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Glutathione and its associated enzymes in peripheral blood cells in different stages of chronic renal insufficiency

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Summary. Reduced glutathione (GSH) levels and glutathione reductase (GR) and glutathione S-transferase (GST) activities were investigated in the erythrocytes and lymphocytes of non-dialyzed patients with varying degrees of chronic renal insufficiency, and also of patients on regular hemodialysis treatment. GSH, GR and GST levels were higher in erythrocytes and lymphocytes of examined patients as compared to their corresponding age-matched healthy controls. A correlation was found between the degree of renal insufficiency and the above parameters tested. A routine hemodialysis did not significantly affect erythrocyte and lymphocyte GSH content and activities of its associated enzymes. The increased GSH levels as well as GSH-linked enzyme activities of blood cells in uremia may be a protective mechanism for the cells due to the accumulation of toxic, oxidizing, wastes in the blood as a result of the uremic state. This view is supported by the results of *in vitro* experiments, which have shown that GR and GST activities of normal human lymphocytes are increased when incubated with plasma from uremic patients.

Keywords: Amino acids – Glutathione – Glutathione reductase – Glutathione S-transferase – Chronic renal insufficiency

Introduction

The tripeptide glutathione (GSH) is the principle low molecular weight non-protein thiol in most cells. It is important in free radical scavenging, maintenance of redox state, conjugation of toxic substances, and amino acid transport (Meister and Anderson, 1983). Reduced glutathione (GSH) is generally considered indispensable in the oxidant defense and survival of erythrocytes and other cells (Beutler and Dale, 1989).

When GSH serves as a reductant in oxidation-reduction reactions, this function results in the formation of glutathione disulfide (GSSG, oxidized

glutathione). The reduction of GSSG by glutathione reductase (E.C.1.6.4.2) is subsequently of fundamental importance for regeneration and maintenance of high level of reduced glutathione in the cells (Schrimer et al, 1989).

Reduced glutathione, mediated by glutathione S-transferase (E.C. 2.5.1.18), also acts as a detoxicant in eliminating different electrophilic compounds of exogenous and endogenous origin (Jakoby, 1978).

Uremic symptomatology is widely attributed to the toxic action of endogenous and exogenous compounds accumulating in the blood. The syndrome of chronic renal insufficiency is recognized as a complex problem, involving all the body's systems and reflecting biochemical alterations in all aspects of the constitution of the internal environment (Wills, 1990). So, it might be expected that these retained substances affect the intracellular as well as extracellular levels of glutathione and its associated enzyme activities. The possible role of GSH in erythrocyte detoxicant mechanisms in chronic renal failure has not, as yet, univocally been established. A few reports, with conflicting results, are available on the effect of chronic uremia on GSH content. In the patients with chronic uremia on regular hemodialysis treatment erythrocytes reduced glutathione content were reported to be normal (Chanhau et al, 1982) or increased (El-Rashidy et al, 1984, Mimic-Oka et al, 1988) or decreased (Vanella et al, 1983). Besides, relatively little information exists on the glutathione-related enzyme activities in erythrocytes of patients on the regular hemodialysis treatment (Mellisinos et al, 1981, Ferrone et al ,1970, Yawata et al, 1971, Carmagnol et al, 1981). Previous studies from this laboratory have documented a significant correlation between the degree of renal insufficiency and increase in erythrocyte reduced glutathione levels (Mimic-Oka et al, 1988). Furthermore, the influence of different stage of chronic renal insufficiency on the above parameters in both erythrocytes and lymphocytes has not been examined.

The present study is designed to examine the levels of reduced glutathione (GSH) and glutathione-linked enzymes, glutathione reductase (GR) and glutathione S-transferase (GST), in erythrocytes and peripheral blood lymphocytes of non-dialyzed patients with varying degrees of chronic renal insufficiency, as well of patients with end-stage renal failure on regular hemodialysis. We present here, also, the results of the *in vitro* studies on influence of uremic plasma on GSH level and GR and GST activities in normal peripheral blood lymphocytes.

Materials and methods

Subjects

The study included 3 groups of subjects. The first group was composed of 33 patients (16 males and 17 females), with a median age of 40 years (range 20 to 56), suffering from chronic renal failure of variable etiology. According to their serum creatinine clearance (Ccr), patients were divided into three subgroups: Ccr <20 ml/min, Ccr 20–50 ml/min and Ccr 51–80 ml/min. The second group included ten patients (6 males and 4 females), with a median age of 45 years (range 27 to 61), with end-stage renal insufficiency undergoing 3 times a week hemodialysis session. Mean time of hemodialysis was 62 ± 10 months. Exclusion criteria were subjects with intercurrent diabetes, chronic respiratory insufficiency, alcoholics and smokers. The third (control) group consisted of 14 healthy volunteers (9 males and 5 females),

aged 37 years (range 24 to 50 years). Both patients and volunteers gave their informed consent to enter the study.

Collection of blood samples

Five ml peripheral venous blood for analysis was collected in the morning in EDTA (1 mg/ml). In hemodialyzed patients, blood samples were taken immediately before hemodialysis and additional blood samples were drawn from the dialyzer venous line at the end of the dialysis.

Isolation of blood cell

The erythrocytes and lymphocytes were separated from 5 ml of blood samples by the Ficoll-Hypaque separation using a modification of the method of Boyum, 1968. The blood was carefully layered over 5 ml of Ficoll-Hypaque and centrifuged at 1700 rpm for 30 min at 4°C. The white ring (lymphocytes) was removed from the aqueous-ficoll interface. The isolated lymphocytes were resuspended in 15 ml of ice physiological saline solution and centrifuged at 7,000 rpm and 4°C for 10 min. The procedure was repeated three times. This method yields $1-2 \times 10^7$ cells/10 ml of whole blood. After removing the lymphocytes, the plasma and ficoll aqueous phases were removed by aspiration, and the erythrocytes were resuspended and washed three times in 15 ml of 0.9% NaCl solution and separated by centrifugation at 4,000 rpm at 4°C for 5 min. After separation, the cells were diluted to 10 ml with 0.9% NaCl. The cell counting was carried out using a Coulter Counter. For the biochemical assays, the suspension of erythrocytes and lymphocytes were lysed with distilled water (1:5 v/v) and three freeze-thaw cycles. The supernatant solution, obtained by centrifugation of cells lysate for 20 min at 10,000 g, was used for the determinations.

In vivo study

Glutathione determination

Reduced glutathione concentration in erythrocytes and lymphocytes was determined by the fluorimetric method of Hissin and Hilf (1976).

Enzyme assays

The glutathione reductase (GR) activity was measured by the method of Carlberg and Mannervik (1985). One unit of GR activity is defined as the amount of enzyme which catalyzes the oxidation of 1 μmol NADPH/min. The glutathione S-transferase (GST) activity was measured by spectrophotometric assay procedure of Habig (1974), using 1-chloro-2,4-dinitrobenzene as the substrate. One unit of GST activity is defined as the amount of enzyme which catalyzes the formation of 1 μmol of product/min. The aliquot of the lymphocyte and erythrocyte lysate supernatant from 6×10^5 cells was used for enzyme activities determinations and each sample was assayed in triplicate.

Protein content

Erythrocytes and lymphocytes lysates were assayed for total protein by the method of Lowry (1951), using bovine serum albumin as standard.

In vitro study

Cross-incubation experiments were performed under sterile conditions. Peripheral blood lymphocytes from healthy subjects were isolated and cultured in RPMI 1640 medium (GIBCO) at a cell concentration of 1×10^6 cells/ml, in the presence of predialysis uremic plasma or autologous plasma (10% v/v) in a humid 5% CO_2 atmosphere at 37°C for 24

hours. At the end of incubation period, lymphocytes were collected and cell viability was assessed by evaluating cell morphology under phase-contrast microscopy and by the trypan blue exclusion test (Freshney, 1987). Reduced glutathione, glutathione reductase and glutathione S-transferase were estimated in 10,000 g supernatant fractions of cell lysates.

Statistics

All results are presented as the mean value ± 1 standard deviation (SD). When two groups were compared, unpaired Student's t test was employed. The limit of significance was set up at $p < 0.05$. The correlation in the various groups were studied by a regression analysis.

Results

The reduced glutathione levels were from 19 to 70% higher in erythrocytes and from 20 to 200% in lymphocytes of patients with different degree of chronic renal insufficiency than in the corresponding age-matched health control (Fig. 1). The results presented in Fig. 1 also show that GSH content of peripheral blood cells increases with renal deterioration. Patients with a creatinine clearance of less than 50 ml/min have a significantly increased erythrocytes and lymphocytes GSH levels. The highest levels of reduced GSH were found in subjects with creatinine clearance <20 ml/min (Fig. 1). A significant negative correlation was found between the creatinine clearance and the erythrocytes and lymphocytes GSH levels ($r = -0.770$, $p < 0.01$; and $r = -0.686$, $p < 0.01$, respectively). No significant differences were noted in comparing the GSH contents of the two cell fractions pre- and post-hemodialysis of the uremic patients (Table 1). The

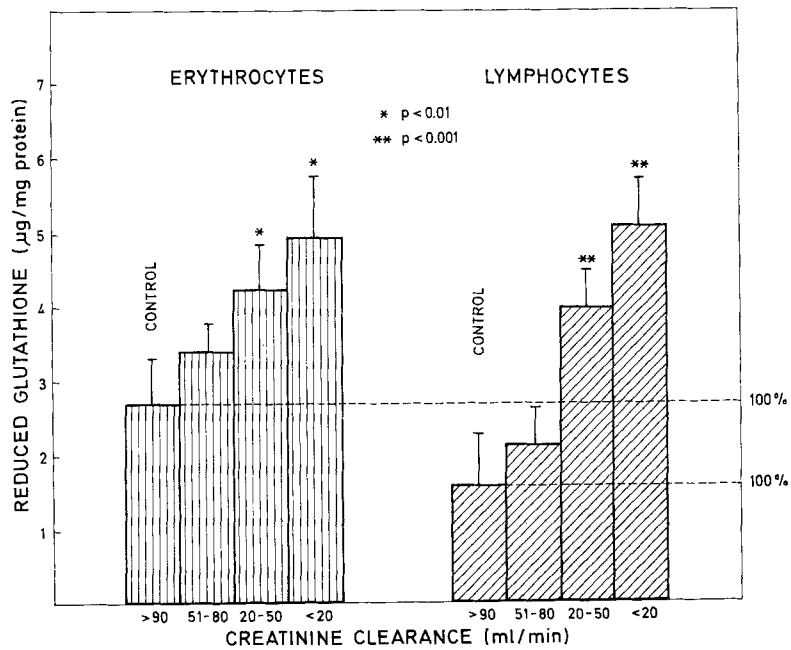

Fig. 1. Glutathione levels in erythrocytes and lymphocytes of healthy persons (control) and patients with chronic renal insufficiency. Each value is the mean $\pm$ SD; p-value is calculated to the age-matched control group. Control levels = 100%

Table 1. Glutathione, glutathione S-transferase and glutathione reductase activities in the blood cells of uremic patients pre- and post-hemodialysis

	Glutathione (nmol/mg prot)	Glutathione S-transferase ($U/10^6$ cells)	Glutathione reductase ($U/10^6$ cells)
A. Erythrocytes			
Control	8.52 ± 1.07 (14) 100%	2.93 ± 0.82 (14) 100%	3.54 ± 0.71 (14) 100%
Pre-dialysis	13.60 ± 1.79* (10) 159%	7.30 ± 1.15** (10) 249%	8.50 ± 0.98** (10) 240%
Post-dialysis	13.27 ± 1.63* (9) 155%	6.95 ± 0.65** (9) 237%	8.03 ± 0.31** (9) 227%
B. Lymphocytes			
Control	5.21 ± 1.01 (14) 100%	3.52 ± 0.70 (14) 100%	4.25 ± 0.78 (14) 100%
Pre-dialysis	19.36 ± 3.58** (10) 371%	6.60 ± 0.86** (10) 187%	6.10 ± 0.46* (10) 143%
Post-dialysis	16.43 ± 2.47** (9) 315%	6.24 ± 0.90** (9) 177%	5.80 ± 0.68* (9) 136%

One unit (U) of glutathione S-transferase activity is defined as the amount of enzyme catalyzing the formation of 1 μmole product/min; a unit (U) of glutathione reductase activity is 1 μmole of NADPH/min.
Each value is the mean ± SD of the number of samples given in parentheses.
* $p < 0.01$; ** $p < 0.001$

activity of glutathione reductase (GR) in erythrocytes and lymphocytes from control subjects (with creatinine clearance >90 ml/min) and patients with varying degrees of chronic renal failure is presented in Fig. 2. Erythrocytes GR activity was 33 to 79% higher in patients groups. An increase in lymphocyte GR activity was less pronounced than in erythrocytes (Fig. 2) and it accounts for 11 to 40% higher values in uremic subjects than in healthy persons (Fig. 2). Statistically significant negative correlation between erythrocyte and lymphocyte glutathione reductase activities and creatinine clearance have been observed.

Glutathione S-transferase (GST) activity in erythrocytes and lymphocytes from the control group and subjects suffering from chronic renal impairment is shown in Fig. 3. Enzyme activity was 30 to 180% higher in the erythrocytes of the patient groups as compared to the healthy individuals. With respect to the lymphocytes, GST activity was from 22 to 188% higher in patients groups

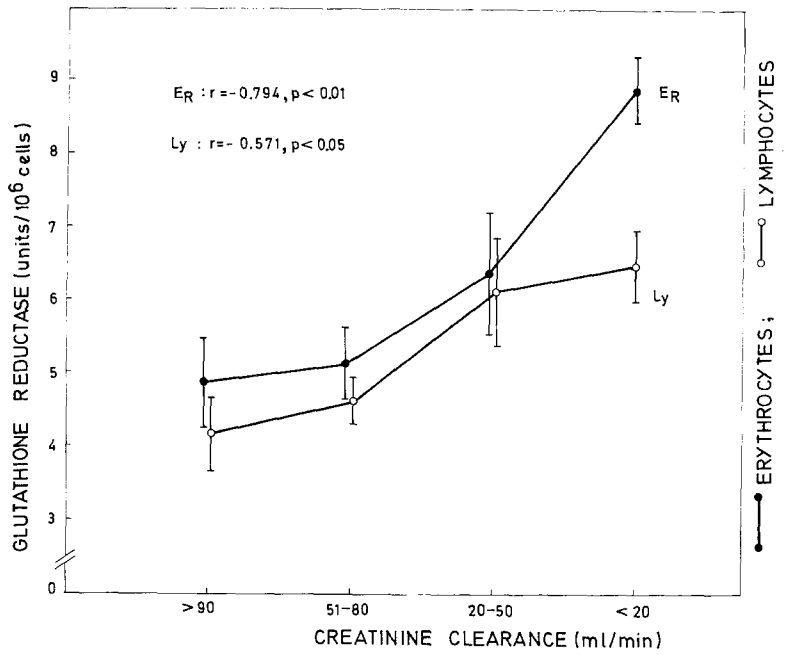

Fig. 2. Glutathione reductase activity in erythrocytes and lymphocytes of healthy persons (with creatinine clearance > 90 ml/min) and non-dialyzed patients with various degrees of chronic renal insufficiency. Each value is the mean ± SD; one unit of the enzyme activity is defined as the amount of enzyme which oxidizes 1 μmole of NADPH/min; r = coefficient of correlation between the enzyme activity and creatinine clearance

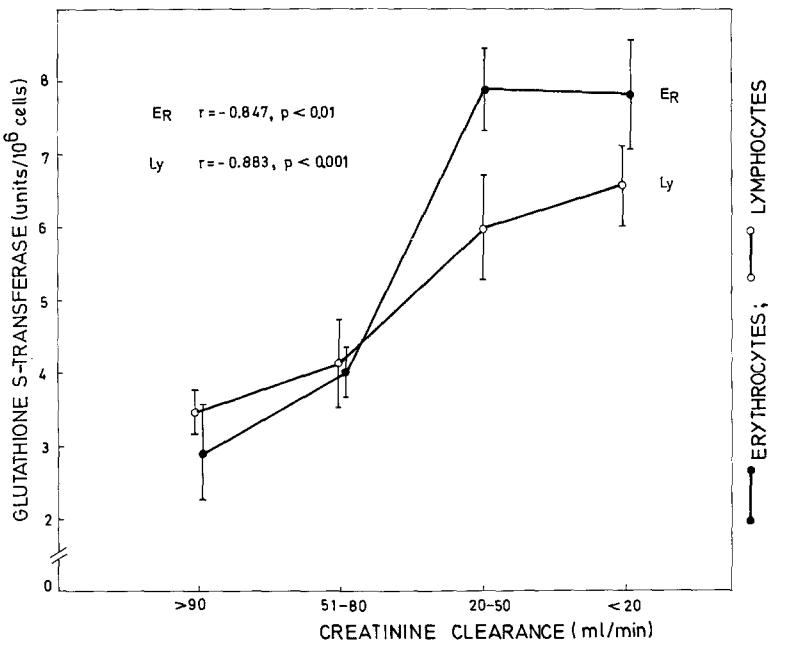

Fig. 3. Glutathione S-transferase activity in erythrocytes and lymphocytes of healthy persons (with creatinine clearance > 90 ml/min) and non-dialyzed patients with various degrees of chronic renal insufficiency. Each value is the mean ± SD; one unit of the enzyme activity is defined as the amount of enzyme catalyzing the formation of 1 μmole product/min; r = coefficient of correlation between the enzyme activity and creatinine clearance

Table 2. Glutathione content, glutathione reductase and glutathione S-transferase activities in normal peripheral blood lymphocytes after 24h of culture without and with uremic plasma

	Reduced glutathione (nmol/10^6 cell)	Glutathione reductase (U/10^6 cells)	Glutathione S-transferase (U/10^6 cells)
Control[a] PBL	1.61 ± 0.29 100%	4.14 ± 1.03 100%	3.91 ± 0.85 100%
PBL + AP	0.96 ± 0.12 59%	4.52 ± 1.51 109%	3.79 ± 0.90 97%
PBL + UP	0.66 ± 0.18 41%	7.32 ± 2.03 176%	5.83 ± 1.32 149%

Peripheral blood lymphocytes (*PBL*) were incubated for 24h in the presence of autologous (*AP*) or uremic (*UP*) plasma (10% v/v).
[a] Control PBL represented cells freshly prepared in which GSH, GR and GST were measured before culture under the same conditions used for cultured cells.
All results are expressed as a mean of 4 experiments ± SE and each sample was measured in triplicate.

(Fig. 3). Statistically significant increases in the GST activities in the both the cells' fractions were observed in patients with creatinine clearance less than 50 ml/min. There is also significant negative correlation between the creatinine clearance and GST activities in the both types of peripheral blood cells examined ($r = -0.847$, $p < 0.01$; and $r = -0.833$, $p < 0.001$, respectively). Therefore, a correlation between the degree of renal insufficiency and all parameters tested was found. Five hours hemodialysis session did not significantly alter erythrocyte and lymphocyte glutathione reductase and glutathione S-transferase activities (Table 1).

Incubation of peripheral blood lymphocytes from healthy subjects with uremic plasma samples, taken before hemodialysis from chronic uremic patients, increased glutathione reductase and glutathione S-transferase activities (Table 2). However, significant decrease in reduced GSH level in normal lymphocytes incubated in the presence of either uremic or autologous plasma, as compared to the background GSH levels in cells, before the onset of culture, were observed. Table 2 also shows that the decrease in GSH levels is more pronounced in the presence of uremic plasma. In these experiments, the morphology of the peripheral blood lymphocytes, evaluated by phase-contrast microscopy, was similar in cells exposed to uremic plasma and autologous plasma. More than 90% of the cells incubated in the presence of uremic or autologous plasma excluded the Trypan blue dye.

Discussion

Reduced glutathione levels, glutathione reductase and glutathione S-transferase activities were measured in erythrocytes and lymphocytes of healthy persons and

subjects suffering from chronic renal failure to determine the effect of the degree of renal insufficiency on the above parameters. Glutathione levels, as well glutathione reductase and glutathione S-transferase activities, in peripheral blood cells from uremic patients were significantly higher than in corresponding age-matched healthy control group. The more pronounced enhancement of the parameters tested have been found in the cells from the patients at the end-stage of renal failure. There is also statistically significant correlation between the degree of renal insufficiency and reduced glutathione levels, glutathione reductase and glutathione S-transferase activities in both types of blood cells. No significant reduction of GSH levels as well as GST and GR activities was obtained after a five hour hemodialysis session. At the same time, no variations in plasma reduced glutathione levels in the relation to the degree of chronic renal failure was observed (Mimic-Oka et al., 1988). Regular hemodialysis, over a long period of time, did not significantly alter erythrocyte and lymphocyte GSH contents and its associated enzyme activities, despite the efficiency in elimination of low molecular toxic metabolites.

The results presented in this study are in agreement with the results of Mellisinos et al. (1981), Ferrone et al. (1970) and Yawata et al. (1971) who have reported higher glutathione reductase activities in erythrocytes and serum from uremic patients. Carmagnol et al. (1981) have measured GST activity in erythrocytes of uremic patients and also found higher enzyme activity in uremic subjects as compared to healthy individuals.

However, our results are in contrast to those of some other (Bernard et al., 1966; Madec et al., 1966), where no differences in erythrocytes GR activity have been found. The discrepancies between the various studies may be due to a technical factor such as the sensitivity of the method used, and in the case of GR activity to the different riboflavin nutritional status of erythrocytes (Beutler and Dale, 1989). All those results mentioned have been obtained for erythrocytes of subjects at the end-stage of renal failure. In our knowledge, there is no evidence that the influence of the degree of chronic renal insufficiency on the above parameters in both lymphocytes and erythrocytes has been investigated.

Our results obtained in *in vitro* experiments confirm the *in vivo* findings. In peripheral blood lymphocytes from healthy persons, after 24h of incubation in the presence of 10% uremic plasma, increased GR and GST activities were observed. These results seem to demonstrate that in uremic plasma some factor(s) exist(s) which are able to induce glutathione-linked enzyme activities with a protective role against toxic injury. Similar results have been reported by Brunetti et al. (1965) for erythrocyte glutathione reductase activity.

Because the RPMI 1640 culture medium is lacking in cysteine, and because lymphocytes are deficient in the activity to synthesize cysteine from methionine (Ishii et al, 1987), the GSH level of lymphocytes incubated in our *in vitro* experiments was lowered. The results obtained in this study also suggest that, besides the deleterious effects of culture itself on glutathione synthesis, a loss of reduced glutathione in the presence of uremic plasma is, at least partly, increased by its utilization in the processes of conjugation and detoxification of uremic toxins.

Our results show only slightly lower GSH levels in the erythrocytes of

regularly hemodialyzed patients than in the erythrocytes of nondialyzed patients with a creatinine clearance of <20 ml/min. On the contrary, in the peripheral blood lymphocytes such a difference was not observed. At present, we are not able to explain the reason(s) for the inefficiency of the dialysis over a time period of several years in reduction of intracellular GSH content and its associated enzymes activities. According to a very recent findings of Richard et al. (1991), the possible explanation is an increased production of free radicals in dialyzed subjects by polymorphonuclear leukocytes, caused by an activation by the dialysis membrane itself or by a compound released by this membrane. The possibility that higher molecular weight retention products also contribute to the elevation of intracellular GSH content, by affecting glutathione-related enzyme activities, can not be ruled out.

Since in the chronic renal failure the human red blood cells and lymphocytes are perforce in an environment in which they are exposed to both extracellular and intracellular high concentrations of different uremic toxins, it is not surprising that they are equipped with mechanisms to defend themselves against these toxins. One of the major protective mechanisms for detoxification is the glutathione redox cycle (Meister and Anderson, 1983). Glutathione reductase is an enzyme critical for maintaining the proper proportions of reduced and oxidized glutathione in cells. Thus, the overexpression in GR activity in erythrocytes and lymphocytes may represent an attempt by these cells to replenish their reduced glutathione levels as a response to supraphysiological oxidant stress due to the uremic state.

One of the very important roles of GSH is its participation in conjugation of toxic compounds. GSH forms S-conjugates with a number of electrophilic compounds of endogenous or exogenous origin, through a reaction catalyzed by glutathione S-transferase (Jakoby, 1978). Under normal conditions, glutathione synthesis is mainly regulated by a feedback inhibition by glutathione (Meister and Anderson, 1983). Conjugates of GSH can disrupt this feedback mechanism and cause an elevated level of GSH synthesis (Kondo et al, 1984). Therefore, the possibility that glutathione conjugates of uremic toxic ligands, might cause an increase in GSH synthesis by this mechanism, should also be considered.

Taken together, the results of *in vivo* and *in vitro* studies indicate that enhancement of glutathione and glutathione-linked enzyme activities, glutathione reductase and glutathione S-transferase, could be a compensatory protective mechanisms for the cells due to the accumulation of toxic, oxidizing, wastes in the blood in uremia. This supports the hypothesis of Beutler (1989), that peripheral blood cells may serve as a small detoxifying packets in removal harmful substances from the plasma, utilizing GST to detoxify them by conjugation with GSH. Besides, evidence for an active red blood cell transport system for GSH S-conjugates has been obtained, and it has been suggested that this system may function as an emergency mechanism that protects cells from toxic injury (Beutler, 1983).

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