possibly not limited to glutamine and  $\omega$ -3 PUFAs.

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**Conflicts of interest:** Melanie Denking and Ewald Schlotzer are employees at Fresenius Kabi.

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## Introduction

The anthracycline antibiotics have been one of the most effective antitumor agents against a broad spectrum of malignancies, including carcinomas, sarcomas, hematopoietic malignancies, and various childhood malignancies. Their antineoplastic spectrum of action compares favorably with the alkylating agents and the taxanes.<sup>1,2</sup> Doxorubicin (DOX) or epirubicin (EPI)-based anthracycline chemotherapy is the cornerstone of many chemotherapy regimens.<sup>3,4</sup> However, cardiotoxicity related to the cumulative dose of an anthracycline is the chronic dose-limiting toxicity that limits anthracycline use in the clinical setting. The overall incidence of anthracycline-related congestive heart failure (CHF) was 3% with a cumulative dose of 300 mg/m<sup>2</sup> and 5% at 450 mg/m<sup>2</sup>, and after exceeding this dose, the increase was actually linear, with up to 50% incidence of CHF with 950 mg/m<sup>2</sup>.<sup>5</sup> As a result, a cumulative dose limit of 450 mg/m<sup>2</sup> has been suggested to reduce the potential for the chronic cardiotoxicity and resultant heart failure.<sup>6</sup> Anthracycline antibiotics produce acute and chronic cardiotoxicity. Of far greater concern is the chronic cardiotoxic effect, with patients exhibiting irreversible cardiomyopathic changes, progressive left ventricular dysfunction, and ultimately CHF.<sup>7</sup> Anthracycline-induced cardiomyopathy may persist or progress after the chemotherapy has been discontinued and may evolve into a chronic dilated cardiomyopathy in adult patients.<sup>8</sup>

A variety of dietary factors are hypothesized to modulate the therapeutic index of anticancer drugs by attenuating toxicities and/or enhancing efficacy, which seems to offer an affordable and safe approach to influence the outcomes associated with cancer chemotherapy.<sup>9</sup> Glutamine is a functionally versatile amino acid involved in a diverse range of physiological processes (ie, interorgan nitrogen transport/exchange, pH homeostasis, regulation of protein synthesis and cell swelling).<sup>10</sup> Anthracycline-induced cardiotoxicity (AIC) is primarily caused by increased oxidative stress related to free radical overproduction and reduction in endogenous antioxidant reserves.<sup>11–13</sup> The redox cycling of anthracyclines and their iron chelates generates highly reactive metabolites, such as superoxide, hydroxyl radicals, and hydrogen peroxide, which subsequently lead to membrane lipid peroxidation with disturbance of the membrane structure and mitochondrial dysfunction.<sup>12–14</sup> This understanding creates a niche for using nutrients that can potentially maintain redox homeostasis during stress as an adjunct to anthracycline chemotherapy. Glutamine (via glutamate) is a precursor for glutathione synthesis and has been suggested to be limiting for glutathione synthesis during stress.<sup>15–19</sup> Glutamine not only preserves the intestinal glutathione stores during oxidative stress related to irinotecan, 5-FU, and methotrexate chemotherapy<sup>16–19</sup> but also maintains normal cardiac glutathione stores during anthracycline treatment and further prevents cardiac lipid peroxidation.<sup>20,21</sup> In exploring

mechanism(s) underlying glutamine's protection during stress, we found a marked induction of heat shock proteins (HSPs) in various tissues and organs associated with the protection conferred by enteral or parenteral bolus glutamine treatment.<sup>16,22–24</sup> The induction of HSP is an important innate universal cytoprotective mechanism<sup>25</sup> and is especially vital for cardiomyocytes to preserve their structural and functional integrity during stress/injury conditions.<sup>26</sup> Nonetheless, the potential protective role of glutamine via HSP induction/potential in AIC has never been explored.

A large amount of preformed long-chain  $\omega$ -3 PUFAs (ie, eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]) can be found in fatty cold-water fish.<sup>27</sup> Dietary  $\omega$ -3 PUFAs are recently reported to show therapeutic promise as an adjunct to cancer chemotherapy. They can both enhance the toxicity of drugs to tumor cells at low doses and/or offer protection to nontarget tissues and thus augment the therapeutic index of anticancer drugs.<sup>28,29</sup> We and others have shown that EPA and DHA alone or in combination with fish oil are reported to enhance the cytotoxicity of several widely used antineoplastic agents in a variety of models.<sup>16,19,30,31</sup> One crucial issue related to the utility of  $\omega$ -3 PUFAs in cancer chemotherapy is that its chemosensitizing effect has to be substantially confined to tumor tissue but not aggravating injury to host tissues. However, the results described in current literature are considerably mixed. Some studies demonstrate  $\omega$ -3 PUFAs aggravate AIC,<sup>32</sup> while others showed neutral<sup>33,34</sup> or conversely cardioprotective<sup>35–37</sup> effects during anthracycline chemotherapy. It is daunting to comparatively interpret all these results and pinpoint a clinically translatable conclusion due to the widely varied control diet used, diverse surrogate markers used for cardiac injury, and the fact that none of the aforementioned studies investigated  $\omega$ -3 PUFAs' effects on both antitumor efficacy and cardiotoxicity related to anthracyclines on the same model system.

Available literature on nutrition modulation of anticancer treatments is generally focused on a single dietary factor in a relatively discrete manner. Currently, we lack a crucial understanding of how these different nutrients act or counteract and whether additivity or subtractivity can be achieved when they are provided in combination.<sup>38</sup> In contrast to the considerably large and rapidly increasing number of nutrition formulas featuring combinations of various nutrients,<sup>39,40</sup> there is a distinct lack of rigorous and systematic experimental evidence to justify these combinations and to support the way in which these commercial products are formulated. Here, we further explored the higher order of nutrient-nutrient interactions by comparing the relative efficacy of combined supplementation of glutamine and  $\omega$ -3 PUFAs with the single supplementation of each individual factor alone. This provides a key understanding on how individually beneficial immune-nutrients interact and an important framework for future work on examining a higher order of nutrient interaction in the context of cancer and cancer chemotherapy.

**Table 1.** Composition of the Parenteral Solutions.

Component Solution (vol/vol, %)	Control Parenteral Treatment	Single Glutamine Parenteral Treatment	Single $\omega$ -3 PUFA Parenteral Treatment	Combined Glutamine/ $\omega$ -3 PUFA Parenteral Treatment
Dipeptiven 20%	0	11.3	0	11.3
Aminosyn 10%	56.5	28.8	56.5	28.8
Omegaven 10%	0	0	43.5	43.5
Intralipid 20%	21.7	21.7	0	0
Lactate Ringer's	21.7	38.2	0	16.4
Total	100	100	100	100

Animals of each parenterally treated group received an identical volume of parenteral solution of 23.0 mL/kg/dose, which delivered glutamine at 0.35 g/kg/dose for glutamine-supplemented parenteral treatments and long-chain  $\omega$ -3 polyunsaturated fatty acids (PUFAs) (0.19 g/kg/dose eicosapentaenoic acid and 0.18 g/kg/dose docosahexaenoic acid) for  $\omega$ -3 PUFA-supplemented parenteral treatments.

## Experimental Methods

### Animal Drug-Tumor Model

Animal use was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Colorado. To closely recapitulate the clinical scenario, the antitumor activity and cardiotoxicity of anthracycline treatment need to be evaluated simultaneously in the same model to address whether a certain agent can protect the heart while affecting the antitumor efficacy of the drug in the same animal. Female Fisher344 rats (Charles River Laboratories, Wilmington, MA) bearing syngeneic mammary adenocarcinoma xenograft treated with DOX were used as the drug-tumor model.<sup>41</sup> Rats were housed 2 per cage in a temperature- (22°C) and light-controlled (12-hour light) room; water and food were available for ad libitum consumption. A suspension of  $1 \times 10^6$  rat mammary adenocarcinoma MatBIII cells (ATCC, Manassas, VA) was injected into the flank of female Fisher344 rats (3 months old, 130–150 g of body weight). DOX treatment was initiated when the tumor mass reached about 1.2 cm<sup>3</sup> (~0.75% of body weight). DOX (1 mg/kg/dose, intravenous [IV] injection) was given on 6 consecutive days and then once weekly for 6 weeks with a cumulative dose of 12 mg/kg, which has been consistently proven to be the maximal tolerated dose for rats.<sup>42</sup> Our pilot data show that this dose regimen led to significant tumor regression while producing substantial chronic cardiomyopathy, which allows a sufficient window to evaluate the effects of both antitumor efficacy and cardiotoxicity related to DOX chemotherapy. Animals were terminated 50 days after initiation of chemotherapy, a time window sufficient for animals to develop clinically and pathologically substantial cardiomyopathy and significant tumor regression.<sup>43</sup>

**Nutrition treatments.** Animals were fed a semi-purified diet throughout the whole study period, which has been well established as the dietary platform for our nutrition modulation of chemotherapy studies.<sup>16,19,24</sup> This diet was formulated to meet or exceed nutrient requirements of laboratory rats and is based on the American Institute of Nutrition (AIN)-76 modified

basal diet with 40% of calories from fat. The modified fat component is formulated to be similar to typical North American dietary patterns in humans (40% of calories, polyunsaturated/saturated fat ratio of 0.35).<sup>44</sup> This diet contained 262 g protein, 3700 kcal of calories per kilogram, and 1.1% of total fatty acids as C18:3(3) and had an  $\omega$ -6: $\omega$ -3 ratio of 21.0.<sup>45–47</sup> The diet was available for ad libitum consumption.

Parenteral supplementation with single and combined glutamine and  $\omega$ -3 PUFAs was compared in this animal drug-tumor model system. Rats were randomly assigned to 1 of the following 6 treatment groups:

1. Non-DOX-treated reference group (REF) receiving saline (sham to DOX) (n = 10)
2. DOX alone without any parenteral intervention (D, n = 10)
3. DOX + parenteral control treatment (D+C, n = 12)
4. DOX + parenteral glutamine treatment (D+G, n = 12)
5. DOX + parenteral  $\omega$ -3 PUFA treatment (D+F, n = 12)
6. DOX + parenteral combined glutamine/ $\omega$ -3 PUFA treatment (D+G+F, n = 12)

The parenteral solutions were composed of varied component amino acid and fat solutions as detailed in Table 1. For glutamine-supplemented parenteral treatments, glutamine was supplied by combining a commercially available amino acid solution (10% Aminosyn; Hospira, Lake Forest, IL) with L-alanyl-L-glutamine dipeptide (Dipeptiven 20%; Fresenius Kabi, Bad Homburg, Germany). Glutamine is unstable and poorly soluble for addition to existing preparations in its native form, whereas synthetic glutamine-containing dipeptides (eg, L-alanyl-L-glutamine) are stable and highly soluble, can readily be hydrolyzed following intravenous administration, and have been proven to be a superior source of glutamine for parenteral supplementation.<sup>48</sup> Alanyl-glutamine was given at 0.52 g/kg/dose, which was equivalent to glutamine to be given at 0.35 g/kg/dose. For  $\omega$ -3 PUFA-supplemented parenteral treatments, soybean-based standard lipid emulsion not enriched in  $\omega$ -3 PUFAs (Intralipid 20%;

Fresenius Kabi) was substituted by lipid emulsion enriched in  $\omega$ -3 PUFAs (OMEGAVEN 10%; Fresenius Kabi) with an equal amount of energy provided by fat. The  $\omega$ -3 PUFA-supplemented parenteral treatments delivered approximately 0.19 g/kg/dose EPA and 0.18 g/kg/dose DHA. All these parenteral solutions are isonitrogenous and isocaloric. For each parenterally treated group, animals received an identical volume of solution of 23.0 mL/kg/dose. With the parenteral supplementation accounted for, each rat received 167.9 kcal/kg of calories (4.5% provided by the parenteral treatment), 12.0 g/kg of protein (5.2% provided by the parenteral treatment), and 9.2 g/kg of fat (5.5% provided by the parenteral treatment) each day. All parenteral solutions were administered via tail vein of anesthetized rats at a rate of 0.5 mL/min<sup>16,22</sup> every other day, starting on day -6 (6 days before initiation of chemotherapy) until the end of the study (day 50). All parenteral solutions were freshly prepared immediately before use on each injection day, and all infusions were completed within 2 hours on the injection days. No creaming or coalescence occurred by visual examination for emulsion status within this time frame.<sup>49</sup> Our parenteral formulations should be stable given the fact that the pH range is within 6.1–6.8, that there is nil phosphate, that calcium level are low (<0.6 mmol/L) in all the formulations, and that the formulations are used within 2 hours after compounding.<sup>50–52</sup>

To measure DOX accumulation in tumor tissues, another set of animals randomized into groups 2–6 (ie, D, C+C, D+G, D+F, D+G+F,  $n = 6$ ) was killed on day 7 (1 day after the first consecutive doses of DOX), and tumors from these animals were harvested.

## Outcome Measures

Body weight and food intake and other symptomatic variables related to DOX-induced toxicities (ie, respiratory rate, extremity/facial edema, ascites sign, behavioral abnormalities) were monitored every second day.

Tumor volume was measured at time points indicated in the figures and in 3 dimensions—length ( $L$ ), width ( $W$ ), and height ( $H$ )—with a caliper. Tumor volume was calculated: tumor volume ( $\text{cm}^3$ ) =  $0.5 \times L(\text{cm}) \times W(\text{cm}) \times H(\text{cm})$ .<sup>16</sup> Tumor response was expressed as relative tumor volume, calculated relative to the volume at the start of chemotherapy, for each rat. Calculation of tumor growth inhibition was as described previously.<sup>16</sup>

## Sample Collection and Assays

Fifty days after initiation of chemotherapy, animals were killed by  $\text{CO}_2$  asphyxiation followed immediately by exsanguination by cardiac puncture. Volume of ascites and pleural effusion, if any, was estimated by syringe. Heart, lung, liver, and tumor were weighed wet. Samples from the left ventricle were fixed in 10% buffered formalin or snap-frozen and cryopreserved in

liquid nitrogen until subsequent analysis. Tumor tissue was snap-frozen and stored in liquid nitrogen for subsequent analysis. The livers and lungs were then chopped into small pieces and dried in an oven until a stable weight was recorded. An additional set of animals from groups 2–6 was killed on day 7 (1 day after the first consecutive doses of DOX) and tumors were snap-frozen and stored in liquid nitrogen to determine DOX accumulation in the tumor tissue.

Blood was collected by cardiac puncture into heparinized tubes at the end of the study (day 50) and plasma was separated. Plasma cardiac troponin I (cTnI) levels were determined by a Rat Plasma cTnI assay kit (Life Diagnostics, West Chester, PA).

**Echocardiography.** Under anesthesia, left ventricular (LV) function was evaluated with transthoracic echocardiography using a 30-MHz transducer (Vevo 770; VisualSonics, Toronto, Ontario, Canada) at day 50 (before euthanization).<sup>53</sup> High-quality 2-dimensional images and M-mode images of the left ventricle were recorded. A parasternal short-axis view was obtained for LV M-mode imaging at the papillary muscle level. Three independent M-mode images were used for 6 measurements of LV end-diastolic internal diameter (LVEDD) and LV end-systolic internal diameter (LVESD) in 2 consecutive beats.<sup>54</sup> Fractional shortening (FS) was calculated as  $\text{FS}\% = [(LVEDD - LVESD)/LVEDD] \times 100$ .

## Morphological Analysis

The fixed heart samples were embedded in paraffin. Sections (4  $\mu\text{m}$ ) of 3 different LV locations were made and stained with hematoxylin-eosin (HE) and observed microscopically. The severity and extension of myocardial lesions induced by DOX were assessed by light microscopic examination on a qualitative/quantitative morphological grading scale as outlined in Table 2.<sup>55</sup> The pathologist who performed the histopathological examination and scoring was blinded to the tissue sample codes. The score obtained for each section was calculated as the product of severity multiplied by extension of damage based on Della Torre et al<sup>55</sup> with adaptations. All images were acquired under  $\times 400$  magnification with MetaMorph 6.0 (Universal Imaging, Bedford Hills, NY).

**Endogenous antioxidant defense systems.** Reduced glutathione (rGSH) and glutathione disulfide (GSSG) levels in the heart and tumor tissues were determined with the Oxford rGSH/GSSG kit (Oxford Biomedical Research, Rochester Hills, MI), which features 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate as a scavenger of rGSH.<sup>24</sup>

**Enzymatic system.** Activity of the cardiac enzymatic antioxidants (ie, superoxide dismutase [SOD], glutathione peroxidase [GPx], and catalase [CAT]) was measured in the heart homogenates by using EnzyChrom GPx (Bioassay Systems, Hayward,

**Table 2.** Criteria for Morphological Evaluation of Cardiotoxicity.

Score	Hallmarks of Degree Severity or Extension
Degree severity	
1	Sarcoplasmic microvacuolizations and/or interstitial cellular edema
2	Same as 1 plus sarcoplasmic macrovacuolizations or atrophy, necrosis, endocardial lesions, and thrombi
Extension	
0	No lesions
0.5	<10 single altered myocytes in the whole heart section
1	Scattered single altered myocytes
2	Scattered small groups of altered myocytes
3	Widely spread small groups of altered myocytes
4	Confluent groups of altered myocytes
5	Most cells damaged

CA), SOD (Cayman Chemical, Ann Arbor, MI), and CAT (Cayman Chemical) assay kits.

**Lipid peroxidation.** Lipid peroxidation in cardiac tissue was assayed by the method of Placer et al,<sup>56</sup> in which malondialdehyde (MDA) released was the index of lipid peroxidation.

### Tumoral DOX Accumulation

DOX was quantified by a solvent extraction and fluorescence detection assay as detailed previously.<sup>57</sup> Briefly, tumor tissues were weighed and homogenized to a 10% homogenate (w/v in H<sub>2</sub>O). Then, 100  $\mu$ L of 10% (v/v) Triton X-100 and 1500  $\mu$ L of acidified isopropanol (0.75 N HCl) were added to the homogenate (400  $\mu$ L). DOX extraction was achieved by vigorously vortexing and freezing at  $-20^{\circ}\text{C}$  overnight. The next day, the samples were thawed, vortexed, and centrifuged at  $15,000 \times g$  for 10 minutes, and the organic phase was collected for analysis. DOX was quantified by determining the fluorescence of the organic phase (excitation wavelength, 500 nm; emission wavelength, 550 nm) with a luminescence spectrometer (Synergy 2 Multi-Mode Microplate Reader; BioTek Instruments, Winooski, VT).

### Western Blotting

Rat heart tissue was homogenized in a solution containing 10 mM Tris (pH 7.2–8), 5 mM MgSO<sub>4</sub>, DNase, and RNase, per the manufacturer's instructions (New England Biolabs, Ipswich, MA), plus protease inhibitors (Roche, Indianapolis, IN). Lysates were quantified for total protein using the BCA protein assay (Pierce, Rockford, IL), separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidenedifluoride (PVDF) membranes. The membrane was incubated with a primary antibody against cleaved caspase-3 (Cell Signaling, Danvers, MA), cleaved poly (ADP-ribose) polymerase (PARP; Cell

Signaling), Hsp70 (Stressgen, San Diego, CA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, Dallas, TX). Blots were then stained with the appropriate horseradish peroxidase-conjugated secondary antibody. Reactive bands were detected with enhanced chemiluminescence (ECL) reagents (Pierce) and exposed by using a UVP chemiluminescent darkroom system (UVP, Inc, Upland, CA). Quantification of images was done by scanning densitometry using *LabWorks 4.0* Image Acquisition and Analysis Software (UVP, Inc). B-actin was used as a loading control.

Arginase activity was determined in heart tissue by using an arginase activity assay kit (Sigma, St Louis, MO). One unit of arginase is the amount of enzyme that converts 1.0 mmol of L-arginine to ornithine and urea per minute at pH 9.5 and  $37^{\circ}\text{C}$ .

**Statistical analysis.** Statistical analysis was performed using univariate or multivariate linear models of SPSS 12.0 (SPSS, Inc, an IBM Company, Chicago, IL). Unless specified in the text, treatment effects on echocardiography and all postmortem measures were analyzed using 1-way analysis of variance (ANOVA) followed by the post hoc Tukey test.  $P < .05$  was accepted as being statistically significant. All parameters were tested for normal distribution. Values not normally distributed were log transformed before statistical analysis.

## Results

### Effects of Parenteral Treatments on DOX-Induced Cardiotoxicity

**Clinical signs.** At the end of the study, there was no mortality in any of the groups, but 75% of rats in the D+C group (9 of 12) and 80% of rats in the D group (8 of 10) developed 1 or multiple clinical symptomatic signs of cardiac dysfunction or heart failure (ie, enlarged abdomen, ascites, and dyspnea). Only 33.3%, 58.3%, and 33.3% of rats, respectively, from the D+G (4 of 12), D+F (7 of 12), and D+G+F (4 of 12) groups developed 1 or more of these signs. However, due to the small sample size, neither comparison to vehicle group reached a level of significance via Kaplan-Meier analysis.

In the D+C group, there was substantial fluid retention due to cardiac dysfunction or heart failure, as suggested by a marked increase in the wet-to-dry weight ratio in the liver and lung. Single glutamine treatment (D+G) significantly attenuated lung and liver congestion ( $P < .05$ , Table 3), whereas single  $\omega$ -3 PUFA treatment (D+F) only partially reduced lung congestion and had no effect on liver congestion. Combined glutamine/ $\omega$ -3 PUFA treatment (D+G+F) significantly reduced lung congestion but only partially corrected the liver congestion.

**Cardiac function.** We next used echocardiography to determine cardiac function deterioration as a result of DOX chemotherapy, which is the most specific and predictable indication of the development of DOX-induced cardiotoxicity (DIC).

**Table 3.** Effects of Single and Combined Parenteral Treatments of Glutamine and  $\omega$ -3 Polyunsaturated Fatty Acids on Organ Congestion Related to Cardiac Dysfunction in Tumor-Bearing Rats Treated With Doxorubicin.

Organ, Wet-to-Dry Weight Ratio	REF	D	D+C	D+G	D+F	D+G+F
Liver	3.02 $\pm$ 0.03 <sup>a</sup>	3.58 $\pm$ 0.05 <sup>b</sup>	3.56 $\pm$ 0.05 <sup>b</sup>	3.10 $\pm$ 0.04 <sup>a</sup>	3.48 $\pm$ 0.06 <sup>b</sup>	3.32 $\pm$ 0.04 <sup>ab</sup>
Lung	4.12 $\pm$ 0.04 <sup>a</sup>	4.62 $\pm$ 0.04 <sup>b</sup>	4.68 $\pm$ 0.05 <sup>b</sup>	4.30 $\pm$ 0.04 <sup>a</sup>	4.39 $\pm$ 0.06 <sup>ab</sup>	4.28 $\pm$ 0.05 <sup>a</sup>

Data presented as mean  $\pm$  SEM. Means within a column not sharing a common letter are significantly different ( $P < .05$ ). n = 10–12. D, doxorubicin (DOX) alone without any parenteral intervention; D+C, DOX + parenteral control treatment; D+F, DOX + parenteral  $\omega$ -3 PUFA treatment; D+G, DOX + parenteral glutamine treatment; D+G+F, DOX + parenteral combined glutamine/ $\omega$ -3 PUFA treatment; REF, non-DOX-treated reference group receiving saline (sham to DOX); SEM, standard error of the mean.

**Table 4.** Effects of Single and Combined Parenteral Treatments of Glutamine and  $\omega$ -3 Polyunsaturated Fatty Acids on DOX-Induced Left Ventricular Dilation and Function Deterioration in Tumor-Bearing Rats Treated With DOX.

Echocardiographic Measures	REF	D	D+C	D+G	D+F	D+G+F
LVFS, %	55.5 $\pm$ 1.3 <sup>b</sup>	38.2 $\pm$ 1.1 <sup>a</sup>	36.6 $\pm$ 1.0 <sup>a</sup>	48.2 $\pm$ 1.5 <sup>b</sup>	42.5 $\pm$ 1.6 <sup>ab</sup>	46.6 $\pm$ 1.1 <sup>b</sup>
LVEF, %	78.2 $\pm$ 1.7 <sup>b</sup>	60.2 $\pm$ 1.5 <sup>a</sup>	58.6 $\pm$ 1.6 <sup>a</sup>	72.6 $\pm$ 2.0 <sup>b</sup>	66.3 $\pm$ 2.5 <sup>ab</sup>	69.0 $\pm$ 2.0 <sup>ab</sup>
LVEDD, mm	5.72 $\pm$ 0.12 <sup>a</sup>	6.76 $\pm$ 0.15 <sup>b</sup>	6.84 $\pm$ 0.15 <sup>b</sup>	6.12 $\pm$ 0.15 <sup>ab</sup>	6.38 $\pm$ 0.20 <sup>ab</sup>	6.26 $\pm$ 0.18 <sup>ab</sup>
LVEDS, mm	3.20 $\pm$ 0.08 <sup>a</sup>	5.28 $\pm$ 0.15 <sup>b</sup>	5.35 $\pm$ 0.13 <sup>b</sup>	3.98 $\pm$ 0.12 <sup>a</sup>	4.32 $\pm$ 0.16 <sup>ab</sup>	4.20 $\pm$ 0.12 <sup>ab</sup>

Echocardiography was performed in tumor-bearing rats treated with doxorubicin (DOX) at the end of study (day 50). Data are presented as mean  $\pm$  SEM. Means within a column not sharing a common letter are significantly different ( $P < .05$ ). n = 10–12. D, DOX alone without any parenteral intervention; D+C, DOX + parenteral control treatment; D+F, DOX + parenteral  $\omega$ -3 PUFA treatment; D+G, DOX + parenteral glutamine treatment; D+G+F, DOX + parenteral combined glutamine/ $\omega$ -3 PUFA treatment; LVEF, left ventricular ejection fraction; LVEDD, left ventricle end-diastolic diameter; LVEDS, left ventricle end-systolic diameter; LVFS, left ventricular fractional shortening; REF, non-DOX-treated reference group receiving saline (sham to DOX); SEM, standard error of the mean.

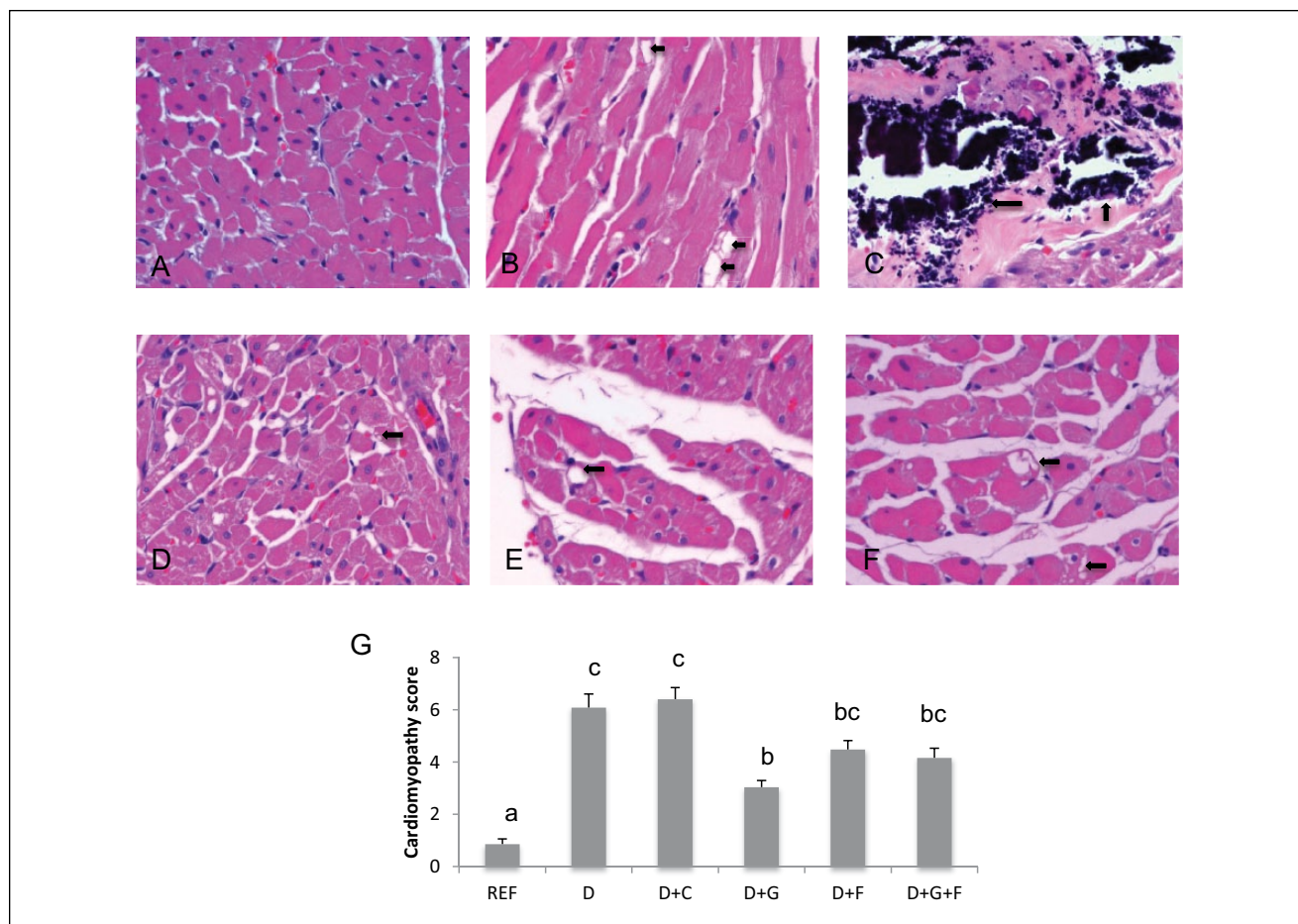
Rats in the D+C group developed a moderate dilation of the LV chamber, indicated by an increase in both LVEDD and LVEDS (day 50). LV performance was markedly compromised, as indicated by a substantial reduction in both LVFS (a 34.1% decline vs REF group,  $P < .05$ ) and LVEF (a 25.1% decline vs REF group,  $P < .01$ ). This LV dilation and performance decline were comparable with rats in the D group, suggesting parenteral manipulation per se did not affect cardiac function. Single glutamine treatment (D+G) significantly improved LVFS and LVEF and prevented LV dilation, whereas single  $\omega$ -3 PUFA treatment (D+F) only partially improved LV dilation and function, yet without any statistical significance achieved with the examined parameters. Combined glutamine/ $\omega$ -3 PUFA treatment (D+G+F) significantly improved LVFS and only partially improved the other parameters, which were, however, not significantly different from the means of the D+C group (Table 4).

**Morphological changes.** Rat myocardium from the REF group showed no pathological changes (Figure 1A). However, treatment with DOX, irrespective of parenteral manipulation, was characterized by prominent and diffuse vacuolization (Figure 1B,C). In contrast, only microvacuolar morphological changes were observed in a few isolated areas in the sections from D+G hearts (Figure 1D), whereas groups of cells with marked macrovacuolization were observed in areas of D+F and D+G+F sections (Figure 1E,F). The scores recorded for the myocardial lesions resulting from DOX administration are presented in Figure 1G. The D and D+C groups had the most severe

myocyte damage according to the recorded scores. However, the cardiomyopathy score of the D+G group was significantly lower than that of the D+C group ( $P = .01$ ), whereas the scores for the D+F and D+G+F groups were just intermediate and not significantly different from either D+C or D+G.

**Plasma troponin.** cTnI is a highly sensitive and specific marker of myocardial injury. DOX treatment, irrespective of parenteral manipulation, resulted in a striking increase in plasma cTnI levels. All 3 parenteral treatments—single glutamine (D+G), single  $\omega$ -3 PUFA (D+F), and combined glutamine/ $\omega$ -3 PUFA treatment (D+G+F)—mitigated the increase of plasma cTnI levels in a comparable manner (a 45.4%, 54.7%, and 53.9% decline compared with D+C, respectively, for D+G [ $P < .05$ ], D+F [ $P < .05$ ], and D+G+F [ $P < .01$ ], Figure 2).

**Cardiac redox homeostasis.** Increased oxidative stress plays an essential role in DIC.<sup>11</sup> We next investigated how the 3 parenteral interventions would modulate cardiac redox homeostasis in rats receiving DOX chemotherapy. DOX treatment, regardless of parenteral manipulation, resulted in a pronounced increase in lipid peroxidation with a concomitant decrease in antioxidant enzymatic reserve in the cardiac tissue. These changes were all normalized by single glutamine treatment (D+G), whereas single  $\omega$ -3 PUFA treatment (D+F) did not significantly affect cardiac lipid peroxidation or the cardiac antioxidant enzyme reserves. Combined treatment (D+G+F) was able to keep lipid peroxidation at a comparable low level as



**Figure 1.** Effects of single and combined parenteral treatments of glutamine and  $\omega$ -3 polyunsaturated fatty acids on heart morphological alterations in tumor-bearing rats treated with doxorubicin. (A–F) Representative light micrographs from REF, D, D+C, D+G, D+F, or D+G+F, respectively (original magnification  $\times 400$ ). In B, arrows indicate myocardial necrosis; in C–F, arrows indicate the vacuolization of the myocardium. (G) Cardiomyopathy scores recorded from all the treatment groups. Means within a column not sharing a common letter are significantly different ( $P < .05$ ).  $n = 10$ – $12$ . D, doxorubicin (DOX) alone without any parenteral intervention; D+C, DOX + parenteral control treatment; D+F, DOX + parenteral  $\omega$ -3 PUFA treatment; D+G, DOX + parenteral glutamine treatment; D+G+F, DOX + parenteral combined glutamine/ $\omega$ -3 PUFA treatment; REF, non-DOX-treated reference group receiving saline (sham to DOX).

single glutamine treatment (D+G) but only partially restored the antioxidant enzymatic stores, which were not significantly from the D+C group (Table 5).

Consistently, the rGSH/GSSG ratio was lowered by  $>40\%$  in host heart tissue irrespective of parenteral manipulation (D vs REF,  $P < .05$ ; D+C vs REF,  $P < .05$ ; Table 6). By contrast, this ratio was completely normalized with single glutamine treatment (D+G). Parenteral  $\omega$ -3 PUFAs, either alone or in combination with glutamine, were not able to maintain cardiac glutathione stores after DOX treatment.

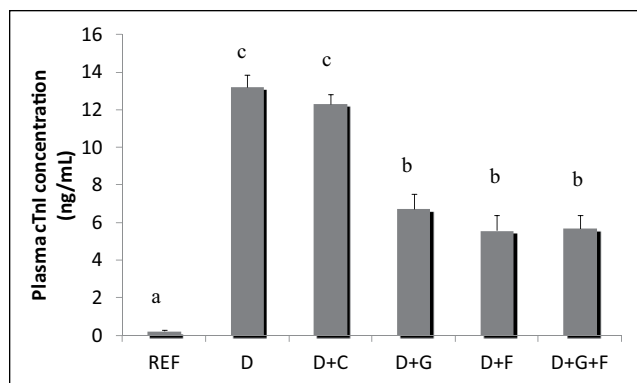
**Cardiomyocyte apoptosis.** In addition, only single glutamine treatment (D+G) seemed to alleviate the apoptosis of cardiomyocytes, as demonstrated by reduced cleaved caspase-3 and PARP (D+G vs D,  $P < .01$ ; D+G vs D+C,  $P < .01$ ; Figure 3). Parenteral  $\omega$ -3 PUFAs, either alone or in combination with

glutamine, was not able to mitigate DOX-induced cardiomyocyte apoptosis (Figure 3).

**Cardiac Hsp70 expression.** We next examined whether any of these parenteral treatments could modulate cardiac HSP expression in association with their different capacity in the modulation of DIC. Only single glutamine-supplemented (D+G) but not single  $\omega$ -3 PUFA treatment (D+F) enhanced cardiac Hsp70 expression. This effect, however, was lost when glutamine and  $\omega$ -3 PUFAs were supplemented in combination (Figure 3).

**Cardiac arginase activity.** Of particular interest to the heart tissue, availability of arginine is the key determinant for the production of nitric oxide (NO),<sup>58</sup> which plays a protective role in DOX-induced cardiomyopathy.<sup>59</sup> Arginase (Arg) plays a crucial role in determining the bioavailability of arginine for





**Figure 2.** Effects of single and combined parenteral treatments of glutamine and  $\omega$ -3 polyunsaturated fatty acids on plasma cardiac troponin I (cTnI) concentrations in tumor-bearing rats treated with doxorubicin. Data presented as mean  $\pm$  SEM. Means within a column not sharing a common letter are significantly different ( $P < .05$ ).  $n = 10$ – $12$ . D, doxorubicin (DOX) alone without any parenteral intervention; D+C, DOX + parenteral control treatment; D+F, DOX + parenteral  $\omega$ -3 PUFA treatment; D+G, DOX + parenteral glutamine treatment; D+G+F, DOX + parenteral combined glutamine/ $\omega$ -3 PUFA treatment; REF, non-DOX-treated reference group receiving saline (sham to DOX); SEM, standard error of the mean.

NO production.<sup>58</sup> Interestingly, our data show that only single  $\omega$ -3 PUFA treatment (D+F) inhibited cardiac arginase activity, which conceivably could lead to preserved arginine availability for NO production. Although not significantly different from other DOX-treated groups, glutamine/ $\omega$ -3 PUFA treatment resulted in an elevated Arg II activity compared with REF ( $P < .05$ ) (Figure 4).

### Effects of Parenteral Treatments on Antitumor Efficacy of DOX Chemotherapy

Concurrently with the evaluation of DIC, we examined whether single and combined parenteral treatments of glutamine and  $\omega$ -3 PUFAs altered DOX-induced antitumor efficacy in the

same animals. Parenteral manipulation alone did not affect the antitumor efficacy of DOX, as indicated by the identical tumor inhibition of D and D+C groups. Single  $\omega$ -3 PUFA treatment (D+F) significantly enhanced antitumor activity of DOX chemotherapy compared with D+C (2-way ANOVA,  $P < .005$ ). Single glutamine treatment (D+G) did not significantly change tumor inhibition kinetics but did lead to 26.3% more tumor inhibition at the end of study (D+G vs D+C,  $P < .05$ ). Intriguingly, the combined glutamine/ $\omega$ -3 PUFA treatment (D+G+F) did not affect DOX-related antitumor activity. On the contrary, it seemed to abrogate the potentiation of tumor inhibition by single  $\omega$ -3 PUFAs, and the tumor inhibition kinetics was identical to that of the D+C group (Figure 5).

**GSH stores in tumor tissue.** In tumor tissue (Table 6), single  $\omega$ -3 PUFA treatment aggravated the tumoral oxidative stress by augmenting the production of GSSG, which resulted in a decreased rGSH/GSSG ratio by 51% (vs D group,  $P < .05$ ). Single glutamine treatment (D+G) did not alter the tumoral glutathione store.  $\omega$ -3 PUFAs' capacity to intensify the tumoral oxidative stress, however, was partially lost when combined with glutamine (Table 6).

**Tumoral DOX accumulation.** The fluorescent properties of DOX were used to quantify DOX retention in tumor tissues. Single  $\omega$ -3 PUFA treatment (D+F) remarkably enhanced tumoral DOX concentration ( $P < .01$  vs D+C), whereas single glutamine treatment (D+G) did not significantly change the drug retention. Consistent with the effects on tumor inhibition, the combined treatment of glutamine and  $\omega$ -3 PUFAs (D+G+F) abolished the enhanced drug accumulation conferred by  $\omega$ -3 PUFAs alone (Figure 6).

## Discussion

Both single glutamine and  $\omega$ -3 PUFA treatments exhibit substantial selectivity in their effects on the host and tumor. The key for using a chemotherapy modulator is that the agent has to be substantially differential in its action toward the tumor and

**Table 5.** Effects of Single and Combined Parenteral Treatments of Glutamine and  $\omega$ -3 Polyunsaturated Fatty Acids on Lipid Peroxidation and Enzymatic Antioxidants in Cardiac Tissue of Tumor-Bearing Rats Treated With Doxorubicin.

Measures	REF	D	D+C	D+G	D+F	D+G+F
MDA	8.0 $\pm$ 0.5 <sup>a</sup>	12.2 $\pm$ 0.4 <sup>b</sup>	12.8 $\pm$ 0.5 <sup>b</sup>	8.6 $\pm$ 0.6 <sup>a</sup>	13.3 $\pm$ 0.7 <sup>b</sup>	8.5 $\pm$ 0.5 <sup>a</sup>
SOD	3.2 $\pm$ 0.2 <sup>b</sup>	2.0 $\pm$ 0.1 <sup>a</sup>	2.2 $\pm$ 0.1 <sup>a</sup>	3.0 $\pm$ 0.3 <sup>ab</sup>	2.1 $\pm$ 0.1 <sup>a</sup>	2.3 $\pm$ 0.2 <sup>ab</sup>
CAT	97 $\pm$ 5 <sup>b</sup>	72 $\pm$ 3 <sup>ab</sup>	68 $\pm$ 3 <sup>a</sup>	98 $\pm$ 6 <sup>b</sup>	80 $\pm$ 4 <sup>ab</sup>	86 $\pm$ 5 <sup>ab</sup>
GPx	0.29 $\pm$ 0.01 <sup>b</sup>	0.19 $\pm$ 0.01 <sup>a</sup>	0.19 $\pm$ 0.01 <sup>a</sup>	0.30 $\pm$ 0.02 <sup>b</sup>	0.18 $\pm$ 0.01 <sup>a</sup>	0.23 $\pm$ 0.03 <sup>ab</sup>

Data are presented as mean  $\pm$  SEM. Means within a column not sharing a common letter are significantly different ( $P < .05$ ).  $n = 10$ – $12$ . Cardiac lipid peroxidation and antioxidant enzymes were analyzed in the host heart and tumor tissue at the end of the study (day 50). MDA released is used as an index of lipid peroxidation, nmol formed per mg protein. CAT,  $\mu$ mol of  $H_2O_2$  consumed/min per mg protein; D, doxorubicin (DOX) alone without any parenteral intervention; D+C, DOX + parenteral control treatment; D+F, DOX + parenteral  $\omega$ -3 PUFA treatment; D+G, DOX + parenteral glutamine treatment; D+G+F, DOX + parenteral combined glutamine/ $\omega$ -3 PUFA treatment; GPx,  $\mu$ g of glutathione consumed/min per mg protein; MDA, malondialdehyde; REF, non-DOX-treated reference group receiving saline (sham to DOX); SEM, standard error of the mean, SOD, amount of enzyme required to give 50% inhibition of pyrogallol auto-oxidation.



**Table 6.** Effects of Single and Combined Parenteral Treatments of Glutamine and  $\omega$ -3 Polyunsaturated Fatty Acids on Glutathione Content in the Heart and Tumor Tissue of Tumor-Bearing Rats Treated With Doxorubicin.

Treatment	tGSH, $\mu\text{mol/g}$ Tissue	GSSG, $\mu\text{mol/g}$ Tissue	rGSH, $\mu\text{mol/g}$ Tissue	rGSH/GSSG
<b>Heart</b>				
REF	1.92 $\pm$ 0.18	0.038 $\pm$ 0.001 <sup>b</sup>	1.59 $\pm$ 0.17	66.6 $\pm$ 3.6 <sup>b</sup>
D	0.96 $\pm$ 0.12	0.033 $\pm$ 0.002 <sup>ab</sup>	0.95 $\pm$ 0.12	38.0 $\pm$ 2.9 <sup>a</sup>
D+C	0.98 $\pm$ 0.10	0.036 $\pm$ 0.003 <sup>ab</sup>	0.97 $\pm$ 0.12	36.6 $\pm$ 3.0 <sup>a</sup>
D+G	1.52 $\pm$ 0.12	0.025 $\pm$ 0.002 <sup>a</sup>	1.51 $\pm$ 0.17	68.2 $\pm$ 5.1 <sup>b</sup>
D+F	1.38 $\pm$ 0.15	0.036 $\pm$ 0.004 <sup>ab</sup>	1.25 $\pm$ 0.10	35.2 $\pm$ 4.0 <sup>a</sup>
D+G+F	1.50 $\pm$ 0.11	0.032 $\pm$ 0.005 <sup>ab</sup>	1.26 $\pm$ 0.11	45.6 $\pm$ 5.2 <sup>ab</sup>
<b>Tumor</b>				
D	1.09 $\pm$ 0.13	0.024 $\pm$ 0.003 <sup>ab</sup>	0.99 $\pm$ 0.12	45.8 $\pm$ 3.9 <sup>b</sup>
D+C	1.02 $\pm$ 0.07	0.025 $\pm$ 0.002 <sup>a</sup>	0.97 $\pm$ 0.10	43.6 $\pm$ 4.0 <sup>ab</sup>
D+G	1.26 $\pm$ 0.10	0.030 $\pm$ 0.003 <sup>ab</sup>	1.03 $\pm$ 0.09	46.2 $\pm$ 3.6 <sup>b</sup>
D+F	1.00 $\pm$ 0.09	0.043 $\pm$ 0.003 <sup>b</sup>	0.97 $\pm$ 0.12	22.5 $\pm$ 2.8 <sup>a</sup>
D+G+F	1.06 $\pm$ 0.12	0.036 $\pm$ 0.006 <sup>ab</sup>	1.05 $\pm$ 0.11	30.7 $\pm$ 5.0 <sup>ab</sup>

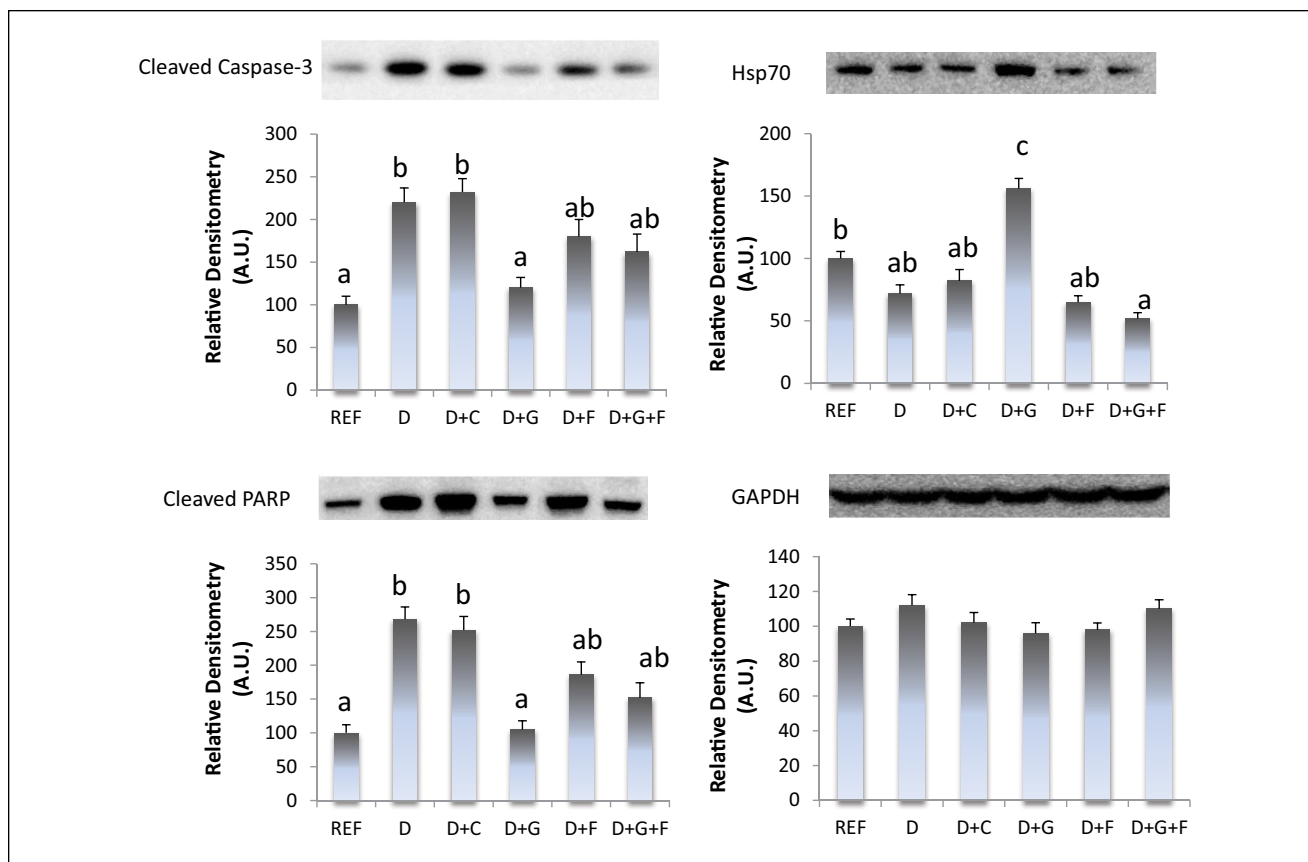
For both colonic mucosa and tumor tissues, means within a column not sharing a common letter are significantly different ( $P < .05$ ).  $n = 10-12$ .

GSH concentration was analyzed in the heart and tumor tissue at the end of the study (day 50). D, doxorubicin (DOX) alone without any parenteral intervention; D+C, DOX + parenteral control treatment; D+F, DOX + parenteral  $\omega$ -3 PUFA treatment; D+G, DOX + parenteral glutamine treatment; D+G+F, DOX + parenteral combined glutamine/ $\omega$ -3 PUFA treatment; GSSG, glutathione disulfide; REF, non-DOX-treated reference group receiving saline (sham to DOX); rGSH, reduced glutathione; SEM, standard error of the mean; tGSH, total glutathione.

the host. A toxicity antagonist confers protection to host tissues but does not supposedly protect the tumor or renders the tumor more chemoresistant, whereas with the use of a chemosensitizer, the potentiated cytotoxic effect should be confined to tumor tissue but does not extend to critical dose-limiting host tissues. In line with this concept, our data demonstrate an interesting differentiality or even dichotomy in the response of tumor and host tissue following DOX treatment to single parenteral glutamine and  $\omega$ -3 PUFA treatments.

Single glutamine treatment clearly protects against DIC with respect to the multileveled end points running the gamut from cardiac function to structural integrity to biochemical markers. Specifically, single glutamine treatment markedly improves host cardiac function, reduces plasma cTnI levels, preserves histological integrity, mitigates cardiomyocyte apoptosis, and lowers cardiac lipid peroxidation associated concomitant enhanced endogenous antioxidant defense. Our findings are supported by the studies from Todorova et al,<sup>20,41</sup> in which oral glutamine supplementation also improved cardiac function, reduced plasma cTnI levels, and preserved cardiac redox homeostasis in an acute DIC model in which mammary tumor-bearing rats received a single bolus dose of DOX at 12 mg/kg. However, of note, the functional, structural, and even transcriptional alterations observed in acute regimen models may not be applicable to patients in the clinic, who receive a considerably lower dose of DOX repeated over many weeks or months.<sup>60</sup> Our study demonstrated for the first time that glutamine is able to improve DIC in a chronic DIC model based on clinically resembling a human patient cyclic regimen, which may demonstrate a more translatable rationale for devising a clinical strategy.

Compared with glucose and fatty acids as metabolic fuels for the heart,<sup>61,62</sup> little attention has been devoted to understanding the role of amino acids as metabolic fuels in the heart, their impact on cardiac gene expression, and consequently potential cardioprotective effects against stress and injury. Emerging evidence suggests that glutamine serves as a significant substrate for energy in the heart.<sup>63,64</sup> The preservation by glutamine of myocardial high-energy phosphates and myocardial glutathione redox state probably could serve as a key mechanism underlying its cardioprotective effects in conditions such as ischemia/reperfusion injury.<sup>65,66</sup> Depletion of a reduced thiol pool plays a vital role in the pathogenesis of DIC, and repletion of glutathione demonstrated cardioprotection during DOX chemotherapy.<sup>67</sup> Our data show that glutamine maintained rGSH/GSSG redox potential during DOX chemotherapy, which is supported by previous work by Cao et al<sup>21</sup> and Todorova et al.<sup>20</sup> Intriguingly, for the first time, we demonstrated a mechanistic link between upregulation of HSP and cardioprotection in the setting of DIC. Associated with the preservation of cardiac functional and structural integrity, a strikingly increased cardiac Hsp70 expression was observed. Increased cardiac HSP expression has been shown to dampen oxidative injury by binding to damaged and misfolded polypeptides, mediating their refolding or degradation, and preventing denaturation and aggregation of intracellular proteins.<sup>68,69</sup> In addition to chaperoning the correct folding of other proteins, Hsp70 has emerged as an antiapoptotic protein by inhibiting caspase-dependent and caspase-independent apoptosis.<sup>70</sup> Our study reveals a potential mechanistic role of heat shock response upregulation in glutamine-mediated cardioprotection during DOX chemotherapy. An *hsp70* gene perturbation model (eg, *hsp70* knockout) would be an asset to



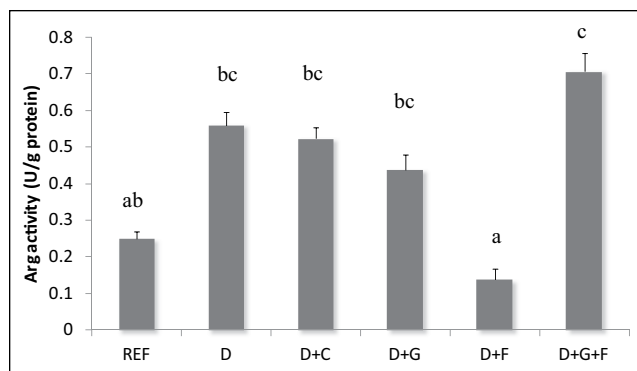
**Figure 3.** Representative Western blot showing effects of single and combined glutamine and  $\omega$ -3 polyunsaturated fatty acid treatments on activation of caspase-3 and poly (ADP-ribose) polymerase (PARP), as well as cardiac Hsp70 expression in the heart tissue of tumor-bearing rats treated with doxorubicin at day 50. Results of relative densitometry are expressed as means  $\pm$  SEM. Means that do not share a common symbol are significantly different ( $P < .05$ ).  $n = 10$ – $12$ . D, doxorubicin (DOX) alone without any parenteral intervention; D+C, DOX + parenteral control treatment; D+F, DOX + parenteral  $\omega$ -3 PUFA treatment; D+G, DOX + parenteral glutamine treatment; D+G+F, DOX + parenteral combined glutamine/ $\omega$ -3 PUFA treatment; REF, non-DOX-treated reference group receiving saline (sham to DOX); SEM, standard error of the mean.

delineate whether glutamine-mediated cardioprotection is Hsp70 dependent. Furthermore, given the safe profile of glutamine use in the oncologic clinical setting,<sup>71,72</sup> it is of significant interest to pursue the potential to develop glutamine as the first clinically relevant heat shock response enhancer used to modulate DIC in clinical care.

Despite these findings, there has been substantial debate regarding the use of glutamine as a standard therapy in oncologic therapy. This is based on the prevalent notion that the tumor is a “glutamine trap” and that exogenous glutamine supply may potentially stimulate tumor growth.<sup>73,74</sup> However, multiple lines of evidence, both in animal studies and in clinical trials, demonstrate that glutamine supply does not promote tumor growth or make the tumor more resistant to chemotherapy.<sup>75–77</sup> Response to chemotherapy, survival, tumor size, or tumoral protein synthesis is not affected by glutamine treatment.<sup>75–77</sup> Glutamine supplementation may conversely exert inhibitory effects on tumor growth.<sup>45,46</sup> Herein, we demonstrate

a striking dichotomy in glutamine’s action toward tumor vs host, which is echoed by our previous findings in a colon tumor model treated with irinotecan chemotherapy.<sup>19,24</sup> Glutamine treatment alone remarkably preserved host cardiac functional and structural integrity in association with preserved redox homeostasis and enhanced cardiac heat shock response while not affecting tumor inhibition kinetics related to DOX chemotherapy or GSH/GSSG redox potential in the tumor tissue. Studies on mechanisms responsible for the observed differential effects in glutamine’s action are warranted. Intrinsic properties in handling exogenous glutamine supply (ie, glutamine uptake and nutrient penetrance) by tumors could contribute to this differential action.<sup>24,47,78,79</sup> In addition, glutamine has been shown to modulate the host’s antitumor immunity by suppressing production of PGE<sub>2</sub> and enhancing natural kill cell activity,<sup>46,80</sup> which could also be an intriguing potential mechanism.

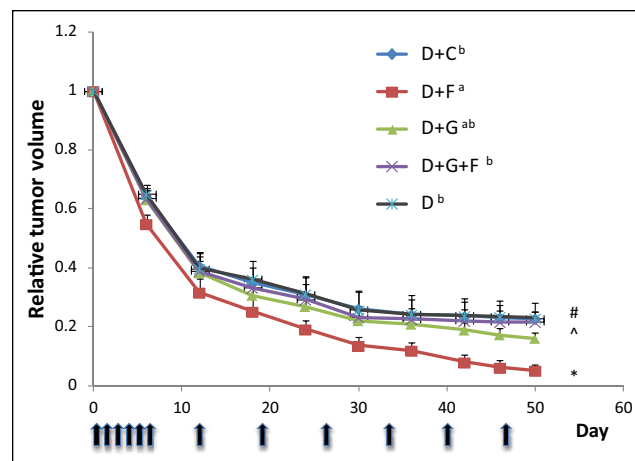
$\omega$ -3 PUFAs have emerged as an anticarcinogenic nutrient with independent chemopreventive effects by inhibiting tumor



**Figure 4.** Effects of single and combined parenteral treatments of glutamine and  $\omega$ -3 polyunsaturated fatty acids on cardiac arginase activity in tumor-bearing rats treated with doxorubicin. Results of relative densitometry are expressed as means  $\pm$  SEM,  $n = 4$ . Means that do not share a common symbol are significantly different ( $P < .05$ ).  $n = 10$ –12. D, doxorubicin (DOX) alone without any parenteral intervention; D+C, DOX + parenteral control treatment; D+F, DOX + parenteral  $\omega$ -3 PUFA treatment; D+G, DOX + parenteral glutamine treatment; D+G+F, DOX + parenteral combined glutamine/ $\omega$ -3 PUFA treatment; REF, non-DOX-treated reference group receiving saline (sham to DOX); SEM, standard error of the mean.

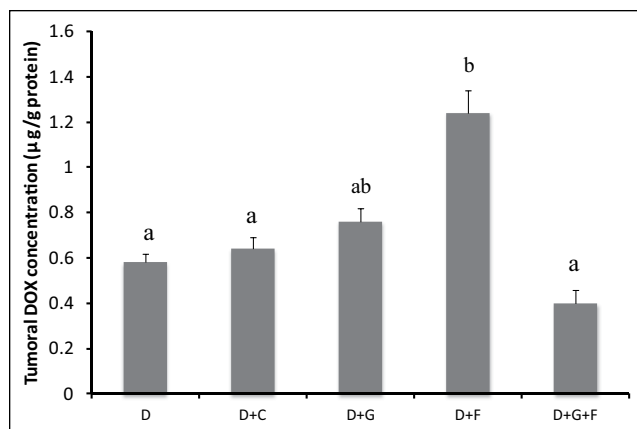
initiation and progression.<sup>81,82</sup> In addition, increasing evidence shows that  $\omega$ -3 PUFAs can potentiate the antitumor efficacy of anticancer drugs when they are used as an adjunct to a variety of chemotherapies, including anthracyclines, platinum-based agents, alkylating agents, tamoxifen, bleomycin, gemcitabine, CPT-11, and 5-FU in both clinical trials and animal studies.<sup>16,19,30,31,83–85</sup> In our study,  $\omega$ -3 PUFA treatment alone clearly enhanced the antitumor efficacy of DOX chemotherapy in mammary tumor-bearing rats, which was associated with aggravated oxidative stress in the tumor tissue. This suggests a prominent mechanism by which  $\omega$ -3 PUFAs increase the degree of unsaturation of tumor lipids and therefore increase the tumor's susceptibility to lipid peroxidation and pro-oxidant chemotherapy such as DOX.<sup>86,87</sup> Interestingly, we further demonstrate that single  $\omega$ -3 PUFA treatment markedly enhanced the DOX accumulation in tumor tissue. This finding sheds insights into additional mechanistic modes of action by which  $\omega$ -3 PUFAs sensitize tumors to anthracyclines. Further studies are needed to elucidate whether  $\omega$ -3 PUFAs are able to facilitate cancer cells to overcome drug resistance by increasing drug uptake<sup>88</sup> and/or reducing drug efflux by downregulating expression or activity of multidrug resistance-related proteins such as P-glycoprotein.<sup>89</sup> Alterations of membrane properties associated with  $\omega$ -3 fatty acid incorporation may critically affect drug transport in tumor cells, which has been suggested to be correlated with the unsaturation index in membrane phospholipids.<sup>87</sup>

The utility of  $\omega$ -3 PUFAs in anthracycline chemotherapy settings is challenged due to the concern that  $\omega$ -3 PUFAs incorporated into the critical dose-limiting tissues such as the heart



**Figure 5.** Effects of single and combined parenteral treatments of glutamine and  $\omega$ -3 polyunsaturated fatty acids on antitumor efficacy of doxorubicin (DOX) chemotherapy. DOX chemotherapy was initiated when rats of all treatment groups had tumors of approximately 1.2 cm<sup>3</sup> in volume. The y-axis represents the relative tumor volume compared with the baseline volume when the chemotherapy was initiated. The x-axis represents time points after initiation of chemotherapy. Each  $\uparrow$  indicates a single DOX injection at 1 mg/kg. Values are means with standard errors represented by vertical bars. In the legend of each figure, treatments with unlike superscript letters are significantly different ( $P < .05$ , post hoc Tukey's). Mean tumor values were compared at the end of the study between treatments, and means that do not share a common symbol are significantly different ( $P < .05$ , Bonferroni posttest). D, DOX alone without any parenteral intervention; D+C, DOX + parenteral control treatment; D+F, DOX + parenteral  $\omega$ -3 PUFA treatment; D+G, DOX + parenteral glutamine treatment; D+G+F, DOX + parenteral combined glutamine/ $\omega$ -3 PUFA treatment; REF, non-DOX-treated reference group receiving saline (sham to DOX); SEM, standard error of the mean.

will equally render normal tissue more susceptible to oxidative injury related to anthracycline chemotherapy.<sup>34</sup> Here in this study, single  $\omega$ -3 PUFA treatment did not further aggravate the cardiac peroxidation or further compromise antioxidant defense in the heart compared with the control treatment. Although incorporation of  $\omega$ -3 PUFAs into the plasma membrane may make the cardiomyocytes more predisposed to oxidation, the anti-inflammatory properties of  $\omega$ -3 PUFAs via reduction of the production of proinflammatory mediators (eg,  $\omega$ -6 series eicosanoids, tumor necrosis factor- $\alpha$ , and interleukin-6)<sup>90,91</sup> may result in an attenuated respiratory burst of immune cells and generation of reactive oxygen and nitrogen species.<sup>92</sup> Notably, single  $\omega$ -3 PUFA treatment significantly reduced the plasma cTnI levels and showed a trend for better persevered LV function and cardiac morphology. Availability of arginine is the key determinant for the production of nitric oxide,<sup>59</sup> which plays a vital cardioprotective role via modulating a diverse gamut of myocardial physiological functions<sup>93</sup>



**Figure 6.** Effects of single and combined parenteral treatments of glutamine and  $\omega$ -3 polyunsaturated fatty acids on tumoral doxorubicin (DOX) concentration in tumor-bearing rats treated with DOX. Data are presented as mean  $\pm$  SEM. Means within a column not sharing a common letter are significantly different ( $P < .05$ ). n = 10–12. D, DOX alone without any parenteral intervention; D+C, DOX + parenteral control treatment; D+F, DOX + parenteral  $\omega$ -3 PUFA treatment; D+G, DOX + parenteral glutamine treatment; D+G+F, DOX + parenteral combined glutamine/ $\omega$ -3 PUFA treatment; REF, non-DOX-treated reference group receiving saline (sham to DOX); SEM, standard error of the mean.

and, particularly in the context of DOX chemotherapy, exerts antioxidant-like actions in the lipid bilayers.<sup>60</sup> Arg is key in determining the bioavailability of arginine for NO production,<sup>59</sup> and furthermore, Arg II has been reported to reciprocally regulate the activity of NO synthase (NOS) I activity in the cardiomyocytes.<sup>94</sup> Herein, we demonstrate that single  $\omega$ -3 PUFA treatment inhibited cardiac arginase activity, which could conceivably modulate NO production by regulating arginine availability and/or NOS activity. This is supported by previous findings by Bansal et al<sup>95</sup> showing that  $\omega$ -3 PUFAs can act as an inhibitor for arginase via production of 3-series prostaglandins, which further lead to a preserved arginine availability for NO production.

Of note, there is a large and disparate candidate list of “good” nutrients that may potentially provide therapeutic benefits in the milieu of a cancer-host-chemotherapy interaction. This is the first direct comparative study of glutamine and  $\omega$ -3 PUFAs in a defined and standardized model featuring anthracycline chemotherapy and breast cancer. To closely recapitulate a patient-oriented clinical scenario, we evaluated the antitumor activity and cardiotoxicity of anthracycline treatment simultaneously in the same model. Single glutamine treatment clearly protects against DIC with respect to the examined end points, whereas single  $\omega$ -3 PUFAs render the tumors more responsive to anthracycline chemotherapy. Nonetheless, it cannot be completely excluded that the observed changes in the study groups are ascribed to a decrease

or lack of component nutrients in the control solutions (ie, amino acids other than glutamine in Aminosyn and long-chain triglycerides in Intralipid), since these nutrients may not be totally neutral themselves.

Overall, our data demonstrate an intriguing differentiality or even dichotomy in the response of tumor and host to single parenteral glutamine and  $\omega$ -3 PUFA treatments. Hence, these 2 individual nutrients show therapeutic promise to be used as an adjunct to DOX chemotherapy as a cardiotoxicity antagonist or chemosensitizer, which can ultimately modulate the balance between tumor and host in a manner that favors the host.

### *Interaction Between Glutamine and $\omega$ -3 PUFAs Seems to Be Negative Rather Than Positive When They Are Combined*

One of the most controversial aspects of immunonutrition formulas is that they usually combine a number of distinct nutrients at the same time based on a prevalent assumption that additivity or even synergism would be achieved by combining different nutrients that are beneficial on an individual basis. The clinical benefits of these individual components have been studied in a relatively extensive manner, yet information regarding potential synergistic or antagonistic interactions between these elements is quite limited. In this study, the combination of these 2 nutrients did not result in a greater or additive benefit in cardiac protection. Of note, these 2 nutrients seem to be antagonistic on a number of end points examined, such as cardiac function and remodeling measured by echocardiography, morphological integrity, cardiac antioxidant enzymatic reserves and rGSH/GSSG redox potential, Hsp70 expression, and cardiac Arg activity. In terms of the tumor’s response to chemotherapy, a clear antagonism has been observed when they are combined. Given the net effects on tumor and heart, our data do not support the idea of achieving greater benefits through combining them into one formula in the DOX chemotherapy setting.

Although information about the therapeutic potential of combining 2 or more factors is limited, of note, it has been suggested that mixtures of nutrients of the same class or different classes may turn out to be antagonistic rather than additive or synergistic in their actions.<sup>19,96</sup> Therapeutic effects of these combined nutrients are dose and end point dependent and may also rely on severity of the illness, timing, and duration.<sup>97–99</sup>

Mechanisms underlying the observed antagonistic interaction between glutamine and  $\omega$ -3 PUFAs are not known. Of particular note,  $\omega$ -3 PUFAs are potent activators of peroxisome proliferator-activated receptors (PPARs).<sup>100,101</sup> Activation of PPAR  $\alpha$ ,  $\gamma$ , and  $\beta/\delta$  transactivating transcription of key genes involved in cardiac lipid metabolism and may prevent the depression in fatty acid oxidation, which commonly occurs in advanced heart failure.<sup>102,103</sup> Furthermore, activating PPARs can promote growth inhibition, apoptosis, and differentiation of

a diverse array of tumor cells, including breast cancer cells.<sup>104,105</sup> Therefore, activation of the PPAR pathway may potentially have a dual benefit in both cardioprotection and promoting tumor inhibition via supplementing  $\omega$ -3 PUFAs. However, PPARs such as PPAR- $\gamma$  are subjected to regulation by O-linked glycosylation (O-GlcNAc) modification. Transactivation activity of PPAR is downregulated by O-GlcNAc.<sup>106</sup> The O-GlcNAc pathway is known to depend on glutamine as a rate-limiting substrate, and glutamine supplementation has been shown to upregulate the O-GlcNAc modifications of proteins in rat heart both in vitro and ex vivo.<sup>107,108</sup> Thus, by upregulating O-GlcNAc and suppressing PPAR activity, glutamine supplementation may conceivably compromise the potential benefits related to activation of the PPAR pathway by  $\omega$ -3 PUFAs.

Our study draws into question the common assumption that there are additive or synergistic benefits of combinations of nutrients, which are beneficial on an individual basis. Given that patients with cancer receiving chemotherapy are a targeted population for immunonutrition treatment, further efforts are surely warranted to understand potential nutrient-nutrient interactions between but possibly not limited to glutamine and  $\omega$ -3 PUFAs, as well as their role in the complex context of host-tumor-drug interactions.

### Statement of Authorship

H. Xue contributed to the conception and design of the research; M. Denking, E. Schlotzer, and P. E. Wischmeyer contributed to design of the research; H. Xue contributed to the acquisition, analysis, and interpretation of the data; W. Ren contributed to the acquisition and analysis of the data; H. Xue drafted the manuscript; and W. Ren, M. Denking, E. Schlotzer, and P. E. Wischmeyer critically revised the manuscript. All authors agree to be fully accountable for ensuring the integrity and accuracy of the work and read and approved the final manuscript.

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