Biomarkers, 2009; 14(5): 321–325

informa
healthcare

ORIGINAL ARTICLE

Markers of oxidative status in a clinical model of oxidative assault: a pilot study in human blood following doxorubicin administration

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Abstract

We used doxorubicin-based chemotherapy as a clinical model for oxidative assault. Study recruited 23 breast cancer patients and collected blood samples before (T0), at 1 (T1) and 24 hours (T24) after treatment administration. Measurements included protein carbonyl content (PPCC), malondialdehyde (MDA), and α- and γ-tocopherols in plasma and total glutathione content in erythrocytes (erGSHt). In all subjects, PPCC and MDA levels did not change. erGSHt levels increased at T24 by 8% (p=0.03). Levels of α, γ, and total tocopherols progressively decreased by 7%-15% (p <0.05). In subjects with low erGSHt levels (below median), PPCC mean levels progressively increased from 0.35 (T0) to 0.56 (T1) and 0.72 nmol carbonyl/mg protein (T24) (p =0.2). These results indicate that (1) plasma MDA is not a sensitive biomarker in humans; (2) PPCC potentially may be used, if antioxidant reserves are taken into account; (3) antioxidant reserves play an important role in the reaction to oxidative stress.

Keywords: *Epidemiology; oxidative stress; chemotherapy*

Introduction

Although the concept that oxidative damage promotes multiple chronic conditions is well-accepted, the dispute on how to measure oxidative status in humans has not been resolved (Halliwell and Gutteridge 2007). There is, however, consensus that existing and newly developed indices of oxidative status should be validated against known oxidative insults in animal models and human studies (Halliwell and Whiteman 2004, Kadiiska et al. 2000). In response to this well-recognized need, the National Institute of Environmental Health Sciences (NIEHS) has established an initiative to conduct a comparative study of biomarkers of oxidative stress (BOSS). The BOSS project tests responsiveness and specificity of the commonly used oxidative indices in an established model of oxidative stress – carbon tetrachloride (CCl_4) poisoning in rodents. To our knowledge, analogous examination of oxidative indices in response to a known oxidative assault in humans has not been published.

An analogous study in humans is imperative because animal models have serious limitations. Studies in animals use high levels of exposure to oxidative insults that in humans are rarely (if ever) observed. Compared to laboratory animal strains, human populations have greater variation in oxidative status (Block et al. 2002) which confounds assessment of the individual response to oxidative or antioxidant intervention (Block et al. 2008). For example, humans may have higher upper tolerance levels to oxidative assaults. Due to these shortcomings, biomarkers identified as responsive in animals may not be responsive in humans.

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(Received 29 December 2008; accepted 03 April 2009)

ISSN 1354-750X print/ISSN 1366-5804 online © 2009 Informa UK Ltd
DOI: 10.1080/13547500902946757

Although very important, conducting a study like BOSS in humans is problematic for several reasons. First and most important, subjecting humans to oxidative assault for research purposes is unethical. Second, it is difficult to find a population that is subjected to a well-controlled oxidative exposure. Both of these barriers, however, can be overcome by using pharmacological intervention with a known oxidative stressor as a model. One example is doxorubicin (DOX)-based chemotherapy, in which an exact dose is given to each patient. DOX belongs to the anthracyclines, a class of effective chemotherapeutic agents (Minotti et al. 2004, Weiss 1992). The chemical structure of anthracyclines, specifically the quinone moiety, allows participation in one-electron redox cycling, which results in production of reactive oxygen species (ROS) (Minotti et al. 2004). Animal studies confirmed that pharmacological levels of DOX (1 μM) generate hydroxyl radicals; this has been demonstrated by direct measurement with electron spin resonance spectroscopy (Rajagopalan et al. 1988). In clinical studies, anthracycline-based chemotherapy was shown to induce DNA oxidative damage in blood peripheral leukocytes (Doroshow et al. 2001, Olinski et al. 1997). Thus, DOX generates ROS both at pharmacological levels and under clinically-relevant conditions.

We used DOX-based chemotherapy in breast cancer patients as a clinical model of oxidative assault. We hypothesized that in response to DOX injection (a) indices of oxidative damage will increase; whereas (b) indices of antioxidant capacity will decline reflecting reduced antioxidant potential. To test these hypotheses, we measured (a) two oxidative markers, plasma protein carbonyl content (PPCC) and malondialdehyde-like products (MDA), and (b) four antioxidant capacity indices: total glutathione content in erythrocytes (erGSHt) and plasma α-tocopherol, γ-tocopherol, and δ-tocopherol; in addition, we computed total tocopherol levels by summing the individual tocopherols.

Methods

Study subjects

The study recruited women with newly diagnosed breast cancer scheduled to undergo standard chemotherapy (DOX 60 mg/m^2 / Cyclophosphamide 600 mg/m^2 × 4). The eligibility criteria were the following: (1) histologically confirmed invasive breast cancer, (2) no evidence of metastasis, (3) age ≥18 years, (4) ≥ 2 weeks since surgery, (5) adequate bone marrow, hepatic and renal functions, and (6) ability to give informed consent. The exclusion criteria were the following: (1) other anticancer concomitant medications known to cause myelosupression, especially neutropenia and neuropathy, (2) Eastern Cooperative Oncology Group functional status > 2, (3) serious co-morbidities (poorly controlled diabetes mellitus, ischaemic heart disease, uncontrolled hypertension, active infection), (4) pregnancy, and (5) prior treatment with weekly paclitaxel. The study protocol was approved by the Duke University Medical Center Institutional Review Board.

Blood samples

Venous blood was drawn from the participants at three time points: immediately before treatment (T0), and after treatment at 1 hour (T1) and 24 hours (T24). Blood was collected in 6ml EDTA vacutainers preloaded with butylated hydroxytoluene (Sigma) at 5 mM final concentration to prevent auto-oxidation. Samples were centrifuged under refrigeration (10 min, 1300 × g). Plasma and erythrocytes were then separated and frozen at –70ºC.

Measurement of maximal circulating concentration of DOX

DOX in plasma (μg/ml) was measured by high performance liquid chromatography (HPLC) with fluorescence detection using daunorubicin as an internal standard. Mobile Phase A was 16 mM ammonium formate in H_2O (pH 3.5): iPr-OH=95:5, and mobile phase B was acetone. Samples were eluted isocratically (87% A, 13% B, at 1.5 ml/min), on a Luna C18(2) 100 Å, 150 × 3.00 mm column (Phenomenex) at 50°C. The calibration curve was linear from 0.04 to 1 μg/ml.

Plasma protein carbonyl content

Plasma protein carbonyl content (PPCC, nmol carbonyl/mg protein) was determined by absorbance of color product obtained by derivatization with 2,4-dinitrophenylhydrazine (Brady's Reagent) using a Protein Carbonyl Assay Kit (Cayman Chemical) (Reznick and Packer 1994). Absorbance was read at 370 nm. Protein concentration was determined by comparing absorbance at 280 nm to a standard bovine serum albumin (Sigma) curve.

Total erythrocyte glutathione content

Total glutathione content in erythrocytes (erGSHt, nmol/mgHb) was determined by a fluorescence-based microplate assay using 2,3-naphthalenedicarboxyaldehyde derivatization (White et al. 2003). A standard curve of glutathione (GSH, Sigma) from 0.025 mM to 0.75 mM was prepared in 5% 5-sulfosalicylic acid (Ricca Chemicals). Fluorescence was read at λ_{Ex}470 and λ_{Em}530. Hemoglobin concentration was determined in erythrocyte samples using the Hemoglobin Assay Kit (BioAssay Systems).

Plasma malondialdehyde

All samples (including calibrating and quality control standards) were pre-treated with butylated hydroxytoluene (Sigma). Plasma lipoperoxides were hydrolyzed by boiling plasma in phosphoric acid (1.22 M). MDA, a hydrolysis product, reacts with thiobarbituric acid (TBA, 44 mM, Sigma) to form an MDA-TBA colored product. MDA-TBA adducts were quantified (μM) by HPLC with a UV-1000 detector monitoring 532 nm. The mobile phase was 20% acetonitrile in sodium phosphate buffer pH 7.0 (80 mM). We used a Luna 5 μ C18(2) 100 Å, 250 × 4.6 mm (Phenomenex) protected with a Phenomenex SecurityGuard C18, 4 × 3.0 mm column, flow rate 1 ml/min. The entire procedure was conducted at room temperature. For the calibration curve, 18.27 mM MDA stock solution was prepared by dissolving 1-1, 3,3 Tetramethoxypropane (Sigma) in distilled water; pH was adjusted to 2–3 with 0.5% sulfuric acid. For working standards (0.138 to 9.02 μM), the stock solution was diluted with 0.5 % sulfuric acid. Fresh working standards were prepared daily.

Plasma tocopherols

Tocopherols α, γ, and δ were quantified using HPLC as previously described (Craft et al. 1988, Satomura et al. 1992). Quality control samples were analyzed and calibrated against α, γ, and δ tocopherols standards. Total tocopherol levels were calculated as the sum of α, γ, and δ tocopherol levels. Because in many subjects δ-tocopherol levels were below detectable, we used only the levels of α, γ, and total tocopherols in the analysis.

Other variables

Data on age, tumor stage, estrogen and progesterone receptor status, height, and weight were obtained from medical records. Body mass index (BMI) was calculated using the formula weight (kg)/height $(m)^2$. Data on supplement intake was collected using a questionnaire completed by participants at the time of recruitment.

Statistical analysis

We compared change in the mean levels of oxidative indices at the three time points using general linear models that allowed controlling for the covariance structure. Because adjustment for age and BMI did not change our estimates, our final models included only the time effect. Correlations between oxidative indices, the maximum dose of DOX, and subjects' characteristics were evaluated using the Pearson correlation coefficient (for continuous variables) and the Kruskal-Wallis test (for categorical variables).

Results

We enrolled 23 eligible women into the study. Most women were in the peri-menopausal age range, Caucasian, and had been diagnosed with breast cancer tumor stage I or II; six had aggressive tumors (ER/PR negative/negative) (Table 1). The participants were almost equally spread across the conventional obesity categories (Table 1). Approximately one third of the participants were taking antioxidants and/or vitamin and mineral complexes.

The baseline indices (presented in Table 2) did not correlate with each other, except for PPCC and erGSHt levels (r = 0.58, p<0.05). Among the characteristics in Table 1, age correlated positively with α-tocopherol and total tocopherols ($r = 0.55$ and $r = 0.50$, respectively, p<0.05); and BMI correlated positively with γ-tocopherol and total tocopherols ($r = 0.62$ and $r = 0.63$, respectively, p<0.05). Self-reported intake of supplements was associated with lower levels of erGSHt: means were 1.93 (SD=0.65) vs. 1.38 (SD=0.64) in participants who reported no vs. at least one type of vitamin/supplement intake, respectively (p=0.07).

DOX level peaked between 18 and 35 min after injection. Although the dose per body volume was standard, the maximal dose of DOX in plasma varied approximately 4-fold between the participants, from 1.29 to 5.15 μg/ml. The maximal dose did not correlate with any of the measured biomarkers at T1 or at T24.

The levels of PPCC and MDA did not change in samples collected at both T1 and T24 (Table 2). The anti-oxidative indices showed different trends. Whereas erGSHt increased by 8% from the baseline to T24, levels

Table 1. Study subjects.

Characteristic		Number
Age	18-39 (pre-menopausal)	4
	40-54 (peri-menopausal)	14
	55-63[a] (menopausal)	5
Race:	African-American	4
	Caucasian	19
BMI:	<25	8
	25-29.9	7
	≥30	8
Tumor Stage:	I	6
	II	13
	III	4
ER/PR status: [b]	Neg/Neg	6
	Neg/Pos	0
	Pos/Neg	5
	Pos/Pos	12
Use antioxidants and/or vitamin and mineral complexes		8

[a] The oldest patient in this study was 63 years old.

[b] Estrogen receptor/Progesterone receptor status, neg-negative, pos-positive.

Table 2. Oxidative indices before (T0), 1 hour (T1) and 24 hours (T24) after administration of treatment.

	Mean (SD) *N*			
Measurement	T0	T1	T24	p-value
PPCC, nmol carbonyl/mg protein	0.53 (0.65) *20*	0.52 (0.54) *20*	0.55 (0.81) *20*	
T0 vs. T1		*–0.01 (0.71)*		0.9
T0 vs. T24			*0.02 (0.85)*	0.9
MDA, μM	0.30 (0.10) *16*	0.27 (0.08) *15*	0.31 (0.08) *16*	
T0 vs. T1		–0.03 (0.06)		0.08
T0 vs. T24			*0.01 (0.05)*	0.4
erGSHt, nmol/mgHb	1.67 (0.68) *17*	1.63 (0.72) *17*	1.80 (0.66) *17*	
T0 vs. T1		*–0.04 (0.30)*		0.6
T0 vs. T24			***0.13 (0.22)***	0.01
α–Tocopherol, μg/ml	12.61 (3.63) 19	12.05 (4.53) 17	11.68 (3.30) 17	
T0 vs. T1		*–0.56 (1.88)*		0.1
T0 vs. T24			***–0.92 (1.83)***	0.008
γ-Tocopherol, μg/ml	2.59 (1.40) 19	2.43 (1.37) 19	2.20 (1.18) 19	
T0 vs. T1		*–0.16 (0.34)*		0.2
T0 vs. T24			***–0.40 (0.62)***	0.02
Total tocopherol, μg/ml [a]	15.36 (3.97) 19	14.63 (4.97) 19	14.02 (3.47) 19	
T0 vs. T1		***–0.73 (2.20)***		0.03
T0 vs. T24			***–1.34 (2.25)***	0.0004

[a] Total tocopherols include δ-tocopherol which was often below the detectable limit and therefore is not shown separately in the table.

of α, γ, and total tocopherols progressively decreased from the baseline with statistically significant differences at T24 (Table 2). In subjects with low erGSHt levels (below median), PPCC mean levels progressively increased from 0.35 (T0) to 0.56 (T1) and 0.72 nmol carbonyl/mg protein (T24) (p=0.2 for both comparisons). Similar analysis for MDA did not yield interpretable results (data not shown).

Discussion

The objective of this pilot study was to evaluate several indices of oxidative status in a chemotherapy-based clinical model of oxidative assault. As established by earlier studies, DOX promotes generation of ROS (Doroshow et al. 2001, Minotti et al. 2004, Olinski et al. 1997, Rajagopalan et al. 1988, Weiss 1992). However, we found that oxidative indices, MDA and PPCC, were unchanged either at 1 hour (T1) or at 24 hours (T24) after DOX injection (Table 2). Several explanations (not mutually exclusive) are plausible. First, the selected oxidative indices may not be sensitive to oxidative stress. In an animal model, PPCC was not sensitive to CCl_4 poisoning, a classical oxidative stress agent; but in the same model, plasma MDA was (Kadiiska et al. 2005). Thus, our data for PPCC agree with the animal model, but there is obvious discrepancy regarding MDA.

Second, the time window for detecting oxidative effects of DOX (24 hours) may be too short. This time window and the specific sampling time-points (1 and 24 hours) were selected based on the expected peak of DOX in plasma (20–30 minutes) and half-life of DOX and its metabolites in human body (17–28 hours) (Mross et al. 1988). Also, the overall 24-hour time-frame was suggested by the exercise studies; they demonstrated that after exercise oxidative indices increased and returned to basal levels within 24 hours (Hudson et al. 2008, Michailidis et al. 2007). These studies, however, suggested different optimal sampling time-points for MDA and PPCC as MDA and PPCC levels peaked at 1 and 4 hours after exercise, respectively. Thus, the 1 hour time-point in our study should be appropriate to detect increase in MDA levels; whereas for PPCC, we could have missed the peak. On the other hand, the direct comparison between our study and the exercise studies may or may not be valid, because the redox (reduction/oxidation) dynamics of exercise may differ from the dynamics of chemically-induced oxidative assault. Therefore, which time points are optimal for measuring oxidative indices after DOX injection remains unclear.

Third, lack of changes in oxidative markers could also be explained by a relatively lower oxidative stress induced by the clinical application of DOX as compared to oxidative stress produced by CCl_4 in animal studies. This is to be expected as the goal of DOX chemotherapy is to kill cancer cells at the lowest possible dose to minimize the risk of cardiac toxicity induced by oxidative stress (Swain et al. 2003). Future studies should consider longer follow-up to examine oxidative assault produced not only by a single but also by cumulative doses of DOX.

Fourth, lack of changes in oxidative markers may be explained by higher antioxidant protection in humans

compared to animals. In our study, individual antioxidant reserves were estimated by two parameters – endogenous antioxidant, i.e. total GSH in erythrocytes, and exogenous antioxidants, i.e. plasma tocopherols. In the CCl_4 animal model, total blood GSH increased 4-10 fold (Kadiiska et al. 2000). In agreement with the animal data, we observed an 8% increase in erGSHt at T24 (Table 2). Most likely, in both animal and clinical models, increase of total GSH presents a compensatory response. The difference in the magnitude of such response, perhaps, relates to both the specifics of the measurements (total blood vs. total erythrocyte GSH) and the strength of oxidative assault. Also likely, individuals with higher erGSHt levels at baseline were better protected against DOX-induced oxidative assault. Increase in PPCC among participants with low initial erGSHt levels supports this assumption, although this increase did not reach statistical significance, probably due to the low sample size. Unlike erGSHt, the levels of all tocopherols decreased (7–15%) at T24 (Table 2). Perhaps, tocopherols showed a different trend, because these are exogenous antioxidants and cannot be synthesized in human body in response to oxidative assault.

A limitation of this study is its small sample size. This precludes valid interpretation of several unexpected findings, for example, positive correlation between erGSHt and PPCC. Also unexpected were lower erGSHt levels in participants who reported vitamin/supplement intake. These correlations should be further explored in a subgroup analysis with a larger sample size, in which the relationships between antioxidants and oxidative indices examined within the subgroup that reposted no vitamin/supplement intake and compared to those among vitamin/supplement users. In addition, increase of PPCC levels in subjects with lower erGSHt is interesting and potentially important, but also needs confirmation in a subgroup analysis.

In summary, these results indicate that (1) plasma MDA is not a sensitive marker of oxidative stress in humans; (2) PPCC potentially may be used to measure the reaction to oxidative stress in humans, but only if antioxidant reserves are taken into account. Our data clearly demonstrate that evaluation of oxidative indices in animal models can not be directly extrapolated to humans. Human studies are more complex. One of the complexities is individual differences in antioxidant reserves that probably play an important role. Future studies should consider an expanded sampling time for measurements of oxidative indices and larger the sample size to allow subgroup analysis.

Acknowledgements

***Declaration of interest*:** This research was funded by Duke Comprehensive Cancer Center pilot study award and NIH SPORE Grant 5P50 CA68438. The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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