Urinary hydrogen peroxide: a probable marker of oxidative stress in malignancy

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Abstract

Background: Urinary hydrogen peroxide was postulated to be a biomarker of oxidative stress. We estimated urinary hydrogen peroxide along with other established parameters of oxidative stress in malignancies where oxidative stress is well documented.

Methods: The oxidative stress markers tested were concentrations of erythrocyte glutathione, erythrocyte malonaldehyde (MDA) and plasma hydroperoxide, and activities of plasma glutathione-S-transferase (GST) and erythrocyte catalase. Urinary hydrogen peroxide was measured by a modified ferrous ion oxidation xylenol orange version-2 (FOX-2) method on a spot random sample of urine.

Results: In healthy controls (n = 10), erythrocyte glutathione concentration was 4.41 ± 0.057 mg/g of hemoglobin, plasma hydroperoxide was 2.5 ± 0.07 μmol/l, erythrocyte MDA was 0.9 ± 0.15 nmol/ml of packed cell suspension and erythrocyte catalase and plasma GST were 74.66 ± 9.2/s/ml of packed cell suspension and 6.12 ± 0.84 IU/l, respectively. In cancer patients (n = 25), erythrocyte glutathione, plasma hydroperoxide and erythrocyte MDA were 9.32 ± 0.42 mg/g of hemoglobin, 6.2 ± 0.13 μmol/l and 2.3 ± 0.27 nmol/ml of packed cell suspension, respectively; and activities of erythrocyte catalase and plasma GST were 151.04 ± 6.5/s/ml of packed cell suspension and 10.9 ± 0.36 IU/l, respectively. Urinary hydrogen peroxide concentration was 15 ± 9.8 μmol/l in the healthy controls and 56.3 ± 3.9 μmol/l in cancer patients.

Conclusion: Urinary hydrogen peroxide may be a marker of oxidative stress in malignancies.

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Keywords: Biomarker; Oxidative stress; Malignancy; Hydrogen peroxide

1. Introduction

There is a considerable current interest in the development of biomarkers of oxidative stress, which is associated with many disease conditions including malignancy [1]. Hydrogen peroxide (H₂O₂) is a ubiquitous biomolecule [2] and is found in substantial amounts in the urine of normal human subjects [2–4]. It has been suggested that urinary H₂O₂ can serve as a biomarker of oxidative stress [2,3]. There have been no reports comparing urinary H₂O₂ with the classical parameters of oxidative stress in a pathological condition where oxidative stress is well documented. In malignancy, the skeletal muscle cell forms increased reactive oxygen species like diabetes mellitus, and malignant cells are also known to produce ROS [5].
It has been shown that oxidative stress is associated with many disease conditions [1]. Although antioxidants are prescribed with the objective of controlling oxidative stress, they are not routinely tested in clinical practice. This is because estimation of many established parameters of oxidative stress requires sophisticated instruments like electron spin-resonance spectroscopy, high performance liquid chromatography, etc. Measurement of malonaldehyde (MDA) by 2-thiobarbituric acid (TBA) method is probably the most popular parameter of oxidative stress but it lacks specificity [6]. If urinary H$_2$O$_2$ behaves as a parameter of oxidative stress, it can be easily measured by spectrophotometric methods, which are fairly sensitive and specific [3,4].

2. Materials and methods

2.1. Patient selection

Urine and blood samples with heparin as anticoagulant were collected from histopathologically proved case of malignancy (who were planned for radiotherapy) in the Department of Radiotherapy, Kasturba Medical College Hospital, Manipal. Urine and blood samples were also collected from healthy controls. Informed consent was obtained from all subjects. Smokers and individuals consuming any antioxidant vitamins or herbal drugs were excluded from the study. Those who had stopped smoking for at least 2 months were considered as nonsmokers. More details of the healthy controls and cancer patients are shown in Table 1.

2.2. Sample preparation

Urinary H$_2$O$_2$ was estimated within 30 min of sample collection. Other parameters were examined within 6 h of sample collection. A sample with any evidence of hematuria or hemolysis was excluded. Blood was collected in siliconized glass syringe with heparin as anticoagulant. Immediately after collection blood was centrifuged at 3000 × g for 10 min. From the plasma, the established parameters of oxidative stress measured were hydroperoxide concentration and glutathione-S-transferase (GST) activity. The hemolysate was prepared from the packed cell suspension after carefully removing the plasma and Buffy coat and washing thrice with phosphate-buffered (pH 7.4) cold saline (sodium phosphate buffer containing 0.15 mol/l NaCl). The parameters measured from the hemolysate were MDA, glutathione (GSH), and catalase activity.

Xylenol orange, triphenylphosphine (TPP), HPLC grade methanol, butylated hydroxytoluene and catalase were from Sigma. All the other chemicals were at least of reagent grade. Double-distilled water was used throughout the study. All glassware were cleaned with concentrated nitric acid and thoroughly rinsed with double-distilled water and dried before an experiment.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Erythrocyte GSH mg/g of Hb</th>
<th>Plasma GST (IU/l)</th>
<th>Erythrocyte catalase activity/s/ml of packed cell suspension</th>
<th>Plasma hydroperoxide (µmol/l)</th>
<th>RBC MDA mmol/ml of packed cell suspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.41 ± 0.057</td>
<td>6.12 ± 0.84</td>
<td>74.66 ± 9.2</td>
<td>2.5 ± 0.07</td>
<td>0.9 ± 0.15</td>
</tr>
<tr>
<td>Cancer group</td>
<td>9.32 ± 0.42</td>
<td>10.9 ± 0.36</td>
<td>151.04 ± 6.5</td>
<td>6.2 ± 0.13</td>
<td>2.3 ± 0.27</td>
</tr>
<tr>
<td>$r$ of urinary H$_2$O$_2$ with classical parameters of oxidative stress</td>
<td>$r = 0.54$</td>
<td>$r = 0.65$</td>
<td>$r = 0.46$</td>
<td>$r = 0.892$</td>
<td>$r = 0.43$</td>
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<tr>
<td></td>
<td>$p &lt; 0.01$</td>
<td>$p &lt; 0.001$</td>
<td>$p &lt; 0.02$</td>
<td>$p &lt; 0.001$</td>
<td>$p &lt; 0.05$</td>
</tr>
</tbody>
</table>

$^a$ The cancer patient group ($n = 25$, 17 males and 8 females, age 44 – 64 years) comprised of 10 carcinoma esophagus, 9 laryngeal carcinoma, 3 cervical carcinoma and 3 breast carcinoma. The healthy control group ($n = 10$, age 40 – 60 years) comprised of 3 females and 7 males. In the third row, correlation coefficient ($r$) of urinary hydrogen peroxide concentration with the classical parameters of oxidative stress in cancer patients ($n = 25$) is given.
2.3. Estimation of urinary hydrogen peroxide and classical parameters of oxidative stress

Plasma hydroperoxide [7] and urinary H$_2$O$_2$ concentrations [3,4] were measured by ferrous ion oxidation xylenol orange version-2 (FOX-2) method [3,4,7]. Any sample oxidizing agent could cause oxidation of FOX-2 reagent ferrous ion to ferric ion which then bound with xylenol orange present in the reagent to form a colored complex with absorption maximum at 560 nm and measured spectrophotometrically. We have added triphenyl phosphine (TPP) [7] and catalase [4] to the sample prior to the addition of the FOX-2 reagent to determine hydroperoxide and H$_2$O$_2$ concentrations, respectively, to make the assays more specific for hydroperoxide and H$_2$O$_2$ as TPP reduces hydroperoxide and catalase significantly destroys H$_2$O$_2$.

To estimate the urinary H$_2$O$_2$ concentration, 90 $\mu$L of urine was placed in a centrifuge tube followed by addition of 10 $\mu$L of HPLC grade methanol and 900 $\mu$L of FOX-2 reagent. The centrifuge tube was vortexed and kept at room temperature for 30 min. Then it was centrifuged at 15,000 $\times$ g for 10 min and absorbance was noted at 560 nm. The concentration of H$_2$O$_2$ in the urine was calculated from freshly prepared standard curve. This was FOX-2 method [3]. To estimate urinary H$_2$O$_2$ concentration by modified FOX-2 method [4] at first the classical FOX-2 method was repeated. Then the same procedure was followed except 10 $\mu$L of methanol was replaced by 10 $\mu$L of catalase solution. The urinary H$_2$O$_2$ concentration was calculated from the absorbance difference (with and without catalase) at 560 nm using the freshly prepared standard curve.

The FOX-2 reagent [17] contained 100 $\mu$mol/ l xylene orange 250 $\mu$mol/l ammonium ferrous sulfate, 90% methanol (HPLC grade), 4 $\mu$mol/l butylated hydroxytoluene and 25 mmol/l sulfuric acid. The catalase solution [4] contained 2200 U of catalase/ml of 25 mmol/l phosphate buffer pH 7.

Creatinine concentration was estimated from each urine sample by the alkaline picrate method [8] and urinary H$_2$O$_2$ was expressed as micromolar in a spot random sample of urine and also as mmol/l of H$_2$O$_2$ per gram of urinary creatinine.

MDA was measured [9] by TBA method where MDA reacted with TBA to form a colored complex with absorbance maximum at 532 nm and was measured spectrophotometrically. Catalase activity measurement was based on the disappearance of H$_2$O$_2$ in the presence of sample catalase at 240 nm [10]. GST activity was determined spectrophotometrically by measuring the conjugation of 1-chloro 2,4 dinitrobenzene (CDNB) with GSH at 340 nm [11]. GSH concentration was measured by the reactivity of GSH with 5,5 dithio-bis-2-nitrobenzoic acid (DTNB) [12].

2.4. Statistical analysis

The values obtained were statistically analyzed and expressed as mean ± standard error of the mean (S.E.M.). An unpaired Student’s t-test was used to analyze the data and a $p<0.05$ value was assumed as significant. The correlation coefficient ($r$) of urinary H$_2$O$_2$ concentration and the classical parameters of oxidative stress were calculated in the study group of cancer patients, and a significant value of $r$ at the 5% level was considered as significant.

3. Results

In healthy controls, the urinary H$_2$O$_2$ concentration was 29 ± 2.1 $\mu$mol/l in a spot random sample of urine, and when expressed in mmol/l/g of urinary creatinine, it was 215.64 ± 12.5 by FOX-2 method. Through the modified FOX-2 method in healthy controls, the same group had values of 15 ± 0.98 $\mu$mol/l and 111.2 ± 5.88 mmol/l/g of urinary creatinine, respectively. In the study group of cancer patients, the urinary H$_2$O$_2$ concentration was 59.84 ± 4.85 $\mu$mol/l in a spot random urine sample and when expressed in mmol/l/g of urinary creatinine, it was 515.56 ± 15.12 via FOX-2 method. By the modified FOX-2 method, the group of cancer patients showed 56.3 ± 3.9 $\mu$mol/l and 343.25 ± 12.62 mmol/l/g of urinary creatinine, respectively. In the study group of cancer patients, the urinary H$_2$O$_2$ concentration was 59.84 ± 4.85 $\mu$mol/l in a spot random urine sample and when expressed in mmol/l/g of urinary creatinine, it was 515.56 ± 15.12 via FOX-2 method. By the modified FOX-2 method, the group of cancer patients showed 56.3 ± 3.9 $\mu$mol/l and 343.25 ± 12.62 mmol/l/g of urinary creatinine, respectively. This group showed a significantly higher value ($p<0.001$) of urinary H$_2$O$_2$ compared to the healthy control group. The individual values of urinary H$_2$O$_2$ by modified FOX-2 is shown in Fig. 1.

Erythrocyte GSH concentration ($p<0.05$), plasma hydroperoxide concentration ($p<0.01$), erythrocyte concentration ($p<0.01$), plasma GST activity ($p<$
**4. Discussion**

The FOX-2 assay is not specific for hydrogen peroxide, although urinary H$_2$O$_2$ concentration was measured before by FOX-2 assay [3]. Any sample oxidizing agent, irrespective of its chemical nature, can oxidize reagent ferrous to ferric ion which then can bind with FOX-2 reagent xylenol orange to give the colored complex with absorption maximum at 560 nm [3,4,17,18]. In fact, plasma hydroperoxide concentration was estimated by the FOX-2 assay previously [17,19]. FOX-2 assay was made more specific for hydroperoxides by performing it in the presence and absence of TPP, an agent which selectively reduces plasma hydroperoxides to their corresponding alcohols itself being converted into triphenylphosphine oxide [7,18]. In a similar way, the FOX-2 assay was made more specific for H$_2$O$_2$ (modified FOX-2) by performing it in presence and absence of catalase, the enzyme which significantly destroys H$_2$O$_2$ [4]. Therefore, we have presented urinary H$_2$O$_2$ values both by means of FOX-2 and modified FOX-2 method.

The reducing agents [17] like GSH, other thiols, and urate were only active at physiological pH and does not interfere in the acidic environment of the FOX assay. Vitamin C did not interfere with the FOX assay under physiological concentrations [18]. Moreover, H$_2$O$_2$ was reported to be recovered 100% from human urine by FOX assay [4]. Therefore, interference was not a serious problem when estimating H$_2$O$_2$ by FOX assay.

The results clearly demonstrated that patients suffering from malignancy excreted more H$_2$O$_2$ in a spot random urine sample than healthy controls along with an increase in the classical parameters of oxidative stress. The behavior of the classical parameters of oxidative stress proved that in the study group of cancer patients, there was a substantial amount of oxidative stress. Thus, in an environment of oxidative stress, there was presence of increased urinary H$_2$O$_2$. The correlation coefficients ($r$) (Table 1) of H$_2$O$_2$ with the measured parameters of oxidative stress suggested that it was the cause of increased H$_2$O$_2$ concentration in the patient’s urine.

There was more hydrogen peroxide in the patients’ urine despite increased erythrocyte catalase activity in these patients. Increase in the catalase activity was reported in many other malignancies as well as in response to oxidants in cultured cells [13]. This was because the source of urinary H$_2$O$_2$ at least by a part was superoxide, which generated H$_2$O$_2$ in the urine by autooxidation of biomolecules in presence of atmospheric 21% O$_2$ [3]. Therefore, increased catalase activity did not contradict the presence of increased amounts of urinary H$_2$O$_2$ among cancer patients. Moreover, there were reports of decreased superoxide dismutase activity in a number of malignancies [1], which could cause substantial excretion of superoxide, though increase in SOD activity has been reported in some cancers [14]. This can be solved by correlating erythrocyte SOD activity and urinary hydrogen peroxide concentration in cancer patients.
Although GSH concentration was reported to be reduced in oxidative stress, in the cancer patients group there was a significant increase of RBC GSH concentration. The increased GSH concentration in malignancy has been previously reported [15], and may be attributed to oxidative inactivation of glutathione peroxidase in malignancies [16]. Moreover, the increased concentration of TPP-reducible plasma hydroperoxide in the cases supported this possibility. Therefore, erythrocyte GSH, urinary H₂O₂ concentration and plasma hydroperoxide concentration correlated to each other in these patients.

In this study, it is shown that a group of 25 cancer patients showed a greater amount of H₂O₂ in their urine, which correlated well with some classical parameters of oxidative stress. We suggest that urinary H₂O₂ may be a marker of oxidative stress, at least in malignancy. We recommend a detailed study of urinary H₂O₂ in malignancy and other proven cases of oxidative stress.

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References