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A bioactive probe for glutathione-dependent antioxidant capacity in breast cancer patients: Implications in measuring biological effects of arsenic compounds

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Abstract

Introduction—Glutathione, a major cellular non-protein thiol (NPSH), serves a central role in repairing damage induced by cancer drugs, pollutants and radiation and in the detoxification of several cancer chemotherapeutic drugs and toxins. Current methods measure glutathione levels only, which require cellular extraction, rather than the glutathione recycling dependent antioxidant activity in intact cells. Here, we present a novel method using a bioactive probe of the oxidative pentose phosphate cycle, termed the OxPhos™ test, to quantify glutathione recycling dependent antioxidant activity in whole blood and intact human and rodent cells without the need for the isolation and cytoplasm extraction of cells.

Methods—OxPhos™ test kit (Rockland Immunochemicals, USA), which uses hydroxyethyl disulfide (HEDS) as a probe for the oxidative pentose phosphate cycle, was used in these studies. The results with OxPhos™ test kit in human blood and intact cells were compared with total thiol and high pressure liquid chromatography/electrochemical detection of HEDS metabolism.

Results—The OxPhos™ test measured glutathione-dependent antioxidant activity both in intact human and rodent cells and breast cancer patient's blood with a better correlation coefficient and biological variability than the thiol assay. Additionally, human blood and mammalian cells treated with various arsenicals showed a concentration-dependent decrease in activity.

Discussion—The results demonstrate the application of this test for measuring the antioxidant capacity of blood and the effects of environmental pollutants/toxins. It opens up new avenues for an easy and reliable assessment of glutathione-dependent antioxidant capacity in various diseases

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Author contributions

I.S.A conceived the idea, designed the experiments, analyzed data and wrote the paper. J.L. and D.Z. performed all the blood experiments. P.A. J. and K.M.W. performed all the tissue culture experiments.

Competing financial interests

The authors declare no competing financial interests.

such as stroke, blood borne diseases, infection, cardiovascular disease and other oxidative stress related diseases and as a prognostic indicator of chemotherapy response and toxicity. The use of this approach in pharmacology/toxicology including screening drugs that improve the glutathione-dependent antioxidant capacity and not just the glutathione level is clinically relevant since mammalian cells require glutathione dependent pathways for antioxidant activity.

Keywords

OxPhosTM test; glutathione; antioxidant activity; blood; human cells; total thiol; arsenicals; toxins; glutathione recycling; biomarker

1. Introduction

Since the discovery of the importance of glutathione (GSH) and glutathione-dependent pathways in various diseases, xenobiotic detoxification and oxidative stress, several biochemical and high pressure liquid chromatography (HPLC) methods have been used to measure intracellular glutathione (Allen & Bradley, 2011; Ayene, et al., 2000; Jani, Ziogas, Angus, & Wright, 2012; King, Korolchuk, McGivan, & Suleiman, 2004; Melnyk, Pogribna, Pogribny, Hine, & James, 1999; Murias, Rachtan, & Jodynis-Liebert, 2005; Pocernich & Butterfield, 2012; Rao, et al., 1997; Ricketts, Minimair, Yates, & Klaus, 2011; Sekhar, et al., 2011; Wilkins, Kirchhof, Manning, Joseph, & Linseman, 2013). However, the presently available biochemical assays for GSH require preparation of extracts from cells (Ghanizadeh, et al., 2013; Gibson, Korade, & Shelton, 2012; Jenko, Karouna-Renier, & Hoffman, 2012; Lushchak, 2012; Potter, Trappetti, & Paton, 2012; Shungu, 2012; Wright, et al., 2013). We and others have demonstrated that HPLC with an electrochemical detector can detect GSH with better sensitivity than the biochemical assays but this too requires cellular extracts (Ayene, et al., 2000; Iguchi, et al., 2012; Raza & John, 2012). Further, these assays may also underestimate the level since GSH measured by biochemical assays may include loss of GSH during sample preparation. Although such methods have been used to measure GSH level and determine the mechanism of toxicity/side effects of drugs in tissue culture and in vivo models, there has not been a method available to determine glutathione function i.e. glutathione-dependent antioxidant capacity in intact cells. In particular, there has not been a rapid, reproducible and easy method developed for a blood based test for glutathione-dependent antioxidant capacity.

A series of recent studies have also indicated that the glutathione dependent antioxidant system consisting of glutathione-dependent biochemical pathways and enzymes involved in glutathione synthesis could be a better prognostic indicator of chemotherapy response and drug toxicity (Goekkurt, et al., 2006; Nock, et al., 2009; Stoehlmacher, et al., 2004; Tahara, et al., 2011; Yang, Ebbert, Sun, & Weinshilboum, 2006). These studies have used polymorphic genetic markers for the enzymes involved in glutathione dependent antioxidant system. Although polymorphic genetic markers that covers the glutathione dependent antioxidant system is likely to be a better prognostic indicator of toxicity and response than the glutathione level, this approach may require independent measurement of several of the genes that makes it complicated due to the difficulty in sample processing, measurement and data interpretation.

We have recently demonstrated in a series of publications that GSH recycling (GSH → GSSG (oxidized GSH) → GSH), which is dependent on several GSH dependent pathways, is required for the metabolic conversion of hydroxyethyl disulfide (HEDS) into mercaptoethanol (ME) (Ayene, Biaglow, Kachur, Stamato, & Koch, 2008; Ayene, et al., 2000; Ayene, et al., 2002; Biaglow, et al., 2003; Biaglow, et al., 2000; Biaglow, et al., 2006; Biaglow, et al., 1998; Li, Ayene, Ward, Dayanandam, & Ayene, 2009; Li, et al., 2013; Li,

Zhang, Ward, Prendergast, & Ayene, 2012; Tuttle, et al., 2007). Unlike all the previous assays that measured just the glutathione level, a functional assay for glutathione such as HEDS metabolism will measure the “GSH dependent antioxidant capacity” of cells, a process that requires “glutathione recycling” by oxidative pentose phosphate cycle (OPPC) in conjunction with other GSH dependent pathways (Ayene, et al., 2002; Biaglow, et al., 2003; Lushchak, 2012). Defect in GSH recycling due to damage to cytosolic pathways, oxidative stress, decreased GSH synthesis and/or loss of GSH by other mechanisms in cells is expected to affect the conversion of HEDS into ME (Ayene, et al., 2008; Ayene, et al., 2002; Biaglow, et al., 2003; Lushchak, 2012; Raza & John, 2012). This type of assay would be more appropriate in determining an individual’s antioxidant capacity and the efficacy of dietary supplements, new drugs and antioxidants since it can be used to identify those supplements that improve the overall glutathione-dependent antioxidant capacity of cells before and after an oxidative/toxic insult. Although we have demonstrated HEDS to ME bioconversion in cell culture using HPLC/EC detection and Ellman’s reagent, it is not clear whether a reliable HEDS based test could be developed to quantify glutathione function i.e. glutathione-dependent antioxidant capacity in whole blood and cells and its application in measuring environmental pollutants induced damage to GSH dependent antioxidant capacity (Ayene, et al., 2008; Ayene, et al., 2002; Biaglow, et al., 2003; Biaglow, et al., 2006; Lushchak, 2012; Raza & John, 2012).

In this report, we describe a test, termed the OxPhos™, that can accurately measure glutathione-dependent antioxidant capacity in whole blood and human and rodent cells. In addition to testing the sensitivity and consistency of this assay for measurement of glutathione recycling dependent antioxidant capacity in whole blood and human and rodent cells, we have also demonstrated the usefulness of this assay in ex vivo blood and in human cells in vitro by determining the biological effects of environmental pollutants such as arsenicals (Phenylarsine oxide (PAO), arsenic trioxide (As₂O₃), arsenate, arsenite) that are known to cause oxidative stress leading to their toxic, carcinogenic and mutagenic effects in humans and animals.

2. Methods

2.1. Cells

Chinese hamster ovary (CHO) cells (K1, E89, and A1A) were provided by Thomas D. Stamato (Lankenau Institute for Medical Research (LIMR), USA). Glucose -6-phosphate dehydrogenase (G6PD) deficient E89 clones without functional enzyme activity were derived from wild type CHO K1 cells after mild mutagenesis with ethyl methane sulfonate. Hamster G6PD cDNA (AF044676) inserted into pcDNA3.1 construct was transfected in E89 mutant cells by electroporation to generate normal phenotype. Histochemical staining for G6PD activity and Northern analysis identified a single A1A clone with G6PD activity similar to K1 cells. Human colon cancer cells (HCT116 and HT29) originally obtained from ATCC were provided by Dr. Cameron Koch (University of Pennsylvania). Cells were seeded in DMEM containing 15% FCS and 25mM HEPES in 60mm Nunc dishes or six-well plates on the day before the experiment.

2.2. Blood Collection

We have chosen to validate the new Oxphos™ test using blood from breast cancer patients since previous studies have indicated that polymorphic genetic markers for glutathione detoxification system correlated with cancer patient survival, toxicity and disease progression (Goekkurt, et al., 2006; Nock, et al., 2009; Stoehlmacher, et al., 2004; Tahara, et al., 2011; Yang, et al., 2006). As part of a breast cancer study approved by the local Institutional Review Board (IRB), one additional blood sample was collected in EDTA

coated purple top tube at each time the health status was assessed prior to and after each treatment by the oncologist. Blood stored on ice was immediately used for the analysis of whole blood.

2.3. Quantification of glutathione-dependent antioxidant capacity in rodent and human cells in vitro by OxPhos™ test

OxPhos™ test kit (Rockland Immunochemicals, USA), which is based on the HEDS metabolism, was used in these studies. Cells were plated at various densities in 6-wells plate with 1ml normal growth medium a day before the assay. OxPhos™ test reagents were prepared as per the instructions provided in the kit. Briefly, reagent 6 was prepared by mixing 25 µl of Reagent 1 into 1.2 ml Reagent 2 in a sterile, clean amber microfuge tube and vortexed for 30 seconds. Fifty µl of reagent 6 was added to each well, mixed gently and incubated for 2 hours at 37° C in a humidified CO₂ incubator. After 2 hours incubation with reagent 6, 500 µl of the medium from each well of the 6-well plate was transferred into a microfuge tube containing 500 µl reagent 3, vortexed gently for 20 seconds and centrifuged at 9,000 rpm in a microfuge at 4°C for 3 minutes. The supernatant was stored at 4° C for short term storage (2 days) or –20° C for long term storage (10 days). The supernatant was used for the OxPhos™ activity/glutathione-dependent antioxidant capacity by mixing 150 µl of the supernatant with 1200 µl of reagent 4 premixed with 150 µl of reagent 5 in glass tubes. These tubes were vortexed gently, left at room temperature for 3–5 minutes and the absorbance was read at 412 nm using a disposable plastic cuvette in a spectrophotometer. The blank (1350 µl of reagent 4 mixed with 150 µl of reagent 5) was subtracted from the samples and delta O.D. was multiplied with 1471 (dilution factor × extinction coefficient) to determine micromolar GSH-dependent antioxidant capacity.

2.4. Quantification of glutathione-dependent antioxidant capacity in rodent and human cells in vitro by OxPhos™ reagents and High Pressure liquid chromatography (HPLC)/ Electrochemical Detection (EC)

In order to demonstrate that the OxPhos™ test measures the glutathione recycling dependent antioxidant capacity in the metabolic conversion of HEDS into ME, we specifically quantified the ME released into the medium in these cells using HPLC/EC. Cells treated with and without OxPhos™ probe (reagent 6) as described above were cooled on ice. To quantify the bioreduction/ME production, 0.5 ml of extracellular medium was mixed with OxPhos™ reagent 3 in microfuge tubes, centrifuged and the supernatant was used for the HPLC/EC analysis using ESA (USA) system consisting of a single pump (Model: 584), autosampler (Model: 542), guard cell (Model: 5020), analytical cell (Model: 5010) and Colouchem (Model: III). Diluted sample (10µl) was loaded onto a C18 column and analyzed in an isocratic mode using a mobile phase with 50mM phosphate, pH 2.7, 0.05 mM octane sulfonic acid and 2.2% acetonitrile.

2.5. Quantification of glutathione-dependent antioxidant capacity in blood by OxPhos™ test

For activity measurement in whole blood, 0, 10, 20, 40, 60, 80, 100 µl of blood was mixed with 200, 190, 180, 160, 140, 120 and 100 µl saline respectively in microfuge tubes. Ten µl of reagent 6, as prepared above for the test in mammalian cells, was added to each microfuge tube, mixed gently and incubated for 2 hours with gentle mixing on a rocker at room temperature followed by centrifugation at 9,000 rpm in a microfuge at 4°C for 2 minutes. One hundred fifty µl of the supernatant was mixed with 150 µl of reagent 3 in a new microfuge tube, vortexed gently for 20 seconds and centrifuged at 9,000 rpm at 4°C for 3 minutes. The supernatant was stored at 4° C for short term storage (2 days) or –20° C for long term storage (10 days). The supernatant was used for the OxPhos™ activity/ glutathione-dependent antioxidant capacity by mixing 75 µl of the supernatant with

premixed reagent 4 (1275 μ l) and reagent 5 (150 μ l). These tubes were vortexed gently, left at room temperature for 3–5 minutes and the absorbance was read at 412 nm in a spectrophotometer. The blank (1350 μ l of reagent 4 mixed with 150 μ l of reagent 5) was subtracted from the samples and delta O.D. was multiplied with 2942 (dilution factor $\times$ extinction coefficient) to determine micromolar GSH dependent antioxidant capacity.

2.6. Quantification of glutathione level in cells in vitro by intracellular ThiolEZ assays

For the measurement of glutathione level, Intracellular ThiolEZ (Rockland Immunochemicals, USA) was used immediately after the OxPhosTM test in the same cells. Although this kit measures all intracellular non-protein thiol including glutathione and cysteine, our previously published and unpublished results demonstrated that the amount of glutathione is 20 times higher than the cysteine thiol suggesting that this assay kit measures mostly the glutathione level (Ayene, et al., 2008; Ayene, et al., 2000; Ayene, et al., 2002). After removal of 500 μ l of medium for the OxPhosTM test as described above, the cells in the six wells plate were washed three times with Earle's balanced salt solution and 1 ml of reagent 1 was added to each well, gently swirled and placed on ice for 10 minutes. Cells and reagent were then mixed by a cell scraper and transferred to a microfuge tube, vortexed gently for 20 seconds and centrifuged at 9000rpm in a microfuge for 3 min at 4°C. The supernatant (600 μ l) was mixed with 750 μ l of reagent 2 premixed with 150 μ l of reagent 3 in glass tubes. Samples were vortexed gently, left at room temperature for 3–5 minutes and the absorbance was read at 412 nm using a disposable plastic cuvette in a spectrophotometer. The blank (1350 μ l of reagent 4 mixed with 150 μ l of reagent 5) was subtracted from the samples and delta O.D. was multiplied with 312.5 (dilution factor $\times$ extinction coefficient) to determine micromolar thiol/NPSH/GSH level.

2.7. Quantification of glutathione level in blood by intracellular ThiolEZ assays

For the measurement of glutathione level in blood, Intracellular ThiolEZ kit as described above was used immediately after the OxPhosTM test in the same blood. After removal of 150 μ l of the supernatant for the OxPhosTM test, the blood cells in the microfuge tubes were washed three times with saline. Reagent 1 (1ml) was added to each sample, gently swirled, placed on ice for 10 minutes followed by gentle vortex for 20 seconds and centrifuged at 9000rpm in a microfuge for 3 min at 4°C. The supernatant was used for the total thiol/NPSH/GSH level by mixing 600 μ l of the supernatant with 750 μ l of reagent 2 premixed with 150 μ l of reagent 3 in glass tubes. These samples were vortexed gently, left at room temperature for 3–5 minutes and the absorbance was read at 412 nm using a plastic cuvette in a spectrophotometer. The blank (1350 μ l of reagent 4 mixed with 150 μ l of reagent 5) was subtracted from the samples and delta O.D. was multiplied with 312.5 (dilution factor $\times$ extinction coefficient) to determine micromolar thiol/NPSH/GSH level.

2.8. Treatment of mammalian cells and whole blood samples with arsenicals (PAO, As₂O₃, arsenite and arsenate)

For experiments with cells in vitro, a stock solution of 35mM PAO, arsenite, arsenate and As₂O₃ (Sigma, USA) were prepared fresh in DMEM growth medium. Cells were exposed to various doses of PAO or arsenite (0, 1, 2, 4, 6, 8 μ M), and other arsenicals (0, 10, 20, 40, 60, 200 μ M) and incubated at 37 °C in a humidified 5% CO₂ incubator. After 24 h incubation with arsenicals, cells were washed three times to remove extracellular arsenicals and replenished with the fresh growth medium. The arsenicals treated cells were used for OxPhosTM, thiol/NPSH/GSH tests and cell proliferation assay. For experiments with blood, whole blood mixed with an equal volume of saline was exposed to different doses of PAO (0, 2, 10, 20, 100 μ M), and As₂O₃, arsenate or arsenite (0, 2, 10, 40, 200 μ M) and incubated at room temperature on a shaker. After 24 h incubation, blood was used for OxPhosTM test, thiol/NPSH/GSH and hemoglobin release.

2.9. Cell Proliferation Assay

The cellular integrity after arsenicals treatment was determined by Coulter Counter Assay as followed by Li et al. (2012). Cells (800K) plated in 60mm dishes treated with and without arsenicals, as described above for OxPhos™, activity was harvested immediately after treatment by trypsinization and the total number of cells were counted using Coulter Counter. The results were presented as fold increase in cell growth.

2.10. Hemoglobin Assay

The blood cell integrity after arsenicals treatment was determined by the measurement of release of hemoglobin as followed by Noe et al. (2009). Whole blood treated with arsenicals as described above was centrifuged immediately after treatment and the supernatant was used to determine the release of hemoglobin. The supernatant (10 µl) was mixed with 990 µl of saline and the absorbance was read at 415, 380 and 450nm in a microplate reader. The Hb concentration (g/l) in the supernatant matrix was calculated as follows, $Hb(g/l) = (167.2 \times A_{415} - 83.6 \times A_{380} - 83.6 \times A_{450}) \times 1/1000$

The results were presented as % increase of the untreated blood samples.

2.11. Statistical analysis

Values were presented as mean $\pm$ standard error (SE) calculated from three to five independent replicates. The sensitivity and the consistency of the assay were determined from the slope/intercept and the biological variability, respectively. The slope and intercept, which represent the sensitivity of the assay, were calculated using linear or polynomial curve fit. The half maximal inhibitory concentration (IC₅₀) for arsenicals was calculated from the slope and intercept of individual dose response curve. The biological variability i.e. percentage of coefficient of variance (CV) was calculated from the formula $100 \times SD/mean$, where SD is the standard deviation of mean of three to five independent replicates. The statistical significance of the differences between the groups was determined by Analysis of Variance (ANOVA) with the p values presented in Tables.

3. Results

3.1. Comparison of GSH dependent antioxidant capacity and intracellular thiol (thiol/NPSH/GSH) assays in rodent and human cancer cells

We first determined the effectiveness of OxPhos™ test in CHO cells with different GSH dependent antioxidant capacity (Figure 1A) in vitro to determine the accuracy of this assay kit. Wild type CHO K1 cells with normal GSH dependent antioxidant capacity exhibited cell density dependent activity response with a slope of 405 and a maximum activity of 4057µM per million cells (Fig 1A and Table 1). Mutant CHO E89 cells with deficiency in OPPC, which is required for GSH recycling dependent antioxidant capacity, showed much lower OxPhos™ activity in this test with a slope of 30 and a maximum activity of 302µM per million cells. Although E89 has a much lower OxPhos™ activity, the accuracy and the dynamic range of the assay kit were demonstrated even in this cell line with a %CV of 5.4% and an r^2 value of 0.98. These values were comparable to K1 cells (%CV – 2%; r^2 – 0.98) that exhibited a 13 fold higher GSH dependent antioxidant capacity. Results for most commonly measured non-protein thiol (NPSH/GSH) using ThiolEZ assay kit showed cell density dependent levels in K1 and E89 cells (Figure 1B). Wild type CHO K1 cells with normal GSH dependent antioxidant capacity exhibited cell density dependent thiol/NPSH/GSH level with a slope of 5 and a maximum activity of 51µM per million cells (Fig 1B and Table 1). Mutant CHO E89 cells with deficiency in OPPC showed much higher thiol/NPSH/GSH level with a slope of 10.5 and a maximum activity of 105µM per million cells (Fig 1B

and Table 1). The intracellular ThiolEZ kit worked as well as the OxPhos™ test kit in these two cells with a %CV of 2% and 5% but with an r^2 value of 0.83 and 0.98.

Human colon cells with wild type p53 (HCT116) and mutant p53 (HT29) also exhibited a cell density dependent OxPhos™ activity with slopes of 479 and 333 and maximum activities of 4794 μ M and 3338 μ M per million cells respectively (Fig 1A and Table 1). The OxPhos™ test exhibited accuracy in both HCT116 cells and HT29 cells with a %CV of 4.2% and 4.7% and r^2 values of 0.97 and 0.99 respectively. The results with ThiolEZ assay also showed cell density dependent thiol/NPSH/GSH levels in HCT116 and HT29 cells (Figure 1B) similar to that observed for OxPhos™ test. However, unlike the OxPhos™ test that showed a significantly lower value in HT29 cells as compared to HCT116, the thiol/NPSH/GSH level measured by ThiolEZ kit showed a statistically insignificant difference (87.8 μ M vs. 74 μ M) between these cells (Table 1). Further, the %CV (HCT116 - 10.49% and HT29 - 15.4%) was two to three times higher as compared to OxPhos™ test suggesting that the OxPhos™ test worked better than the ThiolEZ test in these cells.

3.2. Validation of OxPhos™ test for the glutathione-dependent antioxidant capacity by HPLC/EC detection in rodent and human cancer cell

Although our results demonstrated that colorimetric quantification of OxPhos™ kit worked well in rodent and human cells, we used a HPLC/EC detection to confirm the metabolic conversion of the bioactive probe used in the OxPhos™ kit since HPLC/EC specifically measures ME, the metabolite of the probe, without interference from other thiols such as GSH and cysteine. Consistent with the OxPhos™ kit, the metabolic conversion of the probe (reagent 6) is much higher in normal CHO K1 cells as compared to mutant CHO E89 cells (Fig. 1C, D). Further, use of G6PD transfected E89 cells (A1A cells) confirmed that the metabolic conversion of this probe is dependent on the OPPC (Fig. 1C, D). In addition, the results with HPLC/EC also confirmed that the activity measured in HCT116 and HT29 by OxPhos™ probe is also due to the G6PD/OPPC/GSH dependent conversion of HEDS to ME (Fig. 1C, D).

3.3. Quantification of OxPhos™ activity in blood from breast cancer patients

We tested the OxPhos™ kit in 0, 10, 20, 40, 60, 80, 100 μ l blood from breast cancer patients to determine the slope (i.e. activity per μ l blood), total activity in 100 μ l blood and correlation coefficient (i.e. accuracy) in each individual patient. Table 2 shows the blood analyzed from patients before treatment/pretreatment (17 samples), after first treatment/cycle 1 (12 samples), second treatment/cycle 2 (11 samples) and third treatment/cycle 3 (12 samples).

A volume dependent linear response was observed for all 52 blood samples independent of sampling time and the treatment regimen demonstrating the accuracy of the OxPhos™ test (Figure 2A, B, C and D). These results were similar to data shown for in vitro cultured human and rodent cells (Figure 1). The mean and error of the mean (S.E.) of the correlation coefficient (r^2 values) for blood samples in the pretreatment group was 0.98 ± 0.005 with a biological variability (%CV) of 2.2% (Figure 2E, Table 2). The mean and SE of the correlation coefficient for blood samples for the groups in cycle 1, 2, 3, were 0.98 ± 0.006 , 0.99 ± 0.002 , and 0.98 ± 0.005 respectively, with a biological variability between 1% and 2.1% (Figure 2E, Table 2). The mean and standard error of the mean of the slope of the blood volume response linear curve (slope) for blood samples in the pretreatment, cycle 1, cycle 2 and cycle 3 were 35.4 ± 1.62 , 34.6 ± 2.02 , 34.6 ± 1.92 and 34.5 ± 1.83 with a biological variability between 18.5% and 20.22% (Figure 2F, Table 2). The total OxPhos™ activity in 100 μ l blood in these groups varied between 3446 μ M and 3538 μ M with statistically no significant difference between these groups (Figure 2G, Table 2).

3.4. Comparison of OxPhos™ and intracellular thiol tests in measuring the effect of environmental pollutants in HCT116 human colon cancer cells

We tested the application of the OxPhos™ test in quantifying the effects of common environmental pollutants with biological and clinical implications. We selected the four most common arsenic pollutants (PAO, As₂O₃, arsenite and arsenate), which induce toxicity, oxidative stress and tumorigenesis and deplete GSH, in ex vivo blood and mammalian cells. The OxPhos™ test quantified concentration dependent effects of these arsenicals in human colon cells (Figure 3A, B). The second order polynomial curve fit exhibited the best curve fits with r^2 values of 0.96, 0.95, 0.95 and 0.98 for PAO, As₂O₃, arsenite and arsenate respectively (Fig. 3A, B, Table 3). In contrast, the r^2 values were lower for the thiol/NPSH/GSH assay with values 0.88, 0.95, 0.89 and 0.89 for PAO, As₂O₃, arsenite and arsenate respectively (Fig. 3C, D Table 3). Similarly, the biological variability (%CV) of the slope for the OxPhos™ test was lower with values 7.4%, 6.3%, 11.3% and 9.9% as compared to thiol/NPSH/GSH assay with values 31.3%, 13.7%, 20.4% and 39% (Table 3). This was consistent with a higher total initial activity for OxPhos™ kit with values 4085±77μM, 3698±130μM, 4701±218μM and 4539±118μM as compared to a lower total initial activity for thiol/NPSH/GSH assay with values 62±14μM, 69.5±5.5μM, 38.8±2μM and 52±10μM for PAO, As₂O₃, arsenite and arsenate, respectively (Table 3). The higher slopes for OxPhos™ test (59, 55, 132 and 46 folds for PAO, As₂O₃, arsenite and arsenate respectively) as compared to thiol/NPSH/GSH level were also consistent with low % CV (1.9%, 3.53%, 4.64% and 2.6%) for the initial total OxPhos™ activity as compared to high %CV (22.96%, 7.89%, 5.22% and 19.13%) for the initial total thiol/NPSH/GSH level (Table 3). The assay was sensitive to measure the effect of arsenicals on the GSH dependent antioxidant capacity at concentrations as low as 1μM of PAO and As₂O₃ with IC₅₀ (1.9μM and 3.5μM) close to that measured by intracellular thiol/NPSH/GSH assay (2μM and 3.6μM). The OxPhos™ test exhibited a better %CV for IC₅₀ with values 5.9% and 7.8% as compared to thiol/NPSH/GSH assay with values 17.9% and 17% (Table 3). Although OxPhos™ and thiol/NPSH/GSH assays exhibited comparable IC₅₀ with 26.79μM vs. 31.36μM for arsenite and 97.42μM vs. 79.13μM for arsenate, the biological variability was lower for OxPhos™ test (8.25% and 8.03%) than thiol/NPSH/GSH assay (20.17% and 47.80%). The proliferation of the cells measured immediately after treatment with arsenicals was comparable to the untreated cells suggesting arsenicals did not significantly affect the growth of the cells (Figure 3E, F).

3.5. Comparison of OxPhos™ and intracellular thiol tests in measuring the effect of environmental pollutants in ex vivo human blood

As the OxPhos™ kit worked well in mammalian cells in vitro, we then tested this kit in ex vivo human blood exposed to these arsenicals. We used similar arsenicals exposure used for human cells in vitro but concentrations were increased up to 100μM for PAO and As₂O₃ and 200μM for arsenite and arsenate. The second order polynomial curve fit exhibited r^2 values 0.99, 0.95, 0.98 and 0.91 for these arsenicals (Fig. 4A, Table 3). In contrast, the r^2 values were lower for the thiol/NPSH/GSH assay with values 0.88, 0, 0.67 and 0.56 (Fig. 4B, Table 3). Similarly, the biological variability (%CV) of the slope for the OxPhos™ test was lower with values 7.5%, 10.96%, 10.7% and 9.9% as compared to thiol/NPSH/GSH assay with values 39.62%, 62.58%, 186.66% and 39.05% (Fig. 4A, B, Table 3). The total initial activities for OxPhos™ were much higher with values 3863±27μM, 4210±184μM, 4011±223μM and 3894±104μM as compared to lower total initial activities for thiol/NPSH/GSH assay with values 1.68±0.1μM, 1.72±0.08μM, 1.5±0.1μM and 1.48±0.17μM. The OxPhos™ test exhibited higher slopes as compared to thiol/NPSH/GSH for PAO (873 fold), As₂O₃ (1900 fold), arsenite (2800 fold) and arsenate (2053 fold). The OxPhos™ test was sensitive to measure the effect of arsenicals on the GSH dependent antioxidant capacity at concentrations as low as 10μM of PAO and As₂O₃ with IC₅₀ values (44.74μM and

114.13 μ M) up to 2 fold difference to that measured by intracellular thiol/NPSH/GSH (22.29 μ M and 174.9 μ M). OxPhos™ test showed a better % CV for IC₅₀ with values 7.57% and 14.08% as compared to thiol/NPSH/GSH assay with values 32.94% and 39.04%. OxPhos™ and thiol/NPSH/GSH tests measured comparable IC₅₀ values for arsenite with 92.56 μ M vs. 81 μ M but with close to 2 fold difference (100.8 μ M vs. 197 μ M) for arsenate. Similar to the human cells in vitro, OxPhos™ test exhibited a better biological variability (11.67% and 8.04%) than thiol/NPSH/GSH assay (115.23% and 47.80%) for arsenite and arsenate respectively. Arsenicals did not induce lysis of the blood cells measured immediately after treatment (Figure 4C). The hemoglobin level in the supernatant of the blood cells treated with arsenicals was no greater than 6% of the untreated control (Figure 4C).

4. Discussion

A GSH dependent antioxidant capacity test (OxPhos™) for blood and mammalian cells presented here is the result of a novel probe (HEDS), improved reagents and buffers and optimal conditions used for blood and mammalian cells. In general, the intracellular thiol homeostasis requires at least five pathways directly or indirectly dependent on glutathione. The use of HEDS as a probe for glutathione-dependent antioxidant capacity is unique since it covers these five major pathways and GSH since the metabolic conversion of HEDS is dependent on recycling of GSH by oxidative pentose phosphate cycle and GSH dependent enzymes such as GSH S-transferase, glutaredoxin, glutathione reductase, thioltransferase and thioredoxin reductase (Ayene, et al., 2008; Ayene, et al., 2002; Biaglow, et al., 2003; Biaglow, et al., 2006; Björnstedt, Kumar, Björkhem, Spyrou, & Holmgren, 1997; Yang, et al., 2006).

Our studies with different cell densities of rodent (CHO K1 and E89) and human colon cells (HCT116 and HT29) clearly demonstrated that the reagents provided in the OxPhos™ kit worked well with a dynamic range from 100,000 cells to 800,000 cells with a lower %CV as compared to the intracellular thiol/NPSH/GSH assay. Additionally, the method is adapted to measure the activity in 10 to 100 μ l human whole blood that exhibited r^2 values greater than 0.98 in all 52 samples tested without the need for the isolation and cytoplasm extraction of cells required for the thiol/NPSH/GSH test.

In general, OPPC was the major component of HEDS metabolism by providing NADPH to either directly converting HEDS into ME (reaction 2) or by recycling oxidized GSH that occurred by HEDS interaction with GSH (reaction 3) or GSH dependent enzymatic pathways (reactions 4 and 5). The depletion of GSH by almost 80% only in E89 cells deficient in OPPC that was required for glutathione recycling confirmed that OxPhos™ test measured the GSH recycling dependent antioxidant capacity.

The continuous recycling of NADP to NADPH by OPPC required for various enzymes for the recycling of GSH from GSSG to metabolize HEDS into ME suggested that this test was the most effective to measure the GSH dependent antioxidant capacity in blood and mammalian cells. The lower %CV, best r^2 values and higher slope for OxPhos™ test as compared to the traditional intracellular thiol assay in rodent and human cells in vitro demonstrated that it is not only better in sensitivity and reproducibility but also demonstrated its potential to measure the glutathione-dependent antioxidant capacity covering at least 5 pathways in a single assay. Although it would be expected in a traditional sense that the thiol/NPSH/GSH level should be lower in E89 cells deficient in GSH recycling, our results indeed demonstrated that thiol/NPSH/GSH level was higher in E89 than its parental K1 cells. These results demonstrated that while the thiol/GSH assay may not identify the deficiency in GSH dependent antioxidant capacity, the OxPhos™ test can easily identify differences in activity in mammalian cells and human blood. It also suggested that OxPhos™ as a clinically relevant test to identify subjects prone to oxidative stress for preventive measures. The best r^2 values for blood samples also suggested that OxPhos™ was an easy and sensitive assay to quantify the glutathione function/GSH dependent antioxidant capacity in humans. The results from the blood samples in Table 2 showed that biological variability for the r^2 values (consistency and sensitivity) of the test was in the range of 1 to 2% while ranging from 18.5 to 20% for the oxidative capacity suggesting a significant difference in antioxidant capacity among these patients. A closer analysis of the data suggested that some patients had 25% lower (patient 2 and 12) or higher (patient 4, 14 and 15) total activity in 100 μ l blood than the mean of total activity of all blood samples (Figure 2G, Table 2). A 30% of the 17 patients with differences lower or greater than 25% but an r^2 values closer to 1 suggested that OxPhos™ clearly identifies the variations in the antioxidant capacity among these patients.

We also validated the application of this test in quantifying the effect of environmental pollutants/toxins on glutathione-dependent antioxidant capacity in ex vivo blood and mammalian cells in vitro. Arsenic has been considered the most dangerous and probably the most common contaminant in the soil, plant and water. Inorganic arsenic commonly exists as arsenate (As5+) and arsenite (As3+) in ground water (Bernstam & Nriagu, 2000; Vahter, 2007). The semimetal form of arsenic oxidizes rapidly in air, and at high temperature produces As₂O₃. In addition, As₂O₃ has been used in humans to treat cancer (Evens, Tallman, & Gartenhaus, 2004). Phenyl arsenic compounds, which are mostly found in groundwater at abandoned sites with arsenic containing chemical warfare agents, are highly toxic. A simple blood based test to measure the biological effects of arsenicals will be highly useful for determining the affected population. Additionally, an assay to measure the biological effect of As₂O₃ using a simple blood based assay will not only be useful for determining the efficacy of this compound but also can be used for determining its pharmacokinetics and pharmacodynamics and screening for antidotes. Several studies have demonstrated that these arsenicals can cause toxicity due to lipid peroxidation, protein oxidation, and depletion of GSH and other antioxidants in mammalian cells (Bernstam & Nriagu, 2000; Ramanathan, Shila, Kumaran, & Panneerselvam, 2003; Vahter, 2007).

The OxPhos™ test demonstrated that the effect of environmental pollutants such as arsenic on GSH dependent antioxidant system can be quantified both in cell culture and ex vivo human blood. Further, it also identified the difference between the arsenicals in terms of their effectiveness in inhibiting the antioxidant capacity. Most interestingly, the OxPhos™ test exhibited a higher IC₅₀ for PAO, As₂O₃ and arsenite in blood as compared to that in

colon cancer cells suggesting the potential beneficial use of arsenic in cancer without blood toxicity. Although the results with arsenicals on the cell proliferation of human colon cells (HCT116) and lysis of blood cells under the current experimental conditions demonstrated that the decrease in OxPhos™ activity is unlikely due to cell death, independent measurements of the cell survival or blood cell lysis should be simultaneously carried out to differentiate the direct effect of toxins on glutathione dependent antioxidant capacity from cell death for studies in vitro.

In conclusion, these results demonstrated the application of this test for measuring the antioxidant capacity of blood and the effects of environmental pollutants/toxins. It opened up new avenues for an easy and reliable assessment of glutathione-dependent antioxidant capacity in various diseases such as stroke, blood borne diseases, infection, cardiovascular disease and other oxidative stress related diseases and as a prognostic indicator of chemotherapy response and toxicity. It also opened up further studies to validate the clinical relevance of this approach in pharmacology/toxicology including screening drugs that improve the glutathione-dependent antioxidant capacity since mammalian cells require glutathione and glutathione dependent pathways for antioxidant activity.

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Abbreviations

G6PD	glucose-6-phosphate dehydrogenase
HEDS	hydroxyethyl disulfide
OPPC	oxidative pentose phosphate cycle
ME	mercaptoethanol
GST	Glutathione S-transferase
GRase	glutathione reductase
GRx	glutaredoxin
GSH	glutathione
GSSG	oxidized glutathione
NPSH	non-protein thiol
PSH	protein thiol
DTNB	dithiobisnitrobenzoic acid

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Figure 1.

Comparison of OxPhos™ test and Intracellular ThioLEZ Assay in mammalian cells. Cell density dependent glutathione based antioxidant capacity and glutathione level measured by OxPhos™ (A) and intracellular ThioLEZ (B) tests respectively in rodent and human colon cancer cells. Representative HPLC/EC tracings of mercaptoethanol production illustrating the measurement of GSH dependent antioxidant capacity by OxPhos™ reagent 6 in K1, E89, A1A, HCT116 and HT29 cells (C). Quantification of antioxidant activities for these cells from data shown in Fig C (D). Each value was the mean $\pm$ standard error (SE) calculated from five independent replicates with SE as shown unless smaller than points plotted. Linear regression analysis of the cell number response curve and ANOVA analysis of differences between the OxPhos™ test and intracellular thiol assay are presented in table 1. The biological variability as determined by % coefficient of variance (%CV) for these results is presented in Table 1.

Figure 2.

Quantification of OxPhos™ activity in the blood from breast cancer patients. Measurement of the antioxidant capacity by OxPhos™ kit in a total volume of 200μl saline consisting of 0, 10, 20, 40, 60, 80, 100 μl blood for each of the 52 blood samples collected from breast cancer patients. The volume dependent activity fitted with the linear curve fit is presented for each blood collected from patients before treatment/pretreatment (A), after first treatment/cycle 1(B), after second treatment/cycle 2 (C) and after third treatment/cycle 3 (D). The r^2 values, slope and total activity per 100μl blood calculated from the linear regression analysis and their biological variability (%CV) are presented in Table 2. The r^2 values (E), slope (F) and total activity per 100μl blood (G) calculated from the linear regression analysis and their biological variability (%CV) for data in Figure A–D are plotted for each patient of the four groups (pretreatment, cycle 1, cycle 2 and cycle 3).

Figure 3.

Comparison of OxPhosTM and intracellular thiol tests in measuring the effect of environmental pollutants in human cancer cells HCT116. Quantification of the effects of 24 hours exposure of arsenicals (PAO, As_2O_3 , arsenite and arsenate) in human colon cells. The concentration dependent effect on the OxPhosTM activity (A, B) and intracellular thiol level (C, D) fitted with the second order polynomial curve fit is presented for each of these arsenicals. The effect on the cell growth is presented for each of these arsenicals (E, F). Each value was the mean $\pm$ standard error (SE) calculated from five independent replicates with SE as shown unless smaller than points plotted. The r^2 value, slope and total activity without treatment from the linear regression analysis (second order polynomial curve fit) and their biological variability (%CV) are presented in Table 3.

Figure 4.

Comparison of OxPhos™ and intracellular thiol tests in measuring the effect of environmental pollutants in ex vivo human blood. Quantification of the effects of 24 hours exposure of arsenicals (PAO, As₂O₃, arsenite and arsenate) in ex vivo human blood. The concentration dependent effect on the OxPhos™ activity (A) and intracellular thiol level (B) fitted with the second order polynomial curve fit is presented for each of these arsenicals. The effect on the blood cell lysis as measured by extracellular hemoglobin level is presented for each of these arsenicals (C). Each value was the mean $\pm$ standard error (SE) calculated from five independent replicates with SE as shown unless smaller than points plotted. The r^2 value, slope and total activity without treatment from the linear regression analysis (second order polynomial curve fit) and their biological variability (%CV) are presented in Table 3.

Table 1

The slope, r^2 values and total activity/million cells and their biological variability (%CV) for the oxphos (GSH dependent antioxidant activity) and intracellular thiol tests calculated from the data shown in Figure 1A, B for rodent and human cells in vitro.

	CHO K1		CHO E89		HCT116		HT29	
	Oxphos Test	NPSH Test	Oxphos Test	NPSH Test	Oxphos Test	NPSH Test	Oxphos Test	NPSH Test
Slope	405+8.3* ψ	5.11+0.044*	30+1.62* ψ	10.5+0.31*	479+6.5* ψ	8.8+0.36*	333.5+11.91* ψ	7.38+0.51*
%CV(slope)	2	1.5	5.4	5.0	4.3	9.9	4.7	15.5
r^2	0.98+0.003	0.83+0.006	0.98+0.003	0.98+0.006	0.97+0.009	0.97+0.014	0.99+0.003	0.97+0.017
%CV(r^2)	1.05	1.32	1.2	1.1	3.2	3.6	0.79	3.98
Activity/million cells	4057+83* ψ	51+0.6*	302+16* ψ	104.96+3.03*	4794+65* ψ	87.8+3.76*	3338+118* ψ	74+5.09*
%CV(Activity/million cells)	2.05	2	5.36	5	4.2	10.49	4.7	15.4

* (p<0.001) Oxphos Vs NPSH tests in each cell line

ψ (p<0.001) K1 Vs E89 and HCT116 Vs HT29.

The mean, standard error (SE) and biological variability (%CV) of the slope (antioxidant capacity activity per μl blood), r^2 values and the total antioxidant capacity in 100 μl blood calculated from the data shown in Figure 2 for 0, 10, 20, 30, 40, 60, 80, 100 μl blood from breast cancer patients before treatment (pretreatment) and after 1st (cycle 1), 2nd (cycle 2) and 3rd (cycle 3) treatment.

Table 2

Group	Slope				r^2 values			Total activity in 100 μl blood		
	Samples analyzed	Mean (μM)	SE	%CV	Mean (μM)	SE	%CV	Mean (μM)	SE	%CV
Pretreatment	17	35.38	1.62	18.89	0.98	0.005	2.2	3538	162	18.89
Cycle 1	12	34.61	2.02	20.22	0.98	0.006	2.1	3461	202	20.22
Cycle 2	11	34.57	1.92	19.26	0.99	0.002	1.0	3457	192	19.26
Cycle 3	12	34.46	1.83	18.49	0.98	0.005	1.7	3446	184	18.49

Table 3

The mean, standard error (SE) and biological variability (%CV) of the slope, r^2 values, initial antioxidant activity and IC50 for arsenicals calculated from the data shown in Figure 3 for human cells (HCT116) in vitro and ex vivo blood from breast cancer patients.

	PAO		As2O3		Arsenite		Arsenate	
	Oxphos Test	NPSH Assay	Oxphos Test	NPSH Assay	Oxphos Test	NPSH Assay	Oxphos Test	NPSH Assay
HCT116 in vitro								
Slope	1086±80 ^{*ψ}	18.4±5.8 [*]	555±35 ^{*ψ}	10.13±1.38 [*]	89±10 ^{*ψ}	0.67±0.13 [*]	24±2.33 ^{*ψ}	0.52±0.20 [*]
%CV(slope)	7.38	31.29	6.30	13.68	11.29	20.39	9.85	39.05
r2	0.96±0.01	0.88±0.04	0.95±0.01	0.95±0.029	0.95±0.03	0.89±0.035	0.98±0.0006	0.89±0.04
%CV(r2)	1.0	3.9	1.3	3.1	2.8	3.9	0.05	4.4
Initial Activity/μM	4085±77 [*]	62±14 [*]	3698±130 [*]	69.5±6 [*]	4701±218 [*]	38.8±2 [*]	4539±118 [*]	52±10 [*]
%CV(Initial activity)	1.9	22.96	3.53	7.89	4.64	5.22	2.6	19.13
IC50/μM	1.89±0.11 ^ψ	2.00±0.36 ^φ	3.47±0.27 ^ψ	3.57±0.61 ^φ	26.79±2.21 ^ψ	31.36±6.32 ^φ	97.42±7.83 ^ψ	79.13±37.83 ^φ
%CV(IC50)	5.91	17.94	7.83	17.01	8.25	20.17	8.03	47.8
Ex vivo blood								
Slope	43.66±3.3 ^{*φ}	0.05±0.02 [*]	19±2.08 ^{*φ}	0.01±0.01 [*]	28±2 ^{*φ}	0.01±0.02 [*]	20.53±2.94 ^{*φ}	0.01±0.01 [*]
%CV(slope)	7.52	39.62	10.96	62.58	10.7	186.7	9.86	39.05
r2	0.99±0.001	0.88±0.07	0.95±0.02	0	0.98±0.01	0.67±0.22	0.91±0.043	0.56±0.26
%CV(r2)	0.15	7.45	1.71	-	1.36	32.99	0.06	4.46
Initial Activity/μM	3863±27 [*]	1.68±0.1 [*]	4210±184 [*]	1.72±0.08 [*]	4011±223 [*]	1.5±0.1 [*]	3894±104 [*]	1.48±0.17 [*]
%CV(Initial activity)	0.70	5.87	4.38	4.59	5.57	6.67	2.61	19.12
IC50/μM	44.74±3.4 ^φ	22.29±7.34	114±16 ^φ	174.9±68	92.56±8 ^φ	81±94	100.8±6.71 ^φ	197±117
%CV(IC50)	7.57	32.94	14.08	39.04	11.67	115.23	8.04	47.80

* (p<0.001) oxphos Vs NPSH for each arsenical

^ψ (p<0.01) between PAO, As2O3, arsenite and arsenate for oxphos.

^φ (p<0.1) between PAO, As2O3, arsenite and arsenate for NPSH

^φ (p<0.1) between PAO Vs As2O3 and arsenite or arsenate for oxphos.