Published in final edited form as:
Cancer Epidemiol Biomarkers Prev. 2010 June ; 19(6): 1506–1510. doi:10.1158/1055-9965.EPI-10-0211.

Urinary Biomarkers of Oxidative Status in a Clinical Model of Oxidative Assault

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Abstract

Background—We used doxorubicin-based chemotherapy as a clinical model of oxidative assault in humans.

Methods—The study recruited newly diagnosed breast cancer patients (n=23). Urine samples were collected immediately before (T0), and at 1 (T1) and 24 hours (T24) after intravenous administration of treatment. Measurements included allantoin and the isoprostanes, iPF(2 alpha)-III, iPF(2 alpha)-VI, 8,12-iso-iPF(2 alpha)-VI along with the prostaglandin 2,3-dinor-iPF(2 alpha)-III, a metabolite of iPF(2 alpha)-III. All biomarkers were quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Results—In all subjects, the levels of the biomarkers increased at T1: allantoin by 22% ($p=0.06$), iPF(2 alpha)-III by 62% ($p<0.05$), iPF(2 alpha)-VI by 41% ($p<0.05$), 8,12-iso-iPF(2 alpha)-VI by 58% ($p<0.05$), 2,3-dinor-iPF(2 alpha)-III by 52% ($p<0.05$). At T24 the F2-isoprostanes returned to their baseline levels; the levels of allantoin continued to increase, although the T24-T0 difference was not statistically significant.

Conclusions—These results indicate that urinary F2-isoprostanes are valid biomarkers and allantoin is a promising biomarker of oxidative status in humans.

Impact—The levels of biomarkers change quickly in response to oxidative assault and can be used to monitor oxidative status in humans in response to treatments related either to generation of free radicals (chemotherapy and radiation therapy) or antioxidants (inborn metabolic diseases and Down syndrome).

Keywords

epidemiology; oxidative stress; oxidative status; chemotherapy; biomarker

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Disclosure of Potential Conflicts of Interest: No potential conflicts of interest were disclosed.

Introduction

There is consensus that existing and newly developed indices of oxidative status should be validated against known oxidative insults in animal models and in human studies (1,2). 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 the responsiveness of commonly used oxidative indices in an established model of oxidative stress – carbon tetrachloride (CCl_4) poisoning in rodents. We developed an analogous approach to validate commonly used oxidative indices in humans; specifically, we used doxorubicin (DOX)-based chemotherapy in breast cancer patients as a clinical model of oxidative assault.

DOX-based chemotherapy satisfies two major requirements for a model of oxidative assault. First, it is based on an established oxidative stressor, as generation of hydroxyl radicals at pharmacological levels of DOX (1 μM) has been demonstrated in animal studies by direct measurement with electron spin resonance spectroscopy (3,4). Second, DOX-based chemotherapy presents a well-controlled oxidative exposure with an exact dose given to each patient at a certain time, which allows for timed collection of biological samples.

We examined the responsiveness of several indices of oxidative damage and antioxidant defense measured in blood and urine. The findings related to the blood measurements are reported elsewhere (5). Here, we report our findings on urinary biomarkers of oxidative lipid modification – four F2-isoprostanes – and one oxidative product of uric acid – allantoin. Urinary F2-isoprostanes are well-studied indices of lipid peroxidation that have been validated by the BOSS project (6,7). Allantoin has recently emerged as a promising biomarker of oxidative status that is specific to humans (therefore, it can not be evaluated in animal models). Humans (as well as other hominoid primates and birds), lack urate oxidase, a peroxisomal enzyme that catalyzes the oxidation of uric acid to allantoin in most mammals (8). Uric acid, the terminal product of purine metabolism in humans, is a potent antioxidant and scavenger of reactive oxygen species (9). Allantoin in human bodily fluids is generated by non-enzymatic oxidation by free radicals. Gruber and colleagues (10) recently demonstrated in a human study that allantoin increases in nasal lavage fluid in response to ozone exposure. In this study, we evaluated responsiveness of allantoin to a systemic oxidative stressor.

Methods

Study Subjects

We recruited 23 women with newly diagnosed breast cancer scheduled to undergo standard chemotherapy (DOX 60 mg/m^2 and 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) more than 2 weeks since surgery, (5) adequate bone marrow, hepatic and renal function, and (6) ability to give informed consent. Exclusion criteria included concomitant anticancer medications with myelosupression effects, low functional status, serious co-morbidities, pregnancy, and prior treatment with weekly paclitaxel. The study protocol was approved by the Duke University Medical Center Institutional Review Board.

Urine Samples

Urine samples were collected from participants at three time points: immediately before treatment (T0), and after treatment at 1 hour (T1) and 24 hours (T24). Urine samples were stored at -80°C.

Urinary Creatinine

Creatinine was assayed by a fast ESI-MS/MS method as described previously (11).

Four Urinary F2-isoprostanes

Four isomers of F2-isoprostanes – iPF(2 alpha)-III, 2,3-dinor-iPF(2 alpha)-III, iPF(2 alpha)-VI, and 8,12-iso-iPF(2 alpha)-VI – were quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (12,13) on Shimadzu 20A series LC and Applied Biosystems API 4000 QTrap MS/MS instruments. Based on creatinine measurements, the urine samples were diluted to 0.65 mg/mL creatinine, and samples with creatinine levels equal to or below this value were analyzed without dilution. Sample preparation included addition of internal standards [iPF(2 alpha)-III-d4, 8,12-iso-iPF(2 alpha)-VI-d11, iPF(2 alpha)-VI-d4] and 10 μL 1M HCl; washing of samples (500 μL) with 1 mL hexane; extraction of the analytes by ethyl acetate/hexane mixture (3/1, v/v); evaporation of the liquid and resuspension of the residue in 150 μL of a mixture containing 70% mobile phase A (0.1% formic acid in water) and 30% methanol. The samples (100 μL) then were injected into the LC-MS/MS system. Two solid core C18 columns (Phenomenex Kinetex C18, 150 × 4.6 mm) in series were used to achieve chromatographic separation of the F2-isoprostane isomers. The mass spectrometer was operated in negative mode with the following MRM transitions (m/z): 353/193 [iPF(2 alpha)-III], 357/197 [iPF(2 alpha)-III-d4], 325/237 [2,3-dinor-iPF(2 alpha)-III], 353/115 [iPF(2 alpha)-VI and 8,12-iso-iPF(2 alpha)-VI], 364/115 [iPF(2 alpha)-VI-d11], and 357/115 [8,12-iso-iPF(2 alpha)-VI-d4]. Calibration samples covering the expected range of concentrations were prepared by adding pure material into pooled human urine, injected before and after the patient samples. Lower limits of quantification (LLOQ, >80 % accuracy) were 0.007, 0.34, 0.25, and 0.12 mg/mL for iPF(2 alpha)-III, 2,3-dinor-iPF(2 alpha)-III, iPF(2 alpha)-VI, and 8,12-iso-iPF(2 alpha)-VI, respectively.

Urinary allantoin

Allantoin was quantified using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) with an Acquity UPLC system and TQD triple quadrupole mass spectrometer equipped with an ESI source (Waters Corporation, Milford, MA). Allantoin (Sigma-Aldrich, St. Louis, MO) and DL-allantoin-5-^{13}C;1-^{15}N (C/D/N Isotopes, Quebec, Canada) were used as the standard and internal standard, respectively. Synthetic urine matrix (14) was used for preparation of samples and calibrants. Sample preparation included initial vortex-mixing and centrifugation (15,000g × 10 min); addition of internal standard (25 μL urine + 25 μL internal standard, 100 μM) and matrix (450 μL); and a second round of vortex-mixing and centrifugation. In addition, 5 μL of sample was injected onto an Acquity UPLCTM BEH HILIC, 1.7 μm, 2.1× 100 mm column (Waters Corporation, Milford, MA) heated to 40° C. Chromatographic separation was achieved by isocratic elution using 0.5% formic acid in acetonitrile: DI-H2O (95:5, v/v) as the mobile phase, with a flow rate of 200 μL/min. Allantoin and the internal standard were detected in positive ion mode, using the following MRM transitions (m/z): 159/116 and 159/61 as primary and secondary, respectively, for allantoin and 161/118 and 161/61 for the internal standard. The primary transitions were used to quantify the allantoin and the secondary transitions were used as qualifier ions. Allantoin calibrants covered concentrations from 1 to 500 μmol/L; LLOQ (accuracy > 80%) was 0.06 pmol.

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 us to control 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 the biomarkers and subjects' characteristics were evaluated using the Pearson correlation coefficient (for continuous variables) and the Kruskal-Wallis test (for categorical variables).

Results

The study population included women from 18 to 63 years of age, with 60% in the perimenopausal age range (40-54 years); participants were almost equally distributed across the conventional obesity categories (Table 1). Most women were Caucasian and had been diagnosed with a stage I or II breast cancer tumor; six had aggressive tumors (ER/PR negative/negative) (Table 1). Approximately one third of the participants reported taking antioxidants and/or vitamin and mineral complexes. None of the urinary biomarkers examined at baseline correlated with any of these patient characteristics.

The levels of all examined urinary biomarkers increased at 1 hour after the DOX injection: allantoin by 22% ($p=0.06$), iPF(2 alpha)-III by 62% ($p<0.05$), iPF(2 alpha)-VI by 41% ($p<0.05$), 8,12-iso-iPF(2 alpha)-VI by 58% ($p<0.05$), and 2,3-dinor-iPF(2 alpha)-III by 52% ($p<0.05$) (Table 2). The 1-hour changes in F2-isoprostane levels were statistically significant, while the 1-hour change in allantoin levels was borderline significant. At 24 hours after DOX injection, the F2-isoprostanes returned to their baseline levels, whereas the levels of allantoin continued increasing, although the difference between allantoin levels at T24 vs. T0 was not statistically significant.

Discussion

The objective of this study was to evaluate urinary indices of oxidative status in a chemotherapy-based clinical model of oxidative assault. We found that all five examined biomarkers increased at 1 hour (T1). At 24 hours (T24) after DOX injection, the F2-isoprostanes returned to their baseline levels, while allantoin levels continued to increase (Table 2). The statistically significant 1-hour increases in F2-isoprostanes were greater than the increase in allantoin; nonetheless, the change in allantoin was sizable (22%) and borderline significant ($p=0.06$). Although allantoin may be a less sensitive marker, this result points to its potential importance in diversifying the panel of oxidative indices.

Our study demonstrated that all five urinary measurements are sensitive markers of oxidative stress in humans. Our data on urinary F2-isoprostanes in humans are consistent with the results of the BOSS study using CCl_4 as an oxidative stressor in animals (6,7). Moreover, our results on allantoin are consistent with a study that tested the effect of 2-hour exposure to 0.2 ppm ozone in healthy volunteers (n=15) (10). The ozone study found increased allantoin levels in nasal lavage at 1 hour into the actual exposure and immediately after the 2 hours of exposure as compared to the pre-exposure levels.

The timeline of biomarker changes in our study and the ozone study are noteworthy (10). Both studies, carried out with humans rather than in animal models, showed an increase in oxidative indices at 1 hour after the exposure, for both systemic (DOX injection) and local (ozone exposure) oxidative stressors. In the ozone study, allantoin levels in nasal lavage had returned to pre-exposure levels at 6 hours after exposure (10). In our study, the urinary F2-isoprostanes returned to baseline levels by 24 hours after DOX injection. Although the timeline of our post-exposure measurements did not allow us to ascertain whether the decrease in these biomarkers

occurred earlier in the 24-hour interval, a consideration of DOX pharmacokinetics suggests that this is unlikely. The peak of DOX in plasma was expected and was seen in our study at 20-30 minutes after injection; however, the half-life of DOX and its metabolites in the human body has been estimated to be 17-28 hours (15). The long half-life of DOX suggests that oxidative stress should persist for 24 hours. In fact, dead cells in urine were seen in 24-hour samples practically invariably, but not in 1-hour urine samples.

Our study raises questions as to why the half-life of DOX and its metabolites and the cell-killing DOX effect do not correspond to the dynamics of the biomarkers with its sharp increase at 1 hour after injection. One plausible explanation is that the body's antioxidant defense response is quick, and biomarker levels in bodily fluids increase and decrease rapidly. Compared to this whole-body antioxidant defense reaction, damaged cells are slaughtered slowly due to the more drawn-out process of cytoxic effects. Obtaining direct evidence to support or disprove this assumption would require timed experiments either with cell cultures or using animal models. It should also be noted that many studies have used plasma levels and urinary levels of F2-isoprostanes interchangeably as biomarkers of oxidative status, despite evidence that they may differ. In short, it is possible that the increase and subsequent decrease in F2-isoprostane plasma levels may occur earlier as compared to what we observed measuring urinary excretion of F2-isoprostanes (16).

In conclusion, we validated four biomarkers of oxidative status in a clinical model of oxidative stress. Urinary F2-isoprostanes, specifically iPF(2 alpha)-III and its metabolite 2,3-dinor-iPF (2 alpha)-III as well as iPF(2 alpha)-VI and 8,12-iso-iPF(2 alpha)-VI, are sensitive indices of systemic oxidative stress in humans. In addition, our results indicate that allantoin is a promising biomarker. Although its response to systemic oxidative stress is not as pronounced as those observed in F2-isoprostanes, this biomarker can serve an important role in diversifying the panel of oxidative indices available for the assessment of oxidative status in humans.

Acknowledgments

We thank Dr. Kirk Kitchin (U.S. Environmental Protection Agency, USA) for his critical review of this work.

Grant Support: This research was funded by a Duke Comprehensive Cancer Center pilot study award, NIH SPORE Grant 5P50 CA68438 and Anna Merills' Fund for Down Syndrome Research Foundation grant.

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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

Measurement	Mean (SD) *N*			p-value
	T0	T1	T24	
Allantoin, mmol/mol creatinine	7.2 (3.7) 20	8.8(4.6) 19	9.27(7.9) 19	
T1 minus T0		1.6		0.06
T1 minus T24			2.1	0.3
iPF(2 alpha)-III, ng/mg creatinine	0.26(0.15) 19	0.42(0.18) 18	0.33(0.22) 18	
T1 minus T0		0.16		0.0004
T1 minus T24			0.07	0.2
2,3-dinor-iPF(2 alpha)-III, ng/mg creatinine	12.0(7.8) 19	18.2(11.8) 18	13.3(7.8) 18	
T1 minus T0		6.0		0.003
T1 minus T24			1.2	0.6
iPF(2 alpha)-VI, ng/mg creatinine	3.4(2.0) 19	4.8(2.3) 18	3.0(1.8) 18	
T1 minus T0		1.3		0.001
T1 minus T24			-0.3	0.6
8,12-iso-iPF(2 alpha)-VI, ng/mg creatinine	8.5(4.6) 19	13.4(5.7) 18	7.5(5.1) 18	
T1 minus T0		4.7		<0.0001
T1 minus T24			-1.1	0.4