Glutathione-S-Transferase and Thiol Stress in patients with acute renal failure

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Abstract:
Introduction: Tubular damage is common finding in acute renal failure (ARF). Various etiologies have been put forth to explain the tubular damage in ARF, one important mechanism among them is oxidative damage to renal tubules. Several biomolecules including low-molecular weight peptides and enzymes in urine have been proposed as early markers of renal failure. Current study has been undertaken to study the thiol stress and glutathione-S-transferase (GST) levels in ARF patients. Method: 58 ARF patients and 55 healthy controls were selected based on inclusion and exclusion criteria. Serum thiols, GST, malonaldehyde (MDA) and urine thiols were determined by spectrophotometer based methods. Results: Serum thiols and urine thiols were significantly decreased (p<0.0001), and serum GST and MDA levels were significantly increased (p<0.0001) in ARF patients compared to healthy controls. Serum GST and MDA correlated positively in ARF cases (r² = 0.6938, p<0.0001). Conclusion: There is significant thiol stress and increased lipid peroxidation in ARF patients which leads to tubular cell membrane damage and release of GST into blood stream and into urine. This may be possible mechanism for the increased presence of GST in urine (enzymuria) found in other studies. Key Words: Glutathione-S-transferase; thiol stress; acute renal failure; urine thiols

Introduction: Acute renal failure (ARF) is characterized by a sudden or gradual decline in glomerular filtration rate (GFR), a slow and steady accumulation of nitrogenous waste products, and an inability of the kidney to regulate the balance of sodium, electrolytes, acid, and water. The ischemic damage in ARF is generally most severe in the early proximal tubule (S3 segment) and the thick ascending limb of the loop of Henle. Poor oxygenation leads to a variety of secondary factors that promote the development of tubular injury, including the intracellular accumulation of calcium, the generation of reactive oxygen species, depletion of adenosine triphosphate, and apoptosis.

is.(2-4) Many tubular enzymes have been studied as markers of the necrotic/apoptotic damage or dysfunction of (proximal) tubular cells. Three major origins have been identified: the lysosomes, the brush-border membrane, and the cytoplasm of the cells.(5,6)

Several studies have demonstrated that increased urinary amounts of enzymes are useful to detect acute tubular damage at a very early stage, but increased enzymuria may also be induced by a reversible mild dysfunction of the cells not necessarily associated with irreversible damage. The usefulness of enzymuria may be obscured by the low threshold for release of tubular enzymes, even in response to injury that may not proceed to ARF.(7) However, enzymes are also released during chronic glomerular diseases, which might limit their use as a marker of tubular injury only.(8-11) Some of the best-characterized tubular enzymes to detect tubular injury are glutathione-S-transferases (GSTs), γ-glutamyl transferase (γ-GT), alkaline phosphatase (AP), lactate dehydrogenase (LDH), NAG, fructose-1,6-biphosphatase, and Ala-(Leu-Gly)-aminopeptidase (8,9) Increased urinary excretion of these proteins implies tubular injury.

GSTs are important in intracellular binding and transport of numerous compounds, and play a central role in human detoxification process. Human GSTs mainly consists of class Pi (GST π), Alpha (GST α), Mu (GST μ) and Theta (GST θ) enzymes, each subdivided into one or more isoenzymes. They catalyze the conjugation of glutathione with wide variety of xenobiotics such as carcinogens, pharmacologically active agents, as well as reactive oxygen species (ROS). The conjugation may result in the formation of more water soluble and less biologically toxic molecules that may be easily excreted. In addition to detoxification, GSH is important in storage and transport of amino acids. The characteristic feature of the tripeptide GSH (γ-glutamylesteineglycine) is the presence of reactive sulphhydryl (–SH) group donated by cysteine in GSH is provided by cysteine, and this dictates the chemistry of GSH.(12)
Reactive oxygen species (ROS) have been implicated in the renal cell injury that occurs with reperfusion after ischemia. Products of lipid peroxidation are generated on reperfusion, and these are presumed to derive from ROS action on membrane lipids.(13,14) Scavengers such as superoxide dismutase (SOD), glutathione, and vitamin E, as well as inhibitors of ROS production, such as the iron chelator deferoxamine, have been reported to protect against ischemic injury.(15,16) Exposure of kidney subcellular organelles or microsomes to ROS-generating systems mimics some features of ischemic injury.(14-17) Lipid peroxidation is frequently used as an indicator of oxidative damage in the kidney.(13,18,19)

In the current study we have determined the GST activity, thiols status along with lipid peroxidation markers in ARF patients and compared them with that of healthy individuals to know the difference and to understand the biochemical basis for the change observed.

Materials and Methods:

Subjects
Fifty eight subjects with ARF were selected as cases. Fifty five healthy controls were participated in this study. Inclusion criteria for ARV cases are: age > 18 years, ARF of any etiology, defined by more than 30% rise in serum creatinine from baseline, patients with renal failure presenting to the hospital for the first time with short history (<3 months duration), and ultrasound showing normal sized kidneys (~8.5cm). Exclusion criteria: age <18 years, obstructive acute renal failure, patients with preexisting history of renal failure (acute on chronic renal failure), patients with history of diabetes mellitus or hypertension, kidney size <8.5cm on ultrasound or evidence of hydronephrosis, patients presenting as sepsis with acute renal failure. Healthy controls aged more than 18 years with no past or present history of any medical illness, not on any kind of medication, non-smokers; non-alcoholics were included in the study.

Under aseptic conditions blood was drawn into plain vacutainers from ARF cases and healthy controls, allowed to clot for 30 min, and then centrifuged at 3000 rpm for 15 min for separation of serum. All assays were performed immediately after serum was separated. Twenty four hour urine sample from 58 ARF cases and 55 healthy controls was collected in a brown bottle containing toluene as urine preservative, urine sample bottle was stored at 4°C during the period of collection. Samples were centrifuged at 3000 rpm for 10 minutes and were analyzed immediately after the collection period. Informed consent from the subjects involved in the study and ethical clearance from institutional review board was taken.

Reagents
Special chemicals like reduced glutathione (GSH), 1-chloro 2,4-dinitrobenzene (CDNB), 5'5' dithio-bis (2-nitrobenzoic acid) (DTNB), 1, 1, 3, 3-tetraethoxypropane and thiobarbituric acid (TBA) were obtained from Sigma chemicals, St Louis, MO, USA. All other reagents were of analytical grade.

Table 1: Independent sample t test for all the determined biochemical parameters in both healthy controls and acute renal failure cases (values expressed as mean ± standard error of mean, both minimum and maximum value observed also shown)

<table>
<thead>
<tr>
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<th>Healthy Controls (n = 55)</th>
<th>Acute Renal Failure Cases (n = 58)</th>
</tr>
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<tbody>
<tr>
<td>Serum Thiols (μmoles/L)</td>
<td>346.18±7.21 Min: 261.83, Max: 439.38</td>
<td>240.03±20.78* Min: 32.50, Max: 750.00</td>
</tr>
<tr>
<td>Urine thiols (μmoles/L)</td>
<td>19.90±1.96 Min: 1.88, Max: 51.25</td>
<td>81.79±14.74* Min: 4.40, Max: 552.50</td>
</tr>
<tr>
<td>Serum GST (IU/L)</td>
<td>0.92±0.02 Min:0.62, Max: 1.25</td>
<td>15.16±2.90* Min: 48, Max: 81.25</td>
</tr>
<tr>
<td>Serum MDA (nmol/L)</td>
<td>156.35±8.05 Min: 121.79, Max: 206.69</td>
<td>385.23±4.73* Min: 111.54, Max:394.34</td>
</tr>
<tr>
<td>Urine Creatinine (gm/L)</td>
<td>0.76±0.06Min:0.8, Max: 1.57</td>
<td>1.24±0.35, Min: 0.04, Max: 15.12</td>
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P <0.0001 compared to healthy controls.

Because of wide variation in the observed parameters, we have also analyzed the above parameters by Mann Whitney rank sum test. As mentioned in Table 2, there was significant decrease in the serum thiols (p<0.0001), and significant increase in serum GST (p<0.0001), serum MDA (p<0.001) and urine thiols (p<0.001) in ARF patients compared to healthy controls. On applying Pearson’s correlation, we have seen serum GST correlated positively with serum MDA (r² = 0.694, p<0.0001) (Figure 1).

**Methods**

**Serum GST and MDA, and serum and urine total thiols were measured using GeneSys 10UV spectrophotometer. Urine creatinine levels were determined by automated clinical chemistry analyzer Hitachi 912.**

**Serum GST assay**

One mL reaction mixture containing 850 μL of 0.1 M Phosphate buffer pH 6.5, 50 μL CDNB 20 mM, 50 μL 20 mM GSH, was preincubated at 37°C for 10 min. Reaction was started by adding 50 μL serum or urine. GST activities were assayed kinetically by noting changes in absorbance at every 1 min interval for 5 min at 340 nm. Serum and urine GST activity was determined by using molar extinction coefficient 9.6 M⁻¹ cm⁻¹ (20-22) and was expressed in IU.

**Serum and urine total thiol assay**

100 μL serum or urine was added to reaction mixture containing 900 μL 2 mM Na₂EDTA in 0.2 M NaHPO₄, 20 μL 10 mM DTNB in 0.2 M NaHPO₄, incubated at room temperature for 5 min and absorbance was read at 412 nm. Similarly absorbance of sample blank and reagent blank was subtracted from serum and urine absorbance values to obtain corrected values. The calibration curve was produced using GSH dissolved in phosphate buffered saline (PBS). Total thiol levels were determined using molar extinction coefficient 1600 M⁻¹ L·cm⁻¹ (12).

**Serum MDA assay**

We have followed Satoh’s method (24), where 100 μl of sample, 1000 μl of 0.67% TBA and 500 μl of 20% TCA were nucrate at 100°C for 20 minutes; transferred the content to Eppendorf tube and centrifuged at 12,000 rpm for 5 minutes. The absorbance of the supernatant was read at 532 nm against water blank. 1, 1, 3, 3-tetraethoxypropane (1 μmol) was used as a standard for MDA standard graph and to obtain extinction coefficient (C) for the malonaldehyde-TBA complex which was 1.56×10⁴ M⁻¹ L·cm⁻¹.

**Statistical analysis**

All statistical analysis was done using statistical package for social sciences (SPSS) version 16. Independent sample t test and Mann Whitney U test was done to compare mean values. A Pearson’s correlation was used to correlate between the parameters. P value <0.05 was considered significant. Microsoft office excel 2 was used to prepare correlation figures.

**Results:**

As depicted in Table 1, we have found significant decrease in the serum thiols in ARF patients compared to healthy controls (p<0.0001), however, urine thiols were increased in ARF cases (p<0.0001). Serum GST activity found to be increased in ARF cases compared to healthy controls (p<0.0001). Membrane lipid peroxidation marker MDA levels were found to be higher in ARF cases compared to healthy controls (p<0.0001). We have observed significant skewed values in all the parameters that we have determined (mentioned in Table 1 as minimum and maximum values).

Materials and Methods:

**Reagents**

Because of wide variation in the observed parameters, we have also analyzed the above parameters by Mann Whitney rank sum test. As mentioned in Table 2, there was significant decrease in the serum thiols (p<0.0001), and significant increase in serum GST (p<0.0001), serum MDA (p<0.001) and urine thiols (p<0.0001) in ARF patients compared to healthy controls. On applying Pearson’s correlation, we have seen serum GST correlated positively with serum MDA (r² = 0.694, p<0.0001) (Figure 1).
In increased presence of urine thiols in ARF patients observed by glutathione and protein bound thiols into urine also explains in ARF patients. Renal tubular damage and possible leak of contents into bloodstream and urine. This may possibly species causing tubular membrane damage and loss of cytosol there is possibility of generation of enormous amount of ROS in combination with increased presence of MDA suggest that stress in these patients. The significant decrease in thiol status in ARF patients indicating the increased presence of oxidative found increase in the levels of lipid peroxidation marker MDA ficant decrease in serum thiols in ARF patients. We have also includes both glutathione and protein thiols, and found signi- tations.(25-29) Thiols are organic sulfur derivatives that are characterized by the presence of sulfhydryl residues at the active site. Halliwell and others (30-32) have demonstrated that protein-associated thiols, particularly in the albumin molecule, constitute a major defense against oxidative stress in plasma. In our previous study we have shown that there is protein thiol oxidation and lipid peroxidation in patients with uremia.(33) Glutathione, normally present in high amounts in tubular cells, can react with and neutralize ROS. Cellular glutathione levels fall with ischemia (34), and reduced cellular glutathione levels sensitize cells to oxidative stress.(35) Protective effects of glutathione have been reported, although it remains controversial as to whether these effects are due to the antioxidant characteristics of this compound or due to the generation of glycine, its metabolic product, independent of ROS scavenging. As with other ROS scavengers, glutathione administration has yielded inconsistent results.(36,37)

In our study, we have determined the total thiol status which includes both glutathione and protein thiols, and found signifi- cant decrease in serum thiols in ARF patients. We have also found increase in the levels of lipid peroxidation marker MDA in ARF patients indicating the increased presence of oxidative stress in these patients. The significant decrease in thiol status in combination with increased presence of MDA suggest that there is possibility of generation of enormous amount of ROS species causing tubular membrane damage and loss of cytosol contents into bloodstream and urine. This may possibly explain the increased presence of serum GST that we have found in ARF patients. Renal tubular damage and possible leak of glutathione and protein bound thiols into urine also explains increased presence of urine thiols in ARF patients observed by us. In total our study, in line with similar previous study with different experimental designs agrees with the fact that ARF causes increased generation of ROS generation and depletion of antioxidants. Furthermore, previous authors have observed inverse association between plasma protein thiol content and the plasma levels of proinflammatory cytokines IL-6, IL-8, and TNF- suggest that inflammation and oxidative stress. Critical phenomena with ARF manifest a marked increase in plasma protein oxidation, including plasma protein thiol group oxidation and carbonyl formation.(38)

ROS can damage tissue in a variety of ways. They can cause lipid peroxidation by abstracting a hydrogen atom from a polyunsaturated fatty acid of membrane phospholipids; a conjugated diene forms after molecular rearrangement of the fatty acid. The diene then reacts with oxygen to form a peroxide radical, which can remove hydrogen atoms from other lipids, generating a chain reaction. Lipid peroxidation can increase plasma and subcellular membranes' permeability (39), impair enzymatic processes and ion pumps (14), and damage DNA. (40,41) In addition, direct oxidation of membrane proteins occurs (42), affecting critical proteins such as the sodium-potassium ATPase and the Ca2 ATPase. The role of ROS in ischemic renal injury remains controversial because investigators do not all agree that antioxidants confer protection (43,44), nor do all agree on the presence of increased lipid peroxidation or ROS generation in ischemia.(43,44)

In conclusion, we have observed increased presence of oxidative stress environment in patients with ARF as denoted by depletion of thiol status and increased presence of MDA causing membrane damage and hence leakage of GST and thiols into urine.

**References:**

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