RESEARCH COMMUNICATION

Assessment of 8-isoprostane (8-isoPGF$_{2\alpha}$) in Urine of Non-Small Cell Lung Cancer (NSCLC) Patients Undergoing Chemotherapy

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Abstract

   8-isoprostane (8-isoPGF$_{2\alpha}$) is a reliable marker and considered a gold standard for lipid peroxidation. There are very few reports of 8-isoprostane levels in cancer patients, and in patients undergoing chemotherapy. Oxidative stress is however expected and has been observed in patients with cancer. This study measured 8-isoprostane levels in urine by ELISA of 25 patients undergoing chemotherapy for advanced non-small cell lung cancer, at cycles 1, 2, and 3 of treatment. It considers the creatinine clearance of the patients, and correction of 8-isoprostane levels by creatinine clearance, and overnight urine volume methods. The average 8-isoprostane levels in urine increased more than 6 to 12 fold on chemotherapy treatment, from 532 ±587 pg/mL at cycle 1, 6181 ± 4334 at cycle 2, and 5511 ± 2055 at cycle 3. Similar results were obtained if 8-isoprostane levels were corrected for overnight urine volume, giving averages of 285 ± 244 µg at cycle 1, 4122 ± 3349 at cycle 2, and 3266 ± 1200 at cycle 3. No significant difference was seen in average total overnight urine volume or number of urinations between chemotherapy cycles except for a large variation in urine volume between cycle 2 and 3. Creatinine levels were significantly different only between cycles 1 and 2 (p=0.016). In conclusion, cisplatin therapy has been shown to induce high levels of lipid peroxidation in lung cancer patients and can be assessed from the 8-isoprostane marker in overnight urine, with or without urine volume correction.

Keywords: Lung cancer - 8-isoprostane - 8-isoPGF$_{2\alpha}$ - chemotherapy - oxidative stress - clinical trial – urine

Introduction

   Non small cell lung cancer (NSCLC) is difficult to treat, being relatively insensitive to chemotherapy (Villanueva , 2011). Traditional platinum based drug regimens such as cisplatin and carboplatin drugs act by crosslinking DNA and interfering with cell division. These drugs also result in reactive oxygen/nitrogen species (ROS/RNS) generation (e.g. superoxide and hydroxyl radicals, peroxynitrile and H$_2$O$_2$) (Lieberthal et al., 1996; Baek et al., 2003) that enhance the action of the chemotherapy via ROS/RNS induced apoptosis mechanisms (Chirinoa & Pedraza-Chaverri, 2009). Chemotherapy induced apoptosis generally results in ROS/RNS generation in conjunction with cytochrome C, AIPF-1 and caspases-2, -3, and -9 being released into the cytosol, alteration of mitochondrial transmembrane potential and mitochondrial permeability transition (Kannan & Jain, 2000). However generation of ROS/RNS also gives rise to drug-induced toxicity as a result of damage to healthy tissue. The result is a wide range of adverse events, usually including nausea and vomiting, thrombocytopenia, fatigue, cachexia and nephrotoxicity (Fabbro et al., 2006), followed often by the inability to continue cycles of chemotherapy,

resulting eventually in death. The level of oxidative stress induced by platinum based chemotherapy has not been studied in detail, and individual responses of human to oxidative stress induced by platinum-based therapy needs investigation in order to better inform dosing and regimen strategies, and affect on healthy tissue.

   The F$_2$-isoprotanes are stable compounds that are specific products of free-radical-catalyzed lipid peroxidation of arachidonic acid, independent of the cyclooxygenase enzyme. They are present in detectable quantities in normal biological tissues and fluids, are unaffected by lipid content of the diet, and show significant increase in animal models of antioxidant injury (Roberts & Morrow, 2000). As F$_2$-isoprotanes levels are modulated by antioxidant status they are ideal markers of oxidative stress. These compounds are formed in situ esterified to phospholipids *in vivo* and are then cleaved and released into the circulation before excretion in the urine as free isoprostanes (Morrow & Roberts, 1994).

   The 8-isoprostane (8-epiPGF$_{2\alpha}$, 8-isoPGF$_{2\alpha}$, iPF2α-III, 15-F$_{2t}$-IsoP) is a reliable marker and recently considered a gold standard for lipid peroxidation (Moore & Roberts, 1998; Kadiiska et al., 2005). Levels of 8-isoprostane in excess of normal levels have also been reported in a large

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number of pathologies, for risk factors such as obesity, gender, smoking, alcohol abuse, and for toxic xenobiotics as previously reviewed (Montuschi et al. 2004; Montuschi et al., 2007; Basu, 2008). However there are very few reports of isoprostane levels in advanced cancer patients, or in patients undergoing chemotherapy. Oxidative stress is however expected and has been observed in patients with cancer.

As with other cytotoxic xenobiotics, chemotherapy agents may cause lipid peroxidation and increase in oxidative stress. 8-isoprostane has been determined in cisplatin-induced renal dysfunction in LLC-PK1 renal epithelial cells (Salahudeen et al., 1998) where a dose-related increase in total (free plus esterified) 8-isoprostane was observed.

This study measured 8-isoprostane levels in urine of patients undergoing chemotherapy for advanced NSCLC as a marker of chemotherapy-induced oxidative stress from ROS/RNS over three cycles (courses) of platinum-based treatment. It also considered creatinine clearance of patient undergoing chemotherapy, and correction of 8-isoprostane levels by creatinine clearance versus urine volume methods.

Materials and Methods

Study design

Urine and blood samples were collected from patients participating in a randomized, double-blind, placebo controlled trial conducted in advanced cancer patients receiving treatment from a university teaching hospital in Northeast Thailand. The study was approved by the Khon Kaen University Ethics Committee and conducted in accordance with the International Conference on Harmonization on Good Clinical Practice requirements. All patients provided written informed consent.

Patients

Patients with histologically proven advanced NSCLC were recruited. Additional inclusion criteria included age 18 to 70 years, Eastern Cooperative Oncology Group (ECOG) performance status (PS) ≤ 2, white blood cell count $\geq 3,000$ cell/mm3, serum creatinine ≤ 1.5 mg/dL, bilirubin ≤ 2 mg/dL, AST ≤ 2.5 times upper limit of normal (ULN) for subjects without metastases or ≤ 4 times ULN for those with liver metastasis and New York Heart Association grade ≤ 2. Patients who had received prior chemotherapy or biotherapy, radiotherapy or surgery within 1 month preceding recruitment, or had more than one type of cancer or brain metastasis were excluded from the trial. Additional exclusion criteria included patients with moderate neuropathy (CTCAE grade ≥ 2), an active infection, or uncontrolled complications.

Most patients received cisplatin (75 mg/m2 intravenously) on day 1 of treatment, and etoposide (50-100 mg/m² intravenously) on days 1 to 3, then continuing cycles about every 3 to 4 weeks. Some patients alternatively received paclitaxel and carboplatin regimen.

Data collection

Baseline evaluation included assessment of ECOG PS, standard hematology, chemistry, electrolytes, urinalysis, and physical examination. Collection of blood, saliva, and overnight urine was conducted on the 1st (prior to chemotherapy), 2nd and 3rd chemotherapy cycles (approximately one month apart). Serum and urine creatinine levels were determined by the hospital laboratory as part of normal patient assessment. Overnight urination volumes (19:00-07:00) were carefully measured and recorded, and the volumes combined for analysis.

Urine samples were stored at -20 °C until analysis and analyzed within 6 months. Although plasma samples cannot be stored at this temperature because auto-oxidation can occur, this is not problem with urine because it has only trace quantities of lipid, and thus *ex vivo* formation of F_2-isoprostanes does not apply in the measurement of urine (Morrow et al., 1990; Morrow et al., 1996). Levels of F_2-isoprostanes are not increased by incubation of urine at 37 °C for 1 week or during storage of urine for 6 months at -20 °C (Morrow et al., 1990).

Determination of 8-isoprostane in urine samples

Urine 8-isoprostane was measured using an ACE Immunoassay 516315 test kit (Cayman Chemical Inc, MI USA) as per the manufacturer's instructions. Organic free ultrapure water filtered with a 40 um filter was used for all dilutions (Elga DV25 Purewater OptionQ system). Absorbances of the developed plates were read with an Anthos ELISA reader (Labtec Instruments, Sabzburg, Austria) with ADAP 1.6 software at 420 nm after development of the plates, i.e. after the B_0 wells were more than 0.3 A.U. (blank subtracted), 60-90 minutes. Urine samples from the patient's combined overnight urine voids were centrifuged briefly if necessary to remove sediment and diluted 50 fold to guarantee measurements were within the optimum 20-80% B/B_0 range. 50 uL was added directly to sample wells of the 96 well plates. The majority of samples were analyzed in duplicate. Samples from different cycles were distributed across plates to reduce and bias from variations in individual plates. Detection limit for the Cayman kit is claimed to be 2.7 pg/ml sample (80% B/B_0).

Concentrations were expressed as pg/mL urine, and were also normalized by multiplying by the volume of overnight urine collected to give μg, as this better represents the production of 8-isoprostane under conditions of chemotherapy. The intra- and interassay variations for the enzyme immunoassay were 6 and 10%, respectively. Statistical data analysis was performed with SPSS version 11.

Results

Baseline characteristics

A total of 30 patients were recruited with average age 55 years, but 5 patients withdrew from the study. All 25 remaining patients were advanced non-small cell lung cancer, received platinum-based regimen and reported ECOG performance score of 1.

Urine volumes and creatinine levels

Total volumes of urine collected, number of voids,

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Table 1. Urine Collection and Creatinine Data

Chemotherapy Cycle	1	2	3
Number of patients	25	20	15
Average total overnight urine volume (mL)			
	609.6	683	591.2
Standard deviation total overnight urine volume			
	284	259	323
Average no. of urinations per night	4	4	3.6
Urination Range (no. of times)	2 to 6	2 to 6	2 to 5
Average urine creatinine (mg / dL)	45.8	20.9	20.1
Standard deviation urine creatinine (mg / dL)			
	27.4	22.9	12.9
Range urine creatinine (mg / dL)	9-125	2-76	5-47
Number of patients with urine creatinine < 30 pg/dL (WHO guideline, 1996) [18]	8-25	13/20	10-15
	-32%	-65%	-66%

Table 2. Average Urine 8-isoprostane (corrected and Uncorrected for Urine Volume and Creatinine)

Chemotherapy Cycle	1	2	3
Number of patients	24	19	14
Average 8-isoprostane (pg / mL urine)			
	532 ± 587	6181 ± 4334	5511 ± 2055
Average overnight production of 8-isoprostane (ug)			
	285 ± 244	4122 ± 3349	3226 ± 1200
Average 8-isoprostane (ng/mg creatinine)			
	1.1 ± 0.80	104 ± 145.0	39.0 ± 29.0

* '± values indicate 1 standard deviation, Reference values for healthy population: 1.6 ± 0.6 ng/mg creatinine [9]; 0.25 ± 0.15 ng/mg creatinine [19]

and urine creatinine levels are shown in Table 1. No significant difference was seen between cycles in average total overnight urine volume or number of urinations, but greater variation in urine volume was seen at cycle 2 and 3. Creatinine levels were significantly different only between cycle 1 and cycle 2 (p=0.002).

Urine 8-isoprostane levels

Average values for urine 8-isoprostane (corrected and uncorrected for urine volume and creatinine) are shown in Table 2. Differences between the mean values of each cycle were analyzed by Wilcoxon signed-rank test.

The average 8-isoprostane levels in urine significantly increased up to 12 fold on chemotherapy treatment, from 532 ± 587 pg/mL at cycle one, 6181 ± 4334 at cycle 2, and 5511 ± 2055 at cycle 3. The uncorrected urine 8-isoprostane levels were significantly different between cycles 1 and 2 (p<0.001), and cycles 1 and 3 (p=0.001), but not between cycles 2 and 3 (p=0.91). Figure. 1 shows the average 8-isoprostane levels (and SD) for the three cycles for the individual subjects. A large variation was observed between individuals.

Similar results were obtained if 8-isoprostane levels were corrected for overnight urine volume (Figure. 2), i.e. pg 8-isoprostane/mL urine multiplied by overnight urine volume (shown as overnight production μg isoprostane), giving averages of 285 ± 244 at cycle 1, 4122 ± 3349 at cycle 2 and 3266 ± 1200 at cycle 3. There were significant differences between urine volume corrected 8-isoprostane levels between cycle 1 and 2 (p<0.001) and cycle 1 and
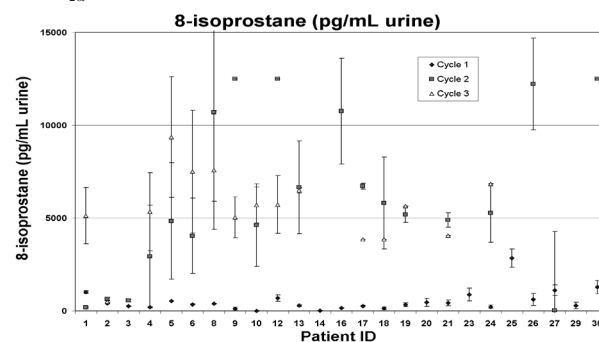

Figure 1. Uncorrected Urine 8-isoprostane Levels (pg/mL) for Individual Patients at Cycles 1, 2 and 3. Error bars show one standard deviation.
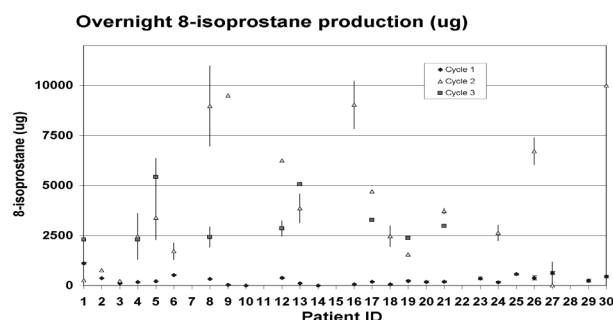

Figure 2. Overnight Urine 8-isoprostane Production (μg) for Individual Patients at Cycles 1, 2 and 3.
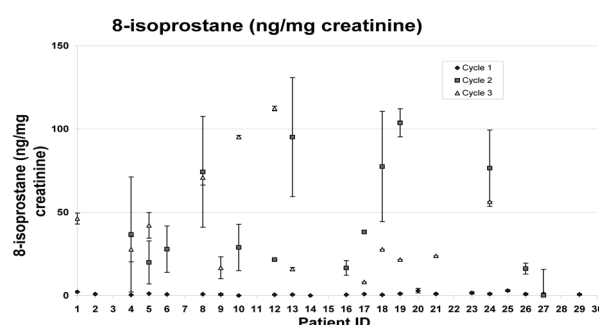

Figure 3. Urine 8-isoprostane Levels Corrected for Creatinine Clearance (ng/mg creatinine) for Individual Patients at Cycles 1, 2 and 3.
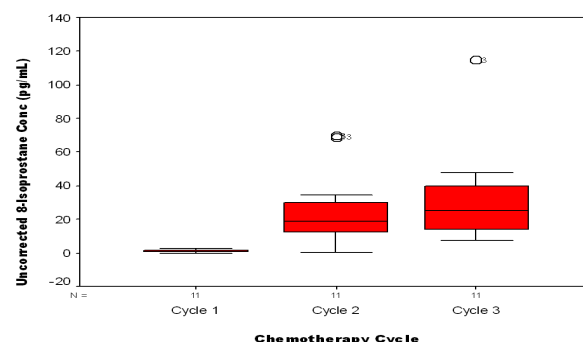

Figure 4. Boxplot of Uncorrected Urine 8-isoprostane Levels (pg/mL) at Chemotherapy Cycles 1, 2 and 3.

3 (p=0.008), but no difference between cycle 2 and 3 (p=0.767).

Creatinine levels were statistically different between cycles 1 and 2 (p=0.003), and cycles 1 and 3 (p=0.019), but not between cycles 2 and 3 (p=0.136). Urine 8-isoprostane corrected for creatinine (Figure. 3) were significantly different between cycles 1 and 2 (p=<0.001), and cycles 1 and 3 (p=0.001), but not between cycles 2 and 3 (p=0.158).

A boxplot of the uncorrected 8-isoprostane levels is shown in Figure. 4. Circles show outliers.

Discussion

Although it is probably essential to clean up tissue or plasma samples by C18 solid phase extraction cartridges or affinity columns (e.g. Cayman affinity column 416358), particularly if good correlation with GC-MS results is desired (Davies et al, 2006; Yan et al., 2007; Zhang & Saku, 2007), there would seem to be no specific requirement for urine samples with immunoassay methods. Immunoassays have been generally demonstrated to work very well in buffer systems that do not contain large amounts of biological substances. Indeed some ELISA kit manufacturers suggest direct analysis of urine samples (Bioxoytech, Urinary 8-Epi-Prostoglandin $F_{2\alpha}$ Enzyme Immunoassay for Urinary Isoprostane, Catalog Number 21048, OxisResearch, Portland, OR, USA).

To verify this for the Cayman kit, a simple check was performed. A previously analyzed urine sample with very low isoprostane level (< 3 pg/mL) was selected. To this sample, test kit standards (0–500 pg/ml of 8-isoprostane) were added, assayed in duplicate and compared to the standard curve produced from the same standards in buffer (also in duplicate). Differences less than 5% in concentration were seen, indicating that the urine matrix causes minimal interfere with the AChE competitive enzyme reaction or quantification of the AChE with the Ellman's reagent (5, 5'-dithiobis-(2-nitrobenzoic acid or DTNB) of the Cayman kit.

Study has shown that there is no diurnal variation in the urinary levels of 8-iso-$PGF_{2\alpha}$ during the day or any statistically significant difference between the 8-iso-$PGF_{2\alpha}$ levels at any time of the day or in morning urine samples compared to 24-h urine samples. Thus all urine samples collected at any time of the day, including morning urine samples can thus be used to obtain reliable and adequate values of the amount of the 8-isoprostane excretion in urine in healthy individuals (Helmersson & Basu, 1999).

Levels of urine 8-isoprostane at chemotherapy cycle 1 (average 2.2 ± 1.6 ng/mg creatinine) were slightly increased above the normal expected range for healthy people (1.6 ± 0.6 ng/mg creatinine) for almost all the cancer patients. The levels increased dramatically (on average more than 12 fold) at cycle 2, and 3 however, assumable due to oxidative stress and lipid peroxidation caused by the cytotoxic agents used (cisplatin). This is unsurprising considering the extreme cytotoxicity of the chemotherapy drugs. The increase in levels seen (10-200 times) are comparable to that observe with acute poisoning of rats by high doses of CCl4 or diquat (Kadiiska et al., 2005).

Reference levels of urine creatinine have been reported for a healthy U.S. population (22,245 subjects) as 130.4 mg/dL (95% CI=128.2-132.7 mg/dL), or 108.1 mg/dL (95% CI=103.8-112.5 mg/dL) for 50-60 years olds (1,823 subjects) (Barr et al., 2005). Similar levels of urine creatinine of 104 ± 48 mg/dL were reported in a group of 22 healthy U.S. patients (Liang et al., 2003). Levels also seem to decrease with increase age.

All of the patients undergoing chemotherapy showed much lower than normal levels of urine creatinine, and some extremely abnormally low levels (2.2 – 9.2 mg/dL urine), although their serum creatinine was within normal range (Table 1). By comparison, only 7.7% of the healthy US population has urine creatinine values less than the WHO guideline range of 30 – 300 mg/dL (Barr et al., 2005), whereas a majority of the patients in this study had levels below this by cycle 2 and 3 of chemotherapy. Adjusting 8-isoprostane concentrations for urine creatinine in these cases produces incredibly high values that are not useful for comparison, even though the unadjusted levels (pg/mL) are within the expected range of the data set.

The question of whether to correct urine 8-isoprostane levels for effects of urine concentration (or volume) arises. Traditionally, levels are reported as pg or ng of 8-isoprostane/mg creatinine. Correcting for concentration with creatinine would seem to be sensible, for example, with drugs that are metabolized and whose appearance in the urine will change with time. In these cases, correction for urine concentration (volume) is therefore essential, particularly for spot samples. Urine 8-isoprostane however is reported to be continuously produced at a constant rate, depending on the level of oxidative stress, and therefore correction may not be necessary. As previously discussed, Liang found that urine concentration did not appear to influence the measurement of 8-isoprostane (Liang et al., 2003). Furthermore there appears to be no diurnal variation in the urinary levels of 8-isoprostane (Helmersson & Basu, 1999). Ideally, the total quantity of the isoprostane of interest produced overnight or per 24 hour period, or concentration per milliliter, uncorrected for volume or creatinine would seem a more useful value. Patients with severe disease, for example cancer, may have renal impairment (renal toxicity of chemotherapy) or other factors that may affect creatinine clearance, but not 8-isoprostane clearance. Furthermore, there was no correlation (Pearson $R^2 < 0.01$) between urine creatinine and overnight urine volume, indicating that for chemotherapy patients, using creatinine to correct for concentration differences due to urine volume, may not be valid.

Grade et al. (2004) also showed that the uncertainty associated with creatinine standardization (19-35%) was higher than the uncertainty related to volume standardization (up to 10%) for 24 hours urine samples, even with average bias of 4% from missed volumes in population studies. Thus correction will contribute to variation and errors in reported levels.

The level of oxidative stress, and therefore 8-isoprostane production expected during chemotherapy would almost certainly be more than that reported for typical pathologies, and might near that reported for severe induced lipo-toxicity e.g. with CCl4 or diquat, i.e. 10-20 times normal healthy levels (Kadiiska et al., 2005).

Gutpa et al. (2010) and Srivastava et al. (2010) have reported elevated levels of lipid peroxidation products (LPO) using MDA-TBA blood plasma assay for 203 non-small cell lung cancer patients undergoing cisplatin+etoposide chemotherapy. The oxidative stress

was elevated more significantly (P<0.01) after the chemotherapy and was more evident in higher stage than lower stage patients.

Il'yasova et al. (2010) also reported increased levels of urinary F2-isoprostanes—iPF(2α)-III (8-isoprostane); iPF(2α)-VI; 8,12-iso-iPF(2α)-VI; and 2,3-dinor-iPF(2α)-III, quantified by LC-MS/MS in 23 women with newly diagnosed breast cancer who underwent four cycles of doxorubicin/cyclophosphamide chemotherapy.

Although several other researchers have reported increased oxidative stress in patients with lung cancer prior to chemotherapy (Zieba et al., 2001; Dalaveris et al., 2009; de Castro et al., 2006), we did not observe increase levels at the beginning of chemotherapy compared with levels previously reported for healthy subjects, although this comparison must be considered carefully due to differences in measurement method as previously discussed.

The role of 8-isoprostane in lung cancer biology and lung pathology is still not clearly understood (Zieba et al., 2001), but there would seem to be important implications for isoprostane levels in human pulmonary pathophysiology, particularly their effect on smooth muscle and their role in mediating free radicals and reactive oxygen species (Janssen, 2001; Janssen, 2008).

In conclusions, the levels of 8-isoprostane as assessed by ELISA in overnight urine collected from lung cancer patients undergoing chemotherapy increased more than 6 to 12 fold from treatment cycle 1 to cycle 2 and 3, indicating significant production of ROS/RNS by chemotherapy drugs, and the resulting lipid peroxidation. Urine creatinine levels were shown to be abnormal for these patients, suggesting that uncorrected 8-isoprostane (pg/mL) or correction by urine volume (μg) is a more suitable measure than correction by urine creatinine (pg 8-isoprostane/mg creatinine).

The effect of cancer chemotherapy on oxidative stress in humans, and 8-isoprostane production, creatinine clearance and urine production has not been previously extensively studied, and future investigation is still warranted.

Further studies should also be conducted to determine whether cancer chemotherapy patients could benefit from antioxidant adjuvant treatment to reduce free radical induced oxidative stress and the associated cellular damage, without compromising the effectiveness of the chemotherapy.

Acknowledgements

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