

Original article

EFFECT OF PRESERVATIVES ON GLUTATHIONE LEVELS IN HUMAN RED BLOOD CELLS *IN VITRO*

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Abstract Glutathione (GSH) is an antioxidant and a biological marker of oxidative stress in the cells. The aims of this study were to measure GSH levels in healthy subjects (n=20) compared to those with chronic renal failure (CRF) (n=10), and investigate the effect of preservatives compared to an oxidizing agent in both normal and CRF human erythrocytes. The red blood cells (RBCs) were incubated with the reagents for various times and then GSH levels were measured. It was found that the mean GSH level in normal people was 53.6±8.7 mg/dL erythrocyte, whereas that of CRF was 52.8±17.1 mg/dL erythrocyte, which was statistically different ($p<0.05$). On incubating normal RBCs with preservatives (i.e. sodium benzoate, sodium metabisulfite) and an oxidizing agent (potassium perchlorate) for 90 min, the mean GSH levels were 47.7±5.4, 39.5±5.7 and 49.3±4.5 mg/dL erythrocyte, respectively. When incubating for 180 min, the mean GSH levels were 43.0±5.3, 50.9±4.7 and 45.8±4.6 mg/dL erythrocyte, respectively. In the same experiment with CRF patients, the mean GSH levels at 90 min were 51.0±5.4, 39.5±5.7 and 49.3±4.5 mg/dL erythrocyte, respectively and at 180 min, 43.0±5.3, 50.9±4.7 and 45.8±4.7 mg/dL erythrocyte, respectively. A significant difference was found between groups of samples and time ($p<0.05$). In this study, when treating the RBCs with preservatives and an oxidizing agent, GSH levels changed, which meant GSH could be activated by preservatives that act as an oxidant. The CRF patients should be aware of taking food that contains preservatives, since they are hazardous to life, and it might be beneficial to use the above experiments as a GSH instability test. **Chiang Mai Med Bull 2004;43(3):105-112.**

Keywords: glutathione (GSH), chronic renal failure (CRF), erythrocytes or red blood cells (RBCs), preservatives, sodium benzoate, sodium metabisulfite, potassium perchlorate

Oxidative stress occurs when there is and the antioxidant defence mechanism
(are) oxidizing agent(s) in the system is impaired. The molecular targets of

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Received 29 January 2004, and in revised form 26 February 2004.

reactive oxygen species include protein, DNA, membrane lipid, carbohydrate (hyaluronic acid), and antioxidants such as glutathione. Free radicals can cause several diseases, e.g. cancer, atherosclerosis, aging and autoimmune diseases.⁽¹⁾

Glutathione (GSH) is a thiol tripeptide antioxidant found in the cells and is composed of glutamic acid, glycine and cysteine. It can come in two forms, which are oxidized glutathione (GSSG) and reduced (GSH). The GSH form is found mostly (95%). Its functions are to (1) normalize the oxidative stress by counteracting free radicals, (2) detoxify toxins by acting in concert with glutathione S-transferase, (3) transport amino acid in the γ -glutamyl cycle, (4) act as a cofactor for glutathione peroxidase enzyme to scavenge hydrogen peroxide, and (5) maintain the viability and integrity of red blood cells (RBCs).⁽²⁾

Preservatives are chemicals added to food in order to prevent it from decaying or inhibit the proliferation of infectious microorganisms. The action mechanism of sodium benzoate affects the wall or enzymes of the microorganisms by inhibiting the growth of yeast, fungi, and bacteria. The maximal amount of sodium benzoate safe for humans is less than 0.1%. Sodium metabisulfite would produce sulfurous acid, bisulfite ion and sulfite ion. Sulfurous acid acts on microorganisms to inhibit their enzyme function, and suppress growth and proliferation.

Chronic renal failure (CRF) is an end stage disease that may be caused by several etiologies, such as vascular diseases, tubular abnormality, interstitial diseases, or nephrotoxins. At the end of the disease, CRF patients develop uremia, which includes symptoms of nausea, vomiting, malaise, seizure, drowsiness, and unconsciousness.⁽³⁾ There were several reports of enhanced oxidative stress found in CRF patients in France,⁽⁴⁾ Poland,^(5, 6) Turkey,⁽⁷⁾ India⁽⁸⁾ and Sweden^(9,10) as plasma aldehyde concentration, 4-hydroxyalkenals, hydroxynonenal and oxidized lipoprotein (oxLDL) increased and GSH decreased.⁽¹¹⁾

It was intriguing to demonstrate whether oxidative stress is present in Thai CRF patients by measuring GSH levels in human RBCs. The aims of this study were to demonstrate the levels of glutathione in CRF patients compared to normal subjects, and illustrate the effect of preservatives on the erythrocytes of stressed CRF patients compared to normal RBCs *in vitro*. The GSH levels changed when the RBCs were incubated with sodium benzoate and sodium metabisulfite, as in the system of incubation with potassium perchlorate, which is an oxidant. This assay showed that the RBCs were activated and it might be used as an instability test for determining glutathione levels.

Materials and methods

Sodium benzoate, sodium metabisulfite, potassium perchlorate, glutathione, 5,5'-dithiobis(2-nitrobenzoic acid) or DTNB and ethylenediaminetetraacetic

acid (EDTA) were obtained from Sigma. Other chemicals were obtained from Merck.

The blood was collected from the CRF patients who attended a special clinic at the Department of Surgery for an arteriovenous shunt operation. Normal blood was obtained from the blood bank and screened for infectious diseases (i.e. hepatitis viruses and HIV). Ten milliliters of whole blood was collected and stored at 4 degrees Celsius until use or for a maximum of 1 week. EDTA was used as anticoagulant. The CRF patients were dialyzed frequently, which made the shunt necessary. Neither normal subjects nor CRF patients received any drugs.

The normal subjects and CRF patients were screened to exclude the status of glucose 6-phosphate dehydrogenase (G6PD) deficiency by methemoglobin reduction assay.⁽¹²⁾ Briefly, whole blood (0.2 mL) was added to glucose/sodium nitrite (0.01 mL) and methylene blue (0.01 mL), mixed well, and incubated at 37 degrees Celsius for 3 h. Distilled water (5 mL) was added and the color was observed. For the G6PD deficient patients, the solution was brown in color and for the normal subjects, red.

Determination of glutathione by the DTNB method⁽¹³⁾

Whole blood (0.4 mL) was added to distilled water (1.6 mL) together with 3 ml of precipitating solution (1.67 g% glacial metaphosphoric acid, 0.7 mM ethylenediaminetetraacetic acid (EDTA) and 5.13 M sodium chloride). Then the

filtrate (1 mL) was added to 0.05 M phosphate buffer, pH 6.4 (4 mL). Finally 1 mM DTNB (0.5 mL) was added, mixed well and the absorbance was read at 412 nm within 4 min.

Statistical analysis

The GSH levels in normal subjects were compared to CRF patients by using the Student-*t*-test. The other data were analyzed by two way ANOVA. Statistical differences were considered at $p < 0.05$.

Results

The mean glutathione level in CRF patients (52.8 ± 17.1 mg/dL erythrocyte) was lower than that of the normal subjects (53.6 ± 8.7 mg/dL erythrocytes) as shown in Fig. 1, which was significantly different ($p < 0.05$). It was reported that oxidative stress was present in CRF patients, with evidence of low levels of antioxidant molecules and antioxidant enzymes in other races.^(5-10,14) This was the first report of Thai end stage renal failure patients that showed GSH levels in RBCs lower than those of normal subjects. Such low levels of GSH might be caused from anemia, which usually coexists in CRF patients due to a low concentration of erythropoietin,^(11,15) and the status of low selenium and erythropoietin.⁽¹⁶⁻¹⁷⁾ After the correction of renal anemia, malondialdehyde (MDA) levels were significantly lower than in anemic CRF patients, which reflected a decreased in free radical generation.⁽¹⁸⁾

The G6PD patients had to be excluded first, since their pentose phosphate pathway was impaired. That made the NADPH

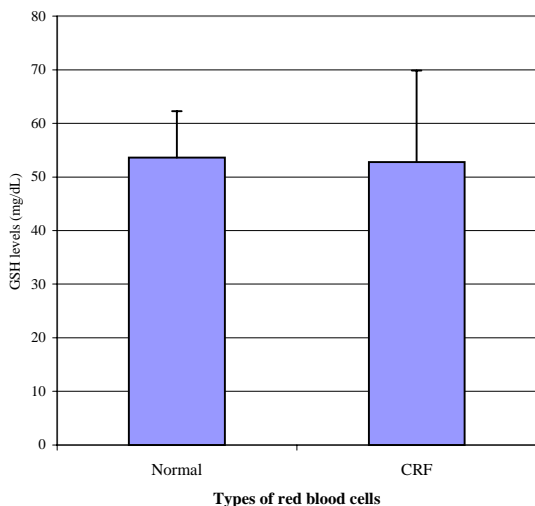


Figure 1. Glutathione (GSH) levels in RBCs of normal subjects (n=20) and CRF patients (n=10). The value represented as mean \pm SEM.

(nicotinamide adenine dinucleotide phosphate) level decrease and cause initially low levels of GSH.⁽¹⁹⁾ Such an event interfered with the response of GSH change in RBCs, due to the preservatives and oxidants. The RBC potential of G6PD patients in response to stress from chemical treatment could be less than in normal human RBCs.

In CRF patients, the patterns of mean GSH levels are shown in Fig. 2. Descriptively, the mean GSH level was first increased when treating erythrocytes with sodium benzoate at 90 min, and then decreased at 180 min. The pattern of erythrocyte changes meant mean GSH levels were the same as those of benzoate when incubated with potassium perchlorate. However, when RBCs were treated with sodium metabisulfite the mean level of GSH was decreased at 90 min and then increased at 180 min.

When the mean GSH levels in RBCs of normal subjects were compared at 0, 90 and 180 min, the mean GSH levels were decreased after activation with sodium benzoate in a time-dependent manner. The condition of RBC incubation with potassium perchlorate was the same as that with sodium benzoate *in vitro*. Whereas the GSH level was first decreased in the condition of treatment with sodium metabisulfite at 90 min, and then increased when incubated at 180 min as shown in Fig. 3. The data of the two types of subjects were analyzed at various times by using two way ANOVA, and they were statistically significant ($p < 0.05$), which means that the GSH levels were significantly different in the two types of hosts at various time points.

Discussion

Preservatives are frequently used to preserve food for a longer period of time. From this study, both sodium benzoate and sodium metabisulfite were able to change the erythrocyte GSH level in more or less the same as potassium perchlorate, which is an oxidant. Hence, this should be made known when people consume various kinds of food that contain preservatives. The two preservatives, i.e. sodium benzoate and sodium metabisulfite, may be considered as oxidizing agents that contain the same effect in changing GSH levels. The change of GSH levels may be used as a screening test for G6PD.⁽²⁰⁾

As mentioned before, there is oxidative stress in various kinds of diseases

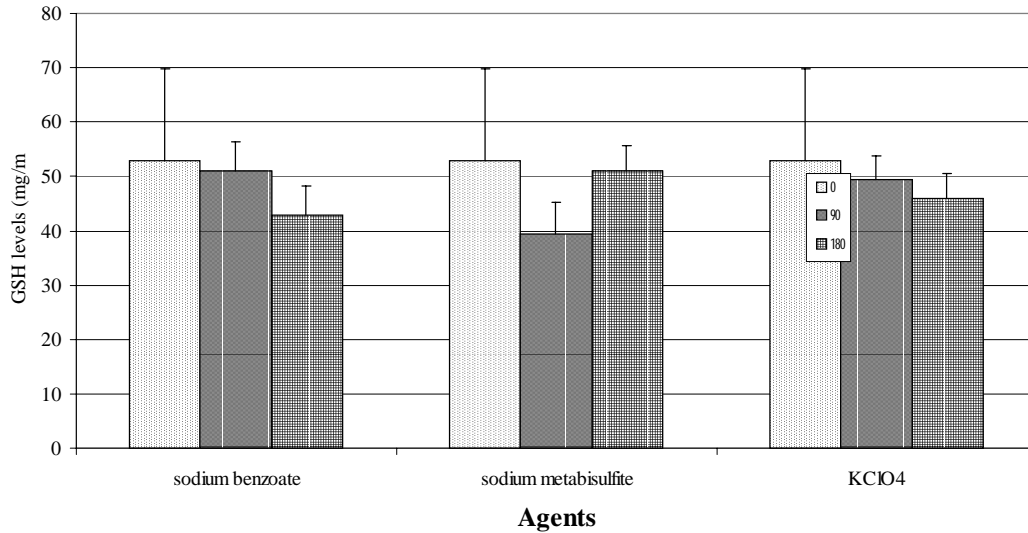


Figure 2. Glutathione (GSH) levels in RBCs of CRF patients after treatment with preservatives (i.e. sodium benzoate, sodium metabisulfite) and potassium perchlorate at 0, 90 and 180 min, respectively. The value represented as mean \pm SEM.

Figure 3. Glutathione (GSH) levels in normal RBCs after incubation with preservatives (i.e. sodium benzoate, sodium metabisulfite) and potassium perchlorate at 0, 90 and 180 min, respectively. The value represented as mean \pm SEM.

such as lung inflammation, amyotrophic lateral sclerosis, malignant diseases, diabetes mellitus and chronic renal failure (CRF). The proximal renal tubular cells are the site in the kidney that has high

oxidative metabolic activity and, in CRF, is associated with adaptive hypertrophy and hypermetabolism.⁽²¹⁾ CRF patients are subjected to increased oxidative stress, as a result of reduced antioxidant

systems (vitamin C and selenium deficiency, reduced intracellular levels of vitamin E, reduced activity of the glutathione system) and increased prooxidant activity (advanced age, high frequency of diabetes, chronic inflammatory state, uremic syndrome, and bioincompatibility of dialysis membranes and solutions).

Oxidative stress and inflammation are deeply inter-related, as different free radicals are generated by phagocytic cells in response to inflammatory stimuli. Both are related to endothelial dysfunction, as the endothelium is a source and target of oxidants and participates in the inflammatory response. There is growing evidence from experimental and clinical studies, that oxidative stress may be implicated in the pathogenesis of atherosclerosis and other complications of CRF, namely dialysis-related amyloidosis, malnutrition and anemia.⁽²²⁾ There was evidence of oxidative stress in CRF patients before hemodialysis, which increased further after hemodialysis, the mechanism of which is not delineated.⁽²³⁾

This finding might make us (normal subjects) and CRF patients become aware of taking food-containing preservatives, since they may decrease antioxidant molecules, i.e. glutathione and other antioxidant vitamins such as vitamin C and E. Thus, when food with preservatives is consumed, more antioxidants should be taken to keep a balance and prevent the status of oxidative stress.

Conclusion

When erythrocytes were treated with preservatives, i.e. sodium benzoate and

sodium metabisulfite, the levels of glutathione changed when incubated for 90 and 180 min. Hence, such preservatives could alter the levels of glutathione and act as oxidants. This assay may be called a "GSH instability test", since the erythrocytes are capable of or contain the potential to respond to oxidative stress by changing the GSH levels. However, the GSH pool (both oxidized and reduced forms) is constant and there is interchange between the two forms. The decrease of GSH level or oxidative stress is recognized as an important risk factor that could be prevented by optimal treatment. Improvement in renal function may be accomplished with antioxidant therapy following the correction of oxidant and antioxidant imbalance.

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ผลของสารกันเสียต่อระดับกลูตาไรโอนินในเม็ดเลือดแดงของมนุษย์

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บทคัดย่อ กลูตาไรโอนินเป็นสารต้านอนุมูลอิสระและเป็นเครื่องหมายของภาวะเครียดออกซิเดชันภายในเซลล์ วัตถุประสงค์ของการศึกษานี้เพื่อวัดระดับกลูตาไรโอนินในคนปกติ (จำนวน 20 คน) เปรียบเทียบกับในผู้ป่วยโรคไตวาย (จำนวน 10 คน) และศึกษาผลของสารกันเสียเปรียบเทียบกับสารออกซิแดนซ์ในเม็ดเลือดแดงของคนปกติ และผู้ป่วยไตวาย เมื่อเม็ดเลือดแดงถูกบ่มด้วยสารดังกล่าวและวัดระดับกลูตาไรโอนินที่เวลาต่างๆ พบว่าค่าเฉลี่ยของระดับกลูตาไรโอนินในเม็ดเลือดแดงของคนปกติเท่ากับ 53.6 ± 8.7 มก./ค.ล. และในผู้ป่วยโรคไตวายเท่ากับ 52.8 ± 17.1 มก./ค.ล. ซึ่งมีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) เมื่อบ่มเม็ดเลือดแดงของคนปกติด้วยสารกันเสีย (คือโซเดียมเบนโซเอทและโซเดียมเมตาไบซัลไฟท์) และสารออกซิแดนซ์ (โปแตสเซียมเปอร์คลอเรท) เป็นเวลา 90 นาที วัดระดับกลูตาไรโอนินเฉลี่ยได้เท่ากับ 47.7 ± 5.4 , 39.5 ± 5.7 และ 49.3 ± 4.5 มก./ค.ล. ตามลำดับ เมื่อบ่มเป็นเวลานาน 180 นาที พบว่าระดับกลูตาไรโอนินเฉลี่ยเท่ากับ 43.0 ± 5.3 , 50.9 ± 4.7 และ 45.8 ± 4.6 มก./ค.ล. ตามลำดับ ในการทดลองแบบเดียวกันในผู้ป่วยโรคไตวาย ระดับกลูตาไรโอนินเฉลี่ยที่เวลา 90 นาทีเท่ากับ 51.0 ± 5.4 , 39.5 ± 5.7 และ 49.3 ± 4.5 มก./ค.ล. ตามลำดับ และที่เวลา 180 นาทีเท่ากับ 43.0 ± 5.3 , 50.9 ± 4.7 และ 45.8 ± 4.7 มก./ค.ล. ตามลำดับ ซึ่งมีความแตกต่างอย่างมีนัยสำคัญในระดับกลูตาไรโอนินระหว่างกลุ่มและเวลาที่ต่างกัน ($p < 0.05$) โดยสรุปเมื่อบ่มเม็ดเลือดแดงด้วยสารกันเสียและสารออกซิแดนซ์ ระดับกลูตาไรโอนินมีการเปลี่ยนแปลงโดยสารกันเสียทำหน้าที่เหมือนเป็นสารออกซิแดนซ์ ผลจากการศึกษานี้จะเป็นประโยชน์ต่อผู้ป่วยโรคไตที่ควรระวังเรื่องการบริโภคอาหารที่ใส่สารกันเสีย เพราะจะทำให้เกิดอันตรายได้ และการตรวจวัดนี้มีประโยชน์สามารถใช้เป็นการทดสอบความไม่เสถียรของกลูตาไรโอนินได้ด้วย **เชียงใหม่เวชสาร 2547;43(3):105-112.**

คำสำคัญ: กลูตาไรโอนิน โรคไตวาย เม็ดเลือดแดง สารกันเสีย โซเดียมเบนโซเอท โซเดียมเมตาไบซัลไฟท์ โปแตสเซียมเปอร์คลอเรท