Original Article *J. Clin. Biochem. Nutr.*, **43**, 210–220, November 2008

Role of Vitamins C and E in Regulating Antioxidant and Pro-Oxidant Markers in Preeclampsia

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Received 5 March, 2008; Accepted 23 June, 2008

Summary **We compared three groups of pregnant women: placebo with normotensive women, group A which included preeclamptics, and group B which comprised preeclamptics who were supplemented their diets with vitamins C and E. MDA increased from 6.22 ± 2.8 (placebo) to 8.48 ± 1.2 (A) and 8.02 ± 1.8 nmol/gHb (B). NO concentrations were enhanced from 19.3 ± 4.2 (P) to 23.8 ± 6.4 (A) and 24.1 ± 5.4 µmol/L (B). GSH contents were decreased from 10.42 ± 2.81 (P) to 8.02 ± 2.92 (A) and 9.39 ± 1.02 µmol/g Hb (B), whereas GSSG concentrations increased from 0.98 ± 0.28 (P) to 1.24 ± 0.29 (A) and 1.08 ± 0.12 µmol/g Hb (B). SOD activity decreased 23% in A and 14% in B; GRx decreased 27% in A and 5.5% in B; GPx decreased 12% in A and 9.6% in B. Catalase activity, however, increased 27% in A and 29% in B as compared to control. Thus, we conclude that the use of vitamins C and E should be considered for the control of certain important biochemical indices during the development of preeclampsia; however, further studies are needed to develop methods for the prevention of preeclampsia in women at high risk.**

Key Words**: vitamin C & E, antioxidants & pro-oxidants, enzymatic & non-enzymatic, preeclampsia**

Introduction

Preeclampsia is a disorder of pregnancy characterized by pregnancy-induced hypertension (≥140 mmHg systolic and/or ≥90 mmHg diastolic blood pressure), new-onset proteinuria (≥300 mg protein/day), and edema occurring in the second half of pregnancy. A significant reduction in the incidence of preeclampsia was found in women at risk who were taking vitamins C and E supplements [*1*]. Antioxidant supplements in these women were also shown to be associated with changes in indices of oxidative stress and placental functions [*2*]. The vitamin C content in both mild and severe preeclamptics was found to decrease significantly [*3*] in association with enhanced peroxidation [*4*] and lowered thiol levels [*5*]. The reason for the decrease in plasma ascorbate is not very clear, but it is consistent with the hypothesis that uteroplacental perfusion induces oxidative stress and ascorbate consumption [*6*]. The functional consequence and the possible role of the decrease in plasma vitamin C reserves in preeclampsia (PE) are consistent with the fact that women who ingested less vitamin C than the recommended daily allowance (8.5 mg) were at a 2-fold increased risk of developing PE [*7*]. Currently, there are several large multicenter trials in progress to determine the efficacy of antioxidant therapy in the prevention of preeclampsia. Some negative results [*8*] have appeared but more results are awaited finally with interest and will determine whether antioxidants are effective in preventing preeclampsia in all populations, or whether such therapy will be population specific. However, in a recent review, antioxidant therapy has been suggested and that the prophylactic supplementation of vitamins C and E to pregnant women is associated with reduced incidence of preeclampsia and decreased placental and endothelial dysfunction [*9*].

In preeclampsia, an inverse correlation between vitamin E

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levels and lipid peroxidation has been found [*10*] suggesting that vitamin E content in plasma may be used as a prognostic marker of the likely development of preeclampsia [*11*]. In addition, there was a relationship found between the extent of vitamin E deficiency and increased lipid peroxidation with enhanced diastolic blood pressure [*12*]. Thus, decreased plasma levels of vitamin E could be a contributory factor of hypertension in preeclampsia, but other studies have not demonstrated significant differences in plasma vitamin E levels between normal and preeclamptic women [*13*]. Moreover, some results are at variance with the prevailing opinion, since increased plasma levels of vitamin E among women with preeclampsia as compared to those in normotensive pregnant women have been reported [*14*]. The latter study does not support the hypothesis of a causal relationship between decreased dietary vitamin E consumption and plasma levels and the development of preeclampsia. Thus, there is not a consensus whether or not these antioxidant vitamins exert a positive effect to control preeclamptic developments, especially as they pertain to oxidative stress, this area needs further study.

During pregnancy, nitric oxide (NO) is one of the most important relaxing factors of myometrium and also no less important in the control of blood flow in uterus and placenta. Oxidative stress developed in preeclampsia may cause endothelial dysfunction, which may lead to hypertension by decreased release of vasodilating agents such as NO [*15*]. NO is a potent vasodilator, and its altered production by the vascular endothelium may influence the pathogenesis of preeclampsia [*16*]. NO regulates leukocyte adhesion to the endothelium [*17*] and inhibits vascular smooth muscle cell proliferation and platelet aggregation [*18*].

All these functions could be impaired by the consumption of NO by superoxide anions produced during oxidative stress, thereby diminishing NO bioavailability. Consistent with this are the findings of a diminished level of NO in plasma of preeclamptic women [*19*]. The status of plasma NO in preeclampsia is, however, still controversial, and different groups have reported an increase [*20*, *21*], a decrease [*22*, *23*] or no change [*24*] in NOx levels (nitrites and nitrates, products of stable degradation of NO). A pathogenic link between serum uric acid is evident from several studies [*25*, *26*] although it has dual roles. It may have beneficial functions [acting as an antioxidant] as well as detrimental actions [to stimulate vascular smooth muscle cell proliferation and induce endothelial dysfunction] [*27*]. Thus, our present study also aimed to evaluate NO and uric acid contents in the plasma of control and preeclamptics with and without vitamin supplements.

There is no satisfactory treatment of this disease, except steps to avoid or reduce complications such as hypertension and eclamptic seizures and early delivery is the only successful treatment. It is hypothesized that preeclamptic condition could be explained by alterations in the function of vascular endothelium and placenta, likely in response to reduced perfusion, produces circulating factor(s) that alters endothelial function. These factor(s) are products of oxidative stress [*28*] and placenta may be the site of the lipid peroxides [*29*]. The maternal circulating components were found to regulate oxidative status of glutathione redox cycle and adhesion molecule expression in endothelial cells (ECs). Reduced cellular glutathione, and glutathione reductase (GRx) activity and altered glutathione peroxidase (GPx) activity accompany increased inflammatory reactions in ECs responding to circulatory toxic factors in preeclampsia [*30*]. Various studies have highlighted the asymmetric alterations in the activities of superoxide dismutase (SOD), catalase, GRx and GPx [*5*, *30*, *31*].

In view of the contradictory reports and the seriousness of preeclampsia, the present study included the determination of the activities of catalase, SOD, GPx and GRx in the blood of normal and preeclamptic women with and without vitamin treatment, along-with the contents of MDA, GSH, GSSG, GSSG/GSH, NO and uric acid, to collectively evaluate the roles of vitamins C and E in the regulation of these factors.

Materials and Methods

Chemicals

ATP, NADPH, GSH, GSSG, Aspergillus nitrate reductase, and GRx, EDTA, TBA and butylated hydroxytoluene (BHT) were obtained from Sigma Chemical Company (St. Louis, MO.). Other chemicals were obtained from E. Merck (Mumbai, India). All other reagents used were of analytical grade, either from BDH or SISCO Chemicals (Mumbai, India).

Subjects

In India, pregnant women are encouraged to book and to attend regular antenatal check ups. Standard antenatal care is defined as monthly visits up to 28 weeks; fortnightly until 34 weeks and weekly visits thereafter. The patients in our study included pregnant women with normal blood pressure, preeclamptic women admitted to our hospital who had been or not under regular care, and also those who were referred from private sectors or primary health centers. The control group (placebo) consisted of 20 normotensive pregnant women who were taking regular prenatal vitamins from the second trimester of pregnancy to delivery.

Group A comprised 22 patients who were not taking any vitamin supplements, as they belonged to rural areas with incomes below the poverty-line; they were not booked cases and they arrived with full term pregnancies just a few days before delivery. Group B initially consisted of a high number of patients who were viewed as being at an increased risk of

preeclampsia because of factors such as preeclampsia in a preceding pregnancy, HELLP syndrome, or eclampsia in any past pregnancy at any gestational stage, and who were taking additional vitamin supplement (1000 mg vitamin C plus 400 IU vitamin E per day) from the second trimester of pregnancy to delivery as mentioned for the controls (placebo). At a later stage, however, some of the women in this group did not appear at the time of full term delivery. For this group, 21 women who had developed severe preeclamptic conditions agreed to donate blood.

The present study was carried out with the prior approval of the local ethical committee. All the patients mentioned above gave their consent in writing, and the objectives of the study were fully explained to them in detail prior to taking consent. They were always instructed to fast between 9 p.m. till retiring in the night before the blood collection. On the morning of the test they were requested to have only a light breakfast such as simple toast avoiding any cooked breakfast, meat, cereals, fruit juices and any nutritional supplements. They were advised to abstain from having coffee, chocolates, etc. for at least 2 h before coming for blood sampling; 8 ml of venous blood was collected in EDTA-treated tubes from the cubital vein.

Clinical examination and history taking excluded women addicted to tobacco, patients with diabetes, ischemic heart disease, a history of stroke, kidney disorders or other conditions of known free radical etiology. The criteria for dividing women into normotensive and preeclamptic groups have been set at a blood pressure of 140/90 mmHg or higher, proteinuria and edema.

Estimation of plasma levels of NO as stable metabolites (nitrite + nitrate) [*21*]

To 300 µl of plasma was added 300 µl of KH_2PO_4/K_2HPO4 buffer (pH 7.5), 50 µl of 2 mM NADPH, 50 µl of 50 µM FAD and 50 µl of I unit/ml aspergillus nitrate reductase. This was incubated at room temperature for one hour followed by the addition of 500 µl of 0.2 M Z_nSO_4 and 70 µl of 2 M NaOH to deproteinize the sample. After centrifugation, 0.75 ml of the supernatant was added to 1 ml of 1% sulphanilic acid (in 4 M HCl). After 10 min at room temperature, 0.75 ml of freshly prepared 1% N-naphthyl ethylene diamine was also added. The resultant color developed was measured at 548 nm using spectrophotometer. Nitrite concentration was calculated using nitrite standard solution.

Uric acid estimation [*32*]

Simple colorimetric method was employed. To 4 ml. N/23 sulphuric acid, 0.5 ml. serum was added and mixed. 0.5 ml. 5.6 % sodium tungstate was then added, mixed, and centrifuged. 3 ml of the decanted supernatant was placed in a test tube, to which was added 0.2 ml PTR (Phosphotungstic acid reaction reagent), mixed well, followed by the addition of 1.0 ml 0.6 N sodium hydroxide. Reading was taken after 15 min, at 720 nm on spectrophotometer.

Isolation of erythrocyte and hemolysate preparation

The blood samples were centrifuged at 1000 g for 15 min at 4°C and the isolated red cells were washed 4–5 times with 0.154 M NaCl to remove plasma and buffy coat. After the final wash, the required packed cells were lysed by hypotonic shock and different dilutions were used as hemolysate.

Hemoglobin estimation [*33*]

Hemoglobin content of the erythrocyte was measured using cyanmethemoglobin method.

Determination of reduced glutathione (GSH) [*34*]

Packed red cells (0.2 ml) were used in the assay. The GSH was made to react with 5'5-dithiobis (2-nitrobenzoic acid) [DTNB], which reacts with sulfhydryl groups, to develop a stable color. The absorbance was measured at 412 nm and GSH content was expressed as µmol/gHb.

Quantitation of oxidized glutathione (GSSG) [*35*]

Erythrocytes lysate was deproteinized with 0.5 M $HClO_4$. Then estimation was made on the basis of reduction of GSSG in presence of NADPH and GRx and decrease of NADPH at 340 nm after initiating the reaction by adding GRx was taken as an index of GSSG content, which was evaluated and expressed as µmol/gHb.

Estimation of lipid peroxidation [*36*]

Packed red cells (0.2 ml) were used for the quantitation of malondialdehyde (MDA) as thiobarbituric acid reactive substances (TBARS). Aliquots of 0.2 ml were mixed thoroughly with 0.8 ml of phosphate buffered saline (pH 7.4) and 25 µl of butylatedhydroxytoulene solution. After adding 0.5 ml of 30% trichloroacetic acid, the samples were placed on ice-bath for 2 h and then centrifuged at 2000 × g at 25°C for 15 min. One ml of supernatant was mixed with 75 µl of 0.1 M EDTA and 250 µl of 1% thiobarbituric acid in 0.05 M NaOH and placed on boiling water for 15 min. After cooling to room temperature, absorbance was measured at 532 nm. MDA contents are expressed as nmol/gHb.

Assay of GPx activity [*37*]

GPx activity was measured spectrophotometrically at 340 nm in 50 mM phosphate, 5 mM EDTA, pH 7.0 containing 0.3 mM NADPH, 0.3 U/ml GRx, 5 mM GSH, 4 mM sodium azide, 75 µM H_2O_2 and 10 µl of erythrocyte lysate in a final reaction mixture of 3 ml. One unit of GPx was considered to be the amount necessary to oxidize 1 µmol NADPH/min. Activity was expressed as U/gHb.

Assay of SOD activity [*34*]

The assay mixture contained 1M Tris, 5 mM EDTA buffer, pH 8.0, and 10 mM pyrogallol and the inhibition of pyrogallol oxidation by SOD was monitored at 420 nm, and the amount of enzyme producing 50% inhibition was taken as one unit of enzyme activity (U/gHb).

Assay of catalase activity [*38*]

The assay mixture contained 1 M Tris, 5 mM EDTA buffer, pH 7.0 and 200 mM hydrogen peroxide (H_2O_2). Disappearance of H_2O_2 was monitored at 240 nm for 30 s and the activity of catalase was evaluated and expressed as kU/gHb.

Assay of GRx activity [*39*]

The main reagent was prepared by combining 18 ml of KH_2PO_4 buffer 139 mM, 0.76 mM EDTA, pH 7.4 and 2 ml of NADPH 2.5 mM. The sample (20 µl of 1:20 hemolysate + 20 µl of KH_2PO_4 buffer), 220 µl of the main reagent and 5 µl of FAD 0.315 mM + 10 µl of KH_2PO_4 buffer were added to the cuvette and the absorbance at 340 nm was monitored for 200 s (step A). Then 30 µl of GSSG 22 mM + 10 µl of KH_2PO_4 buffer were added to start the reaction and the absorbance was followed for 175 s (step B). The final reaction volume was 315 µl. The difference in absorbance per minute between steps B and A was used to calculate the enzyme activity. The unit in µmol of NADPH oxidized/min and the GRx activity was evaluated and expressed as U/gHb.

Statistical analysis

Data were analyzed between placebo-P; and Group A and Group B by the Student *t*-test. The data were expressed as mean ± Standard Deviation. The statistical *t*-test was considered significant with a *p*-value of 0.05 or less. Calculations of the two-tailed probability: *p*-values and *SEM* (Standard Error of Means) were performed using GraphPad QuickCalcs Software.

Results

The systolic and diastolic blood pressures of patients with preeclampsia who were taking or not taking vitamin supplements were higher (statistically extremely significant $p<0.0001$) than those of normotensive patients. This indicates that the preeclampsia in both the cases was of severe nature. The preeclamptics presented with proteinuria & edema, but differences in their average pulse rate were not

Table 1. Demographic and clinical characteristics of three study groups.

Parameters	Control (Placebo) ($n = 20$)	Preeclamptics (PE) without vitamin supplements Group A ($n = 22$)	Preeclamptics (PE) with vitamin supplements Group B ($n = 21$)	*p* values (two tailed probability)	Statistical significance
Age (year)	28.8 ± 7.2	29.2 ± 6.4	27.4 ± 8.4	—	—
Mean gestational age at sampling (weeks)	36.4 ± 1.8	34.4 ± 2.2	33.8 ± 2.4	—	—
BMI at sampling weight/(height)2 Kg/(m)2	21.8 ± 3.4	19.3 ± 1.8	20.2 ± 2.6	—	—
Systolic BP (mmHg)	109.4 ± 8.8 (SEM = 1.968)	158 ± 14.6 (SEM = 3.113)	152 ± 12.4 (SEM = 2.706)	<0.000[a] <0.0001[b] 0.1549[c]	(extremely significant) (extremely significant) (not significant)
Diastolic BP (mmHg)	66 ± 8.2 (SEM = 1.834)	110.4 ± 4.2 (SEM = 0.895)	103.8 ± 16.8 (SEM = 3.666)	<0.0001[a] <0.0001[b] 0.0815[c]	(extremely significant) (extremely significant) (not significant)
Pulse rate (beats/min)	70.6 ± 2.8 (SEM = 0.626)	72.6 ± 3.8 (SEM = 0.810)	70.4 ± 3.6 (SEM = 0.786)	0.0613[a] 0.8442[b] 0.0584[c]	(not significant) (not significant) (not significant)
Proteinuria (gm/day)	Nil	1.78 ± 0.6	0.8 ± 0.4	—	—
Edema	Nil	++ in all cases	++ in all cases	—	—

Values are expressed as mean ± SD *n* = number of subjects studied. [a] (Group A compared to placebo); [b] (Group B compared to placebo); [c] (Group A compared to Group B).

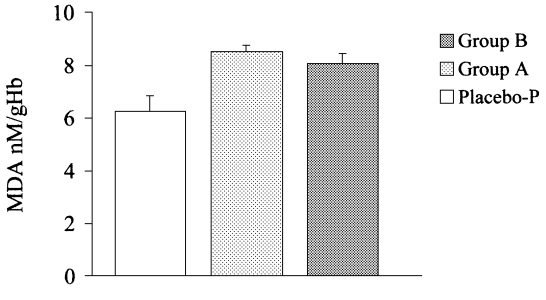

Fig. 1. MDA contents. Columns show mean values. Vertical bars represent SEM.
P = 6.22 ± 2.8 (SEM = 0.6261) A = 8.48 ± 1.2 (SEM = 0.2558) B = 8.02 ± 1.8 (SEM = 0.3928) Values are Mean ± SD.
Group A- PE without Vit. Supplements compared to placebo-P (Difference highly significant, $p = 0.0013$);
Group B- PE with Vit. Supplements compared to placebo-P (Difference significant, $p = 0.0184$);
Group A compared to Group B (Difference not significant, $p = 0.3278$).

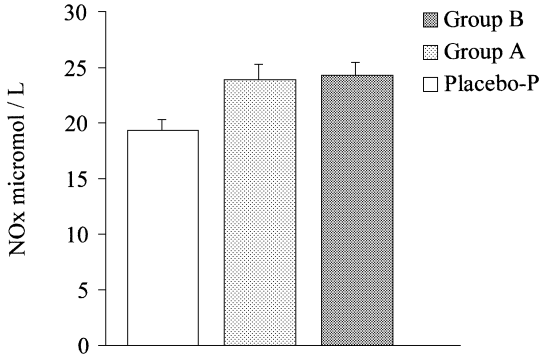

Fig. 2. Nitric oxide contents. Columns show mean values. Vertical bars represent SEM.
P = 19.3 ± 4.2 (SEM = 0.939) A = 23.8 ± 6.4 (SEM = 1.364) B = 24.1 ± 5.4 (SEM = 1.178) Values are mean ± SD.
Group A- PE without Vit. Supplements compared to placebo-P (Difference significant, $p = 0.0111$);
Group B- PE with Vit. Supplements compared to placebo-P (Difference highly significant, $p = 0.003$);
Group A compared to Group B (Difference not significant, $p = 0.8692$).

significant. The demographic and clinical data of these groups are shown in Table 1.

NO, MDA, NO/MDA ratio

Plasma lipid peroxides (MDA) levels were found to be elevated in preeclamptics with and without vitamin supplements. Moreover, the increase was significantly higher in Group A ($p = 0.0013$) than in Group B ($p = 0.0184$) as compared to placebo-P (Fig. 1). The nitric oxide contents were also observed to be at higher levels in preeclamptic women, but, in this case, the elevation was higher in Group B (highly significant, $p = 0.003$) than in group A (significant, $p = 0.0111$) as depicted in Fig. 2. The NO/MDA ratios, however, were not significantly different in all the three cases (Fig. 3).

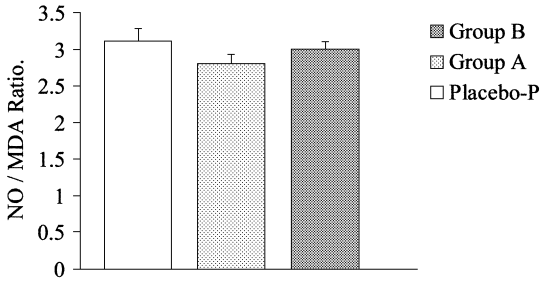

Fig. 3. Ratio of NO/MDA. Columns show mean values. Vertical bars represent SEM.
P = 3.103 ± 0.8 (SEM = 0.1789) A = 2.807 ± 0.6 (SEM = 0.1279) B = 3.005 ± 0.5 (SEM = 0.1091) Values are mean ± SD.
Group A- PE without Vit. Supplements compared to placebo-P (Difference not significant, $p = 0.18$);
Group B- PE with Vit. Supplements compared to placebo-P (Difference not significant, $p = 0.6389$);
Group A compared to Group B (Difference not significant, $p = 0.2477$).

GSH, GSSG, GSSG/GSH and uric acid contents

The amount of reduced glutathione was found to decrease ($p = 0.0099$, highly significant) during the development of preeclamptic conditions. But, this decrease was lesser in Group B with vitamin supplements. The fall in glutathione levels is statistically non-significant ($p = 0.1233$) in Group B when compared to placebo, however comparison of A with B showed significant ($p = 0.0485$) difference (Fig. 4). Oxidized glutathione was higher ($p = 0.0053$, highly significant) in Group A but this increase was not significant ($p = 0.1419$) in Group B (Fig. 5). The ratios of GSSG/GSH were also evaluated which gave interesting results (Fig. 6). The ratio increase was higher in Group A ($p = 0.003$, highly significant), but it was not significant ($p = 0.3483$) in Group B. When groups A and B were compared, however, the difference was significant ($p = 0.0134$). Uric acid contents were found to increase (extremely significant, $p<0.0001$) both in Groups A and B as compared to placebo. But there was not significant difference ($p = 0.1813$) between Groups A and B as evident from Fig. 7.

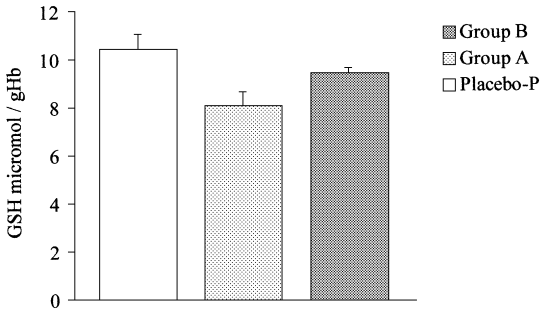

Fig. 4. Reduced glutathione contents. Columns show mean values. Vertical bars represent SEM.
P = 10.42 ± 2.81 (SEM = 0.6283) A = 8.02 ± 2.92 (SEM = 0.6225) B = 9.39 ± 1.02 (SEM = 0.2226) Values are mean ± SD.
Group A- PE without Vit. Supplements compared to placebo-P (Difference highly significant, $p = 0.0099$);
Group B- PE with Vit. Supplements compared to placebo-P (Difference not significant, $p = 0.1233$);
Group A compared to Group B (Difference significant, $p = 0.0485$).

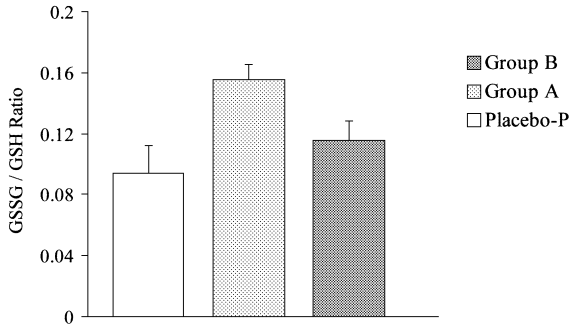

Fig. 6. Ratio of GSSG/GSH in three groups. Columns show mean values. Vertical bars represent SEM.
P = 0.0941 ± 0.08 (SEM = 0.01789) A = 0.155 ± 0.04 (SEM = 0.00853) B = 0.115 ± 0.06 (SEM = 0.01309) Values are mean ± SD.
Group A- PE without Vit. Supplements compared to placebo-P (Difference highly significant, $p = 0.003$);
Group B- PE with Vit. Supplements compared to placebo-P (Difference not significant, $p = 0.3483$);
Group A compared to Group B (Difference significant, $p = 0.0134$).

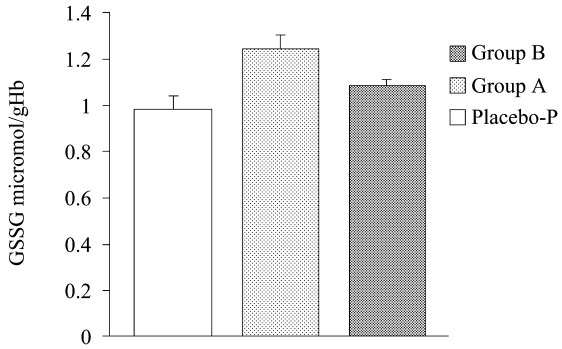

Fig. 5. Oxidized glutathione contents. Columns show mean values. Vertical bars represent SEM.
P = 0.98 ± 0.28 (SEM = 0.0626) A = 1.24 ± 0.29 (SEM = 0.0618) B = 1.08 ± 0.12 (SEM = 0.0262). Values are mean ± SD.
Group A- PE without Vit. Supplements compared to placebo-P (Difference highly significant, $p = 0.0053$);
Group B- PE with Vit. Supplements compared to placebo-P (Difference not significant, $p = 0.1419$);
Group A compared to Group B (Difference significant, $p = 0.0241$).

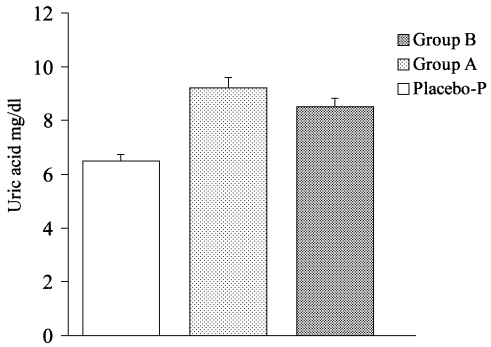

Fig. 7. Uric acid contents. Columns show mean values. Vertical bars represent SEM.
P = 6.5 ± 1.02 (SEM = 0.2281) A = 9.2 ± 1.84 (SEM = 0.3923) B = 8.5 ± 1.51 (SEM = 0.3295). Values are Mean ± SD.
Group A- PE without Vit. Supplements compared to placebo-P (Difference extremely significant, $p<0.0001$);
Group B- PE with Vit. Supplements compared to placebo-P (Difference extremely significant, $p<0.0001$);
Group A compared to Group B (Difference not significant, $p = 0.1813$).

Variations in the activities of key enzymes involved in antioxidative actions

The activity of SOD decreased significantly ($p<0.0001$, extremely significant) in preeclamptics not taking vitamins, whereas the decrease was simply significant ($p = 0.002$) for those taking vitamins. There was a significant difference ($p = 0.0434$) between these two Groups (Fig. 8). Comparison of the activity of GPx in all the three Groups revealed no significant changes (Fig. 9). But, interesting alterations were found in the activities of GRx, which decreased significantly

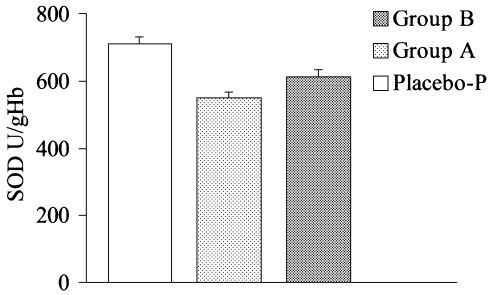

Fig. 8. Superoxide dismutase activities. Columns show mean values. Vertical bars represent SEM.
P = 710 ± 90.6 (SEM = 20.259) A = 548 ± 92.8 (SEM = 19.785) B = 610 ± 102.2 (SEM = 22.302). Values are Mean ± SD.
Group A- PE without Vit. Supplements compared to placebo-P (Difference extremely significant, $p<0.0001$);
Group B- PE with Vit. Supplements compared to placebo-P (Difference highly significant, $p = 0.002$);
Group A compared to Group B (Difference significant, $p = 0.0434$).

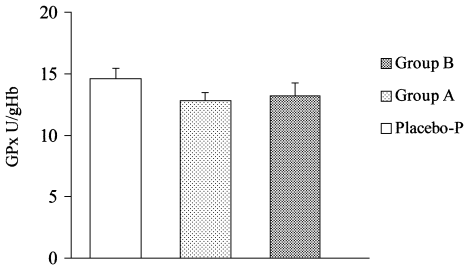

Fig. 9. Glutathione peroxidase activities. Columns show mean values. Vertical bars represent SEM.
P = 14.6 ± 3.8 (SEM = 0.85) A = 12.8 ± 3.2 (SEM = 0.682) B = 13.2 ± 4.8 (SEM = 1.047). Values are Mean ± SD.
Group A- PE without Vit. Supplements compared to placebo-P (Difference not significant, $p = 0.1036$);
Group B- PE with Vit. Supplements compared to placebo-P (Difference not significant, $p = 0.3084$);
Group A compared to Group B (Difference not significant, $p = 0.7484$).

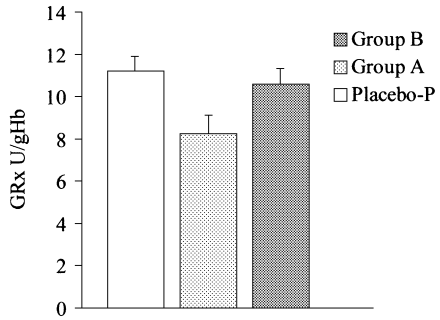

Fig. 10. Glutathione reductase activities. Columns show mean values. Vertical bars represent SEM.
P = 11.2 ± 3.08 (SEM = 0.6887) A = 8.22 ± 4.26 (SEM = 0.9082) B = 10.58 ± 3.32 (SEM = 0.7245). Vales are Mean ± SD.
Group A- PE without Vit. Supplements compared to placebo-P (Difference significant, $p = 0.0138$);
Group B- PE with Vit. Supplements compared to placebo-P (Difference not significant, $p = 0.5395$);
Group A compared to Group B (Difference significant, $p = 0.05$).

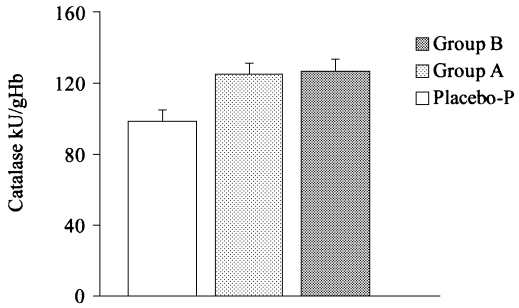

Fig. 11. Catalase activities. Columns show mean values. Vertical bars represent SEM.
P = 98.2 ± 30.4 (SEM = 6.798) A = 124.8 ± 31.6 (SEM = 6.737) B = 126.6 ± 32.8 (SEM = 7.158). Values are Mean ± SD.
Group A- PE without Vit. Supplements compared to placebo-P (Difference highly significant, $p = 0.0084$);
Group B- PE with Vit. Supplements compared to placebo-P (Difference highly significant $p = 0.0066$);
Group A compared to Group B (Difference not significant, $p = 0.8555$).

($p = 0.0138$) in preeclamptics not having used vitamins. Those who consumed vitamin E and C during the gestational period from the second trimester of pregnancy to delivery demonstrated an insignificant ($p = 0.5395$) fall in GRx activities.

However, comparisons between Groups A and B showed a significant ($p = 0.05$) difference in GRx activities (Fig. 10). Catalase activities were elevated in both Groups A and B as compared to placebo, and the increase was found to be highly significant ($p = 0.0084$ and $p = 0.0066$, respectively), whereas no statistically significant difference ($p = 0.8555$) was observed between Groups A and B, as depicted in Fig. 11.

Discussion

NO, MDA and NO/MDA ratio

NO plays an important role in the pathogenesis of PE as

the enhanced level of oxidative stress associated with the conditions increases the concentration of superoxide that reacts with NO to produce peroxynitrite ($ONOO^-$), reducing the level of available NO. Peroxynitrite is a potentially harmful ROS as it causes nitrosylation of tyrosine residues, leading to changes in protein conformation and inactivation. Preeclampsia is associated with increased nitrotyrosine residues in placental villous vascular endothelium [*40*]. The effect of elevation in peroxynitrite and reduced bioavailability of NO, resulting from enhanced production of free radicals and the resultant placental oxidative state, may contribute to the elevation of maternal blood pressure, proteinuria, platelet dysfunction and alteration in the level of thromboxane, prostacyclin and endothelin characteristic of preeclampsia [*41*]. Another common source of placental oxidative damage is the peroxidation of membrane phospholipids that exerts a variety of effects within placenta. Our results on NO contents showed an increase of about 23–25% in preeclamptics, but the bioavailability of NO might have been blocked, as there was an increase of 29–36% in MDA contents.

The oxidative destruction of polyunsaturated fatty acids (PUFAs) of phospholipids, known as lipid peroxidation, can be in fact considered as a hallmark of oxidative stress. PUFAs are important for the normal function of most of the cells. MDA, an end product of lipid peroxidation induced by ROS, is well correlated with the degree of lipid peroxidation [*28*, *29*]. The result mentioned above revealed a highly significant increase in MDA content in preeclamptic patients in comparison to normal placebo. This may result in a greater potential for endothelial damage ultimately leading to elevated diastolic pressure [*19*]. Enhanced ROS in turn can oxidize many other important biomolecules including erythrocyte membrane phospholipids. Thus, lipid peroxides and free radicals may be important in the pathogenesis of preeclampsia. Our results on MDA contents were consistent with the observations of other workers [*31*, *19*] as well. The increase in MDA content in patients who took vitamin (group B) was simply significant (29%) when compared to group A in which it was very significant (36%). This shows the effectiveness of vitamins C and E in restricting the elevation of lipid peroxidation. Our results on NO contents were in agreement with the findings of other investigators [*20*, *21*]. Although levels of NO in preeclamptics, both without and with vitamin had increased significantly and highly significantly, respectively, there was no distinct improvement in blood pressure symptoms for either group. This shows that bioavailability of NO was restricted, perhaps due to its enhanced reaction with superoxide resulting in peroxynitrite ($ONOO^-$) as mentioned above.

The pathogenic link of serum uric acid is evident from other studies [*25*, *26*] and our results are consistent with their reports as the increase in uric acid contents in both groups A and B were found to be extremely significant.

GSH, GSSG, GSSG/GSH ratio

Glutathione and glutathione related enzymes are one of the major antioxidant systems within body. GSH is the most abundant thiol-based antioxidant, and is found primarily in the reduced form, with intracellular concentration up to 11 mM and provides sulfhydryl-buffering capacity. It also conveys an antioxidant power through the direct inactivation of ROS or by acting as an electron donor for GPx that reduces H_2O_2 to water [*42*]. It has been suggested that the high levels of GSH may act as a compensatory mechanism in preeclampsia to prevent excessive lipid peroxidation via membrane bound GPx [*43*]. In our study the erythrocyte GSSG concentration was very significantly elevated in preeclamptics, and we observed a significant increase in the redox potential values for the pair GSSG/GSH as compared to placebo, showing altered antioxidant balance. Our data clearly indicate that GSH in red blood cells decreased profoundly in preeclamptic pathophysiological conditions with a parallel increase in MDA and GSSG concentration. Our findings on MDA, GSH and GSSG are in agreement with those reported earlier [*44*].

SOD, GPx, catalase and GRx

SOD, catalase and GPx are important factors in the antioxidative defense system. SOD protects and revitalizes cells and reduces the rate of cell destruction. It neutralizes some of the most dangerous free radicals, the superoxide radicals, before they can wreak havoc on the body. Superoxide generation also perpetuates oxidative stress and lipid peroxidation through the oxidation of mitochondrial iron-sulphur clusters such as aconitase, which subsequently stimulate membrane phospholipid peroxidation by alkoxyl radicals [*45*]. Deficient SOD activity may also increase oxidative insult by promoting the interaction of NO and superoxide to produce peroxynitrite as mentioned earlier. Superoxide dismutase and nitric oxide compete for superoxide. A consequence of SOD deficiency is an increase in nitric oxide interactions with superoxide, which results in the generation of peroxynitrite, subsequently reducing vasodilation through the impairment of NO and increasing $ONOO^-$ induced lipid peroxidation [*41*]. Our results on SOD activity clearly indicate an extremely significant decline (23%) in its activity in preeclamptic patients (Group A) and a significant decrease (14%) in preeclamptics using vitamin (Group B); a significant difference was observed between these two Groups (10–11%). From our results we infer the positive effect of vitamins C and E on retaining SOD activity during preeclampsia.

The principal function of GPx is to protect against damage from the endogenously produced hydroxyperoxides and to catalyze the reduction of hydroxyperoxides by

glutathione. Catalase promotes the conversion of hydrogen peroxide, a powerful and potentially harmful oxidizing agent, to water and molecular oxygen. It also uses H_2O_2 to oxidize toxins such as phenols, formic acid, formaldehyde and alcohols. Catalase, along-with SOD and GPx, controls the levels of oxygen-derived free radicals in cells. Our results show non-significant alterations in GPx activities. The activity of catalase increased very significantly in both Groups A and B (27% and 29% increase respectively), which shows its compensatory regulation in response to increased oxidative stress. No effect of vitamins C and E, however, was observed on GPx levels. GRx activity declined significantly (27%) in preeclamptics who had not consumed vitamins C and E (Group A), but its activity did not fall significantly (5.5%) in patients who had used these vitamins regularly as mentioned above. A comparison of these two Groups revealed a significant difference (22–29%), which shows that vitamins C and E consumption prevented the fall in GRx activity. This finding shows a clear correlation with the levels of GSH, which is evident from our results on GSH, GSSG and GSSG/GSH.

In conclusion, we hypothesize enhanced oxidative stress in preeclampsia, on the basis of our results showing decreased SOD, GRx and GPx activities, with resulting inefficient control of the higher levels of oxygen free radicals produced. The increased activity of catalase that we observed may be a compensatory regulation in response to increased oxidative stress. Increased catalase activity could be interpreted as a futile effort to counteract the overproduction of reactive oxygen species, and to provide a relief to enhanced oxidative damage in preeclampsia. Lipid peroxides could participate in the cytotoxic mechanism leading to endothelial injury and enhanced blood pressure. The decreased concentration of glutathione supports the hypothesis that lipid peroxidation is an important factor in the pathogenesis of PE. Chappell *et al.* [*1*] have reported that a significant reduction in the incidence of preeclampsia was found in women at risk who were taking a vitamins C and E supplements. They inferred a correlation between the PAI-1/PAI-2 (plasminogen activator inhibitor) ratio with vitamins C and E levels. Furthermore, they [*2*] suggested that antioxidant supplementation in women who were at risk of preeclampsia was associated with improvement in biochemical indices of the disease. Zhang *et al.* [*7*] have reported a 2-fold increased risk of developing preeclampsia in women taking lower vitamin C doses. Several reports reviewed by Rodrigo *et al.* [*9*] suggest the prophylactic use of vitamins C and E before the 20th. week of gestation, after identifying the risk factors on the basis of history of the patient, what would lower the risk of maternal vascular dysfunction and therein the onset of PE.

We found that the patients supplemented with vitamin C 1000 mg plus vitamin E 400 IU daily from the second trimester of pregnancy until delivery showed improvements in certain biochemical indices during the development of preeclampsia. The elevation in MDA content was found to be significantly lower. Oxidation of reduced glutathione to GSSG was inhibited to a greater extent. The activities of antioxidant enzymes were also influenced by the use of vitamins C and E. The reduction of SOD activity during the development of preeclampsia was controlled as a consequence of vitamin consumption: the decrease was found to be 14% whereas it was 23% in patients who had not used vitamins C and E. The most prominent effect of the use of these vitamins was found to be associated with the activity of glutathione reductase. Reduction in its activity was much higher 27% as compared to that in patients who were taking vitamins (5.5%). Thus, we conclude that use of vitamins C and E definitely controls certain important biochemical indices during the development of pre-eclampsia, and that further studies are needed to develop strategies for the prevention of preeclampsia in women at high risk.

Acknowledgments

We are highly grateful to those patients of our hospital who volunteered to donate their blood when needed for this project. The authors would also like to thank the para-medical staff of this hospital for their assistance in collecting and maintaining blood samples.

Abbreviations

BMI, body mass index; U, unit; IU, international unit; Hb, hemoglobin; MDA, malondialdehyde; NO, nitric oxide; ATP, adenosine triphosphate; NADPH, nicotinamide adenine dinucleoltide phosphate reduced; FAD, flavin adenine dinucleotide; GSH, reduced glutathione; GSSG, oxidized glutathione; EDTA, Ethylene Diamine Tetra Acetic acid; TBA, thiobarbituric acid; BHT, butylated hydroxytoluene.

References

[1] Chappell, L.C., Seed, P.T., Briley, A., Kelly, F.J., Lee, R., Hunt, B.J., Parmer, K., Bewley, S.J., Shennan, A.H., Steer, P.J., and Poston, L.: Effect of antioxidants on the occurrence of preeclampsia in women at increased risk: a randomized trial. *Lancet*, **254**, 810–816, 1999.

[2] Chappell, L.C., Seed, P.T., Kelly, F.J., Briley, A., Hunt, B.J., Charnock-Jones, D.S., Mallet, A.I., and Poston, L.: Vitamin C and E supplementation in women at risk of preeclampsia is associated with changes in indices of oxidative stress and placental function. *Am. J. Obstet. Gynecol.*, **187**, 777–784, 2002b.

[3] Mohindra, A., Kabi, B.C., Kaul, N., and Trivedi, S.S.: Vitamin E and carotenes status in preeclamptic pregnant

women from India. *Panminerva Med.*, **44**, 261–264, 2002.

[4] Panburana, P., Phuapradit, W., and Puchaiwatananon, O.: Antioxidants nutrients and lipid peroxide levels in Thai preeclamptic women. *J. Obstet. Gynaecol. Res.*, **26**, 377–381, 2000.

[5] Llurba, E., Gratacos, E., Martine-Gallan, P., Cabero, L., and Dominguez, C.: A comprehensive study of oxidative stress and antioxidant status in preeclampsia and normal pregnancy. *Free Radic. Biol. Med.*, **37**, 557–570, 2004.

[6] Chappell, L.C., Seed, P.T., Briley, A., Kelly, F.J., Hunt, B.J., Charnock-Jones, D.S., Mallet, A.I., and Poston, L.: A longitudinal study of biochemical variables in women at risk of preeclampsia. *Am. J. Obstet. Gynecol.*, **187**, 127–136, 2002a.

[7] Zhang, C., Williams, M.A., King, I.B., Dashow, E.E., Sorensen, T.K., Frederick, I.O., Thompson, M.L., and Luthy, D.A.: Vitamin C and the risk of preeclampsia-results from dietary questionnaire and plasma assay. *Epidemiology*, **13**, 409–416, 2002.

[8] Poston, L., Briley, A.L., Seed, P.T., Kelly, F.J., and Shennan, A.H.: Vitamin C and vitamin E in pregnant women at risk for preeclampsia (VIP trial): randomized placebo-controlled trial. *Lancet*, **367**, 1145–1154, 2006.

[9] Rodrigo, R., Parra, M., Bosco, C., Fernandez, V., Baria, P., Guajardo, J., and Messina, R.: Pathophysiological basis for the prophylaxis of preeclampsia through early supplementation with antioxidant. *Pharmacol. Ther.*, **107**, 177–197, 2005.

[10] El-Salahy, E.M., Ahmed, M.I., El-Gharieb, A., and Tawfik, H.: New scope in angiogenesis: role of vascular endothelial growth factors (VEGF), NO, lipid peroxidation and vitamin E in the pathophysiology of the preeclampsia among Egyptian females. *Clin. Biochem.*, **34**, 323–329, 2001.

[11] Akyol, D., Mungan, T., Gorkemli, H., and Nuhoglu, G.: Maternal levels of vitamin E in normal and preeclamptic pregnancy. *Arch. Gynecol. Obstet.*, **263**, 151–155, 2000.

[12] Madazli, R., Benian, A., Gumustas, K., Uzun, H., Ocak, V., and Aksu, F.: Lipid peroxidation and antioxidant in preeclampsia. *Eur. J. Obstet. Gynecol. Reprod. Biol.*, **85**, 205–208, 1999.

[13] Ben-Haroush, A., Harell, D., Hod, M., Bardin, R., Kaplan, B., Orvieto, R., and Bar, J.: Plasma levels of vitamin E in pregnant women prior to the development of preeclampsia and hypertensive complications. *Gynecol. Obstet. Invest.*, **54**, 26–30, 2002.

[14] Zhang, C., Williams, M.A., Sanchez, S.E., King, I.B., Ware-Jauregui, S., Larrabure, G., Bazul, V., and Leisenring, W.M.: Plasma concentrations of carotenoids, retinal, tocopherols in preeclamptic and normotensive pregnant women. *Am. J. Epidemiol.*, **153**, 572–580, 2001.

[15] Mutlu, T.U., Aykac, T.G., Ibrahimoglu, L., Ademoglu, E., and Uysal, M.: Plasma nitric oxide metabolites and lipid peroxide levels in preeclamptic pregnant women before and after delivery. *Gynecol. Obstet. Invest.*, **48**, 247–250, 1999.

[16] Norris, L.A., Higgins, J.R., Darling, M.R.N., Walshe, J.J., and Bonnar, J.: Nitric oxide in the uteroplacental, fetoplacental and peripheral circulation in preeclampsia. *Obstet. Gynecol.*, **93**, 958–963, 1999.

[17] Kubes, P., Suzuki, M., and Granger, D.N.: Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. USA*, **88**, 4651–4655, 1991.

[18] Radomski, M.W., Palmer, R.M., and Moncada, S.: The antiaggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. *Br. J. Pharmacol.*, **92**, 639–646, 1987.

[19] Aydin, A., Benian, A., Modazli, R., Uloda, S., Uzun, H., and Kaya, S.: Plasma malondialdehyde, superoxide dismutase, sE-selectin, fibronectin, endothelin-1 and nitric oxide levels in women with preeclampsia. *Eur. J. Obstet. Gynecol. Reprod. Biol.*, **113**, 21–25, 2004.

[20] Shaamash, A.H., Elsnosy, E.D., Makhlouf, A.M., Zakhari, M.M., Ibrahim, O.A., and El-Dien, H.M.: Maternal and fetal nitric oxide (NO) concentrations in normal pregnancy, preeclampsia and eclampsia. *Int. J. Gynecol. Obstet.*, **68**, 207–214, 2000.

[21] Smarason, A.K., Allman, K.G., Young, D., and Redman, C.W.: Elevated levels of serum nitrate, a stable end product of nitric oxide in women with preeclampsia. *Br. J. Obstet. Gynecol.*, **104**, 538–543, 1997.

[22] Choi, J.W., Im, M.W., and Pai, S.H.: Nitric oxide production increases during normal pregnancy and decreases in preeclampsia. *Ann. Clin. Lab. Sci.*, **32**, 257–263, 2002.

[23] Davidge, S.T., Stranko, C.P., and Roberts, J.M.: Urine but not plasma nitric oxide metabolites are decreased in women with preeclampsia. *Am. J. Obstet. Gynecol.*, **174**, 1008–1013, 1996.

[24] Diejomaoh, F.M., Omu, A.E., Al-Busiri, N., Taher, S., Al-Othman, S., Fatinikun., T., and Fernandes, S.: Nitric oxide production is not altered in preeclampsia. *Arch. Gynecol. Obstet.*, **269**, 237–243, 2004.

[25] Roberts, J.M., Bodnar, L.M., Lain, K.Y., Hubel, C.A., Markovic, N., Ness, R.B., and Powers, R.W.: Uric acid is as important as proteinuria in identifying fetal risk in women with gestational hypertension. *Hypertension*, **46**, 1263–1269, 2005.

[26] D'Anna, R., Baviera, G., Scilipoti, A., Leonardi, I., and Leo, R.: The clinical utility of serum uric acid measurements in preeclampsia and transient hypertension in pregnancy. *Panminerva Med.*, **42**, 101–103, 2000.

[27] Johnson, R.J., Kang, D.H., Feig, D., Kivlighn, S., Kanellis, J., Watanabe, S., Tuttle, K.R., Rodriguez-Iturbe, B., Harrera-Acosta, J., and Mazzali, M.: Is there a pathogenic role for uric acid in hypertension and cardiovascular and renal disease. *Hypertension*, **41**, 1183–1190, 2003.

[28] Hubel, C.A., Roberts, J.M., Taylor, R.N., Musci, T.J., Rogers, G.M., and McLaughlin, M.K.: Lipid peroxidation in pregnancy: new perspectives on preeclampsia. *Am. J. Obstet. Gynecol.*, **161**, 1025–1034, 1989.

[29] Vanderlelie, J., Venardos, K., Clifton, V.L., Gude, N.M., Clarke, F.M., and Perkins, A.V.: Increased biological oxidation and reduced anti-oxidant enzyme activity in preeclamptic placentae. *Placenta*, **26**, 53–58, 2005.

[30] Zhang, Y., Gu, Y., Lewis, D.F., and Wang, Y.: Reduced cellular glutathione reductase activity and increased adhesion molecule expression in endothelial cells cultured with plasma

of women with preeclampsia. *J. Soc. Gynecol. Investig.*, **13**, 412–417, 2006.

[31] Atamer, Y., Koçyigit, Y., Yokus, B., Atamer, A., and Erden, A.C.: Lipid peroxidation, antioxidant defense, status of trace metals and leptin levels in preeclampsia. *Eur. J. Obstet. Gynecol. Biol.*, **119**, 60–66, 2005.

[32] Buchanan, M.J., Isdale, I.C., and Rose, B.S.: Serum Uric Acid Estimation. Chemical and enzymatic methods compared. *Ann. Rheum. Dis.*, **25**, 285–288, 1965.

[33] Tentori, L. and Salvati, A.M.: Hemoglobinometry in human blood. *Methods Enzymol.*, **76**, 707–715, 1981.

[34] Beutler, E.: Red Cell Metabolism, *A Manual of Biochemical Methods. 3rd*, ed. Grune and Stratton Inc., New York, 1984.

[35] Videla, L.A. and Junqueira, V.B.C.: Metabolism of hepatic glutathione. Laboratory exercise I. 1-6, In: Oxygen radicals in Biochemistry, biophysics and medicine. International training course. School of Pharmacy and Biochemistry, Buenos Aires, University of Buenos Aires, Argentina, 7, -18.3, 1994.

[36] Jain, S.K., McVie, R., Duett, J., and Herbst, J.J.: Erythrocyte membrane lipid peroxidation and glycosylated hemoglobin in diabetes. *Diabetes*, **38**, 1539–1543, 1989.

[37] Paglia, D.E. and Valentine, W.N.: Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.*, **70**, 158–169, 1967.

[38] Aebi, H.: Catalase *in vitro*. *Methods Enzymol.*, **105**, 121–126, 1984.

[39] Goldberg, D.M. and Spooner, R.J.: Glutathione reductase, in *Methods Enzymol*, ed. By Bergmeyer, H.U., Verlag Chemie, Basel, **3**, 258–265, 1983.

[40] Adams, R.H., Porras, A., Alonso, G., Jones, M., Vintersten, K., Panelli, S., Valladares, A., Perez, L., Klein, R., and Nebrada, A.R.: Essential role of p38alpha MAP kinase in placental but not embryonic cardiovascular development. *Molecular Cell*, **6**, 109–116, 2000.

[41] Lowe, D.T.: Nitric oxide dysfunction in the pathophysiology of preeclampsia. *Nitric Oxide*, **4**, 441–458, 2000.

[42] Imai, H. and Nakagawa, Y.: Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells. *Free Radic. Bio. Med.*, **34**, 145–169, 2003.

[43] Knapen, M.F., Peters, W.H., Mulder, T.P., Merkus, H.M., Jansen, J.B., and Steegers, E.A.: Glutathione and glutathione related enzymes in deciduas and placenta of controls and women with preeclampsia. *Placenta*, **20**, 541–546, 1999.

[44] Padmini, E. and Geetha, B.V.: Placental heat shock protein 70 overexpression confers resistance against oxidative stress in preeclampsia. *Turk J. Med. Sci.*, **38**, 27–34, 2008.

[45] Liochev, S.I. and Fridovich, I.: How does superoxide dismutase protect against tumor necrosis factor: a hypothesis informed by effect of superoxide on “free” iron. *Free Radic. Biol. Med.*, **23**, 668–671, 1997.