Promjene ravnoteže prooksidansa i antioksidansa u preeklampsiji – utjecaj na osmotsku rezistenciju eritrocita

Alterations in antioxidant and pro-oxidant balance in preeclampsia – impact on erythrocyte osmotic fragility

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Sažetak

Cilj: Procijeniti korisnost prooksidacijskih i antioksidacijskih biljega, kao i osmotsku rezistenciju eritrocita kod trudnica s preeklampsijom u usporedbi s kontrolnom skupinom. Izmjerene su i uspoređene razine njihovih neenzimskih i enzimskih parametara, kao i pro- i antioksidacijskih parametara.

Materijali i metode: U istraživanje su bile uključene dvije skupine trudnica: kontrolna skupina od 25 normotenzivnih zdravih trudnica i skupina od 27 žena s teškim oblikom preeklampsije. Izmjerene su i uspoređene koncentracije reduciranog glutationa (GSH), oksidiranog glutationa (GSSG) i malondialdehida (MDA) u krvi, koncentracija mokraćne kiseline zajedno s antioksidacijskim enzimskim parametrima u krvi: aktivnosti superoksid-dismutaze (SOD), glutation-peroksidaze (GPx), glutation-reduktaze (GRx) te katalaze, kako bi se procijenila ukupna promjena uslijed preeklampsije. Vrijednosti svakog parametra izmjerene su primjerenom laboratorijskom metodom.

Rezultati: Kod žena oboljelih od preeklampsije koncentracije oksidacijskog biljega MDA bile su povišene za 33% (P = 0,001), GSSG za 19% (P = 0,001), dok se koncentracija GSH u eritrocitima smanjila za 20% (P = 0,001). Koncentracija mokraćne kiseline se u usporedbi s normotenzivnom kontrolnom skupinom povećala za 36,5% (P < 0,001). Smanjenje osmotske rezistencije ukazalo je na smanjenu deformabilnost eritrocita kod teškog oblika preeklampsije. Smanjena je aktivnost antioksidacijskih enzima i to SOD za 23,7% (P < 0,001), GRx za 21,9% (P = 0,014) i GPx za 14,5% (P = 0,109) tijekom razvoja preeklampsije. Jedini biljeg odgovora na oksidativni stres je za 26,8% povećana aktivnost katalaze (P = 0,002).

Zaključak: Zaključujemo kako veće stvaranje reaktivnih kisikovih spojeva (engl. *reactive oxygen species,* ROS) i smanjena aktivnost SOD, GRx, GPx, povećana koncentracija MDA, GSSG s istodobno niskom koncentracijom GSH podupiru hipotezu o snažnijem oksidacijskom stresu u preeklampsiji. Nadalje smo zaključili da smanjena osmotska rezistencija eritrocita u preeklampsiji upućuje na gubitak integriteta stanične membrane, što rezultira kraćim životnim vijekom stanice.

Ključne riječi: preeklampsija; neenzimski antioksidansi i oksidansi; enzimski antioksidansi; osmotska rezistencija eritrocita

Abstract

Objectives: To validate the utility of both pro- and antioxidative stress markers along with erythrocyte osmotic fragility profile in preeclamptic patients compared with normal controls. The levels of nonenzymatic and enzymatic pro- and antioxidative parameters were determined and compared.

Methods: In the present study, we compared two groups of pregnant women: 27 women with severe preeclampsia and 25 normotensive healthy women as a control group. Blood levels of reduced glutathione (GSH), oxidized glutathione (GSSG), malondialdehyde (MDA), uric acid, and of antioxidant enzymatic parameters of superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GRx) and catalase activities were determined by respective laboratory methods and compared to evaluate alterations due to preeclampsia.

Results: In preeclamptic women, the levels of the oxidative markers MDA and GSSG were increased by 33% (P = 0.001) and 19% (P = 0.001), respectively, whereas erythrocyte GSH was decreased by 20% (P = 0.001). The level of uric acid was increased by 36.5% (P < 0.001) as compared with normotensive healthy controls. An increase in osmotic fragility indicated decreased erythrocyte deformability in severe preeclampsia. During the development of preeclampsia, the antioxidant enzymes SOD, GRx and GPx lost 23.7% (P < 0.001), 21.9% (P = 0.014) and 14.5% (P = 0.109) of their activities, respectively. The only relief from oxidative stress was recorded in the catalase activity, which increased by 26.8% (P = 0.002).

Conclusions: We concluded that higher reactive oxygen species (ROS) production, decreased SOD, GRx and GPx activities, and elevated levels of MDA and GSSG along with low GSH supported the hypothesis of higher oxidative stress in preeclampsia. Only the increased catalase activity may have provided compensatory regulation in response to the increased oxidative stress. We also concluded that the increased osmotic fragility of preeclamptic red blood cells suggested a loss in their membrane integrity resulting in their shortened life span.

Key words: preeclampsia; nonenzymatic antioxidants and oxidants; enzymatic antioxidants; erythrocyte osmotic fragility

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Uvod

Preeklampsija je složen višestruki sustavni poremećaj koji se povezuje s hipertenzijom, edemom i proteinurijom tijekom trudnoće. Stečeni mehanizam potiče majčin antioksidacijski obrambeni sustav da se enzimskom indukcijom, kao i neenzimskim hvatačima i zaštitnicima slobodnih radikala poput reduciranog glutationa, suprotstavi učinku slobodnih radikala, čime može spriječiti pojavu oksidacijskog stresa. Međutim, trudnoća je stanje gdje se ta prilagodba može vrlo lako omesti.

Nema jedinstvenog mišljenja o tome povećavaju li se ili smanjuju kod preeklampsije aktivnosti antioksidacijskih enzima, kao što su superoksid-dismutaza (SOD), glutation-peroksidaza (GPx), glutation-reduktaza (GRx) te katalaza. Nekoliko autora (1-4) izvještava o smanjenju aktivnosti SOD, GPx i GRx, dok drugi (5-7) opisuju povećanje njihove aktivnosti. Neki autori izvještavaju o povećanoj aktivnosti katalaze (1,8), dok drugi navode njeno smanjenje (9) kod preeklampsije.

Naš se laboratorij dugo vremena bavi istraživanjima ovog problema i zbog takvih smo proturječnih podataka i ozbiljnosti preeklampsije proveli ovo istraživanje. Željeli smo odrediti aktivnosti katalaze, SOD, GPx i GRx te koncentracije MDA i GSSG/GSH u krvi kontrolne skupine trudnica i trudnica s teškim oblikom preeklampsije, kako bismo procijenili promjene pro- i antioksidacijske ravnoteže. Znanstvena literatura pruža vrlo malo podataka o osmotskoj rezistenciji eritrocita kod preeklampsije, iako je to također bitan parametar za određivanje patofiziološkog statusa. Postoji podatak o smanjenoj koncentraciji GSH u eritrocitima kod osoba s preeklampsijom, što je u korelaciji s smanjenom osmotskom rezistencijom i zbog toga sa smanjenom staničnom deformabilnosti i fluidnosti njezine membrane (10). Stoga smo ovaj parametar uključili u naše istraživanje, što je dalo vrlo zanimljive rezultate.

Ispitanice i metode

Ispitanice

U Indiji se trudnice potiču na redovite trudničke preglede. Standardni pregledi uključuju mjesečne preglede do 28. tjedna trudnoće, preglede jednom u dva tjedna do 34. tjedna te tjedne preglede do poroda. Ovo je istraživanje provedeno uz prethodno odobrenje lokalnog Etičkog povjerenstva. Ispitanice u našem istraživanju bile su zdrave trudnice s normalnim krvnim tlakom i trudnice s preeklampsijom primljene u našu bolnicu, bez obzira na redovitost dolazaka na preglede, kao i one koje su bile upućene iz privatnih ordinacija ili centara primarne zdravstvene zaštite. Odabrano je 25 zdravih trudnica i 27 trudnica s teškim oblikom preeklampsije. Sve su ispitanice dale svoj obaviješteni pristanak, a prije toga im je u potpunosti objašnjen cilj istraživanja. Ispitanicama su izmjerene visina i

Introduction

Preeclampsia is a complex multisystem disorder that is associated with hypertension, edema and proteinuria during pregnancy. An adaptive mechanism enhancing the maternal antioxidant defense system to counteract the effect of free radicals through enzymatic induction as well as through nonenzymatic free radical protectors and scavengers like reduced glutathione can prevent the occurrence of oxidative stress. However, pregnancy is a state where this adaptation may be easily disrupted.

Consensus does not exist whether the activities of the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase increase or decrease in preeclampsia. Several authors have reported a decrease in the activity of SOD, GPx and GRx (1-4), whereas others found their activities to increase (5-7). Similarly, the activity of catalase has been reported to increase (1,8), or suggested to decrease in preeclampsia (9).

Our laboratory has for long been involved in pursuing studies on pathological blood. Considering the contradictory reports and the seriousness of preeclampsia, the present study was carried out to determine the activities of catalase, SOD, GPx and glutathione reductase (GRx) in the blood of normal and severely preeclamptic women, along with the levels of malondialdehyde (MDA) and oxidized glutathione/reduced glutathione (GSSG/GSH) to collectively evaluate the alteration in pro-oxidant and antioxidant balance.

Literature reveals very little information on preeclamptic erythrocyte osmotic fragility, and this parameter is also important to assess the pathophysiological status of preeclampsia. There is a report on decreased GSH content in red blood cells of preeclamptic women, which has been correlated to the increased osmotic fragility and consequently reduced cellular deformability and membrane fluidity (10). Thus, we also included this parameter in our study, which yielded very interesting results.

Subjects and methods

Subjects

In India, pregnant women are encouraged 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 present study was carried out with prior approval from the local Ethics Committee. The study included pregnant women with normal blood pressure as a control group and preeclamptic women admitted to our hospital that had been or had not been under regular care and also those referred from private sectors or primary health centers. Twenty-five normal pregnant women and 27 severely preeclamptic patients with term pregnancy were selected. They

težina kako bi im se izračunao indeks tjelesne mase (engl. *body mass index*, BMI). Kliničkim pregledom i anamnezom iz istraživanja su isključene žene koje puše, ispitanice koje boluju od šećerne bolesti, ishemijske bolesti srca, s anamnezom moždanog udara, poremećajem rada bubrega ili drugim stanjima etiološki povezanim sa slobodnim radikalima. Kriteriji za raspodjelu žena u normalnu skupinu prvorodilja i skupinu prvorodilja s preeklampsijom bili su vrijednost krvnog tlaka od 140/90 mm Hg ili više, proteinurija i pojava edema.

Metode

U istraživanju su se rabili ATP, NADPH, GSH, GSSG, glutation-reduktaza, EDTA, TBA i BHT od proizvođača Sigma Chemical Company (St. Louis, MO, SAD). Druge su kemikalije nabavljene od E. Merck (Mumbai, India). Svi su ostali reagensi kupljeni od BDH ili SISCO Chemicals (Mumbai, India).

Ispitivanje osmotske rezistencije

Ispitivanje osmotske rezistencije (engl. *osmotic fragility*, OF) provedeno je prema metodi Daciea i Lewisa (11). Kao mjera srednje vrijednosti osmotske rezistencije eritrocita (engl. *mean erythrocyte fragility*, MEF) uzeta je koncentracija NaCl kod koje dolazi do 50%-tne hemolize. Kolorimetrijska mjerenja izvedena su na kolorimetru Systronics.
Uzorci krvi su centrifugirani na 1000xg kroz 15 min na 4 °C. Izolirani eritrociti su 4–5 puta isprani s 0,154 M NaCl, kako bi se odstranila plazma i međusloj sa trombocitima i leukocitima (engl. *buffy coat*, BC). Nakon završnog centrifugiranja i ispiranja sediment eritrocita (engl. *packed red cells*) je liziran hipotoničnim šokom, pri čemu su kao hemolizirajuća sredstva upotrebljena različita razrjeđenja.
Koncentracija hemoglobina u eritrocitima izmjerena je Drabkinovim reagensom (12).

Određivanje koncentracije reduciranog glutationa (GSH)

U testu je upotrebljeno 0,2 mL sedimenta eritrocita nakon završnog centrifugiranja. GSH je reagirao s 5,5'-ditiobis-(2-nitrobenzojevom kiselinom) koja reagira sa sulfhidrilnom skupinom kako bi razvila stabilnu boju. Apsorbancija je mjerena na 412 nm, a koncentracija GSH je izražena kao µmol po gramu Hb (µmol/gHb) (13).

Određivanje koncentracije oksidiranog glutationa (GSSG)

Lizat eritrocita deproteiniziran je se s 0,5M $HClO_4$. Načelo reakcije uključuje redukciju GSSG u prisutnosti glutation-reduktaze (GRx). Smanjenje koncentracije NADPH na 340 nm nakon dodavanja GRx je proporcionalno udjelu GSSG koji se nakon toga izražava kao µmol/gHb (14).

Određivanje lipidne peroksidacije

Za određivanje koncentracije malondialdehida (MDA) kao reaktivne supstance tiobarbiturne kiseline (TBARS) upotgave their consent in writing and the objectives of the study were fully explained to them in detail prior to taking consent. Body height and weight of the subjects were measured to calculate their body mass index (BMI). Clinical examination and history taking excluded women addicted to tobacco, patients with diabetes, ischemic heart disease, a history of stroke, kidney diseases or other conditions of known free radical etiology. The criteria for dividing women into normal primipara and preeclamptic primipara groups were set at a blood pressure of 140/90 mm Hg or higher, proteinuria and edema.

Methods

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

Determination of osmotic fragility (OF)

Osmotic fragility (OF) experiments were performed following the method of Dacie and Lewis (11). The NaCl concentration of 50% hemolysis was taken as a measure of mean erythrocyte fragility (MEF). Color measurement was made using Systronics colorimeter.
Blood samples were centrifuged at 1000xg for 15 min at 4 °C and isolated red cells were washed 4–5 times with 0.154 M NaCl to remove plasma and buffy coat. After final wash, the required packed red cells were lysed by hypotonic shock and different dilutions were used as hemolysates.
Hemoglobin content of the erythrocyte was measured by cyanmethemoglobin method of Drabkin (12).

Determination of reduced glutathione (GSH)

Packed red cells (0.2 mL) were used in the assay. GSH was made to react with 5'5-dithiobis(2-nitrobenzoic acid), which reacts with sulfhydryl groups, to develop a stable color. The absorbance was measured at 412 nm and GSH content expressed as µmol/gHb (13).

Determination of oxidized glutathione (GSSG)

Erythrocyte lysate was deproteinized with 0.5M $HClO_4$. Then estimation was made on the basis of reduction of GSSG in the presence of NADPH and glutathione reductase (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 (14).

Determination of lipid peroxidation

Packed red cells (0.2 mL) were used for determination of malondialdehyde (MDA) as thiobarbituric acid reactive

rebljeno je 0,2 mL sedimenta eritrocita prema metodi Jaina i sur. (15).

Određivanje koncentracije mokraćne kiseline

Primijenjena je jednostavna kolometrijska metoda Buchanana i sur. (16).

Određivanje aktivnosti GPx (EC 1.11.1.9)

Aktivnost GPx mjerila se spektrofotometrijski (17) na 340 nm u reakcijskoj smjesi 50 mM fosfata, 5 mM EDTA pri pH 7,0, koja je sadržavala 0,3 mM NADPH, 0,3 U/mL GRx, 5 mM GSH, 4 mM natrijevog azida, 75 μM H_2O_2 i 10 μL lizata eritrocita u reakcijskoj smjesi ukupnog volumena 3 mL. Uzorak je prethodno obrađen Drabkinovim reagensom, kako bi proizveo stabilni cijanmethemoglobin, sprječavajući oksidaciju NADPH posredovanu methemoglobinskom reduktazom (ili neenzimsku oksidaciju). Jedna jedinica GPx uzeta je kao količina potrebna za oksidiranje 1 μmol NADPH/min. Aktivnost je izražena kao U/gHb.

Određivanje aktivnosti SOD (EC 1.15.1.1)

Aktivnost SOD mjerila se prema metodi Beutlera (13). Ukratko, reakcija je ovisna o prisutnosti aniona superoksida koji uzrokuju oksidaciju pirogalola. Promatrana je inhibicija oksidacije pirogalola posredstvom SOD i količina enzima koji proizvode 50% inhibicije definirana je kao jedna jedinica enzimske aktivnosti. Reakcijska smjesa sadržavala je 1M Tris, 5 mM EDTA pufer pri pH 8,0 i 10 mM pirogalola. Inhibicija oksidacije pirogalola promatrana je na 420 nm, a enzimskoj aktivnosti vrijednost je iščitana i zabilježena kao U/gHb.

Određivanje aktivnosti katalaze (EC 1.11.1.6)

Katalaza razgrađuje H_2O_2 te stvara vodu i molekularni kisik. H_2O_2 apsorbira maksimalno svjetlo na 240 nm. Kada katalaza razgradi H_2O_2, smanjuje se apsorbancija. Određivanje aktivnosti katalaze ispituje se spektrofotometrijski promatranjem brzine razgradnje H_2O_2 na 240 nm, prema postupku što ga opisuje Aebi (18). Reakcijska smjesa sadržavala je 0,9 mL 1M Tris, 5 mM EDTA pufera pri pH 7,0 i 0,1 mL uzorka. Reakcija je započeta dodavanjem 1,0 mL 200 mM vodikovog peroksida (H_2O_2) u ispitnu kivetu i dodavanjem istog volumena destilirane vode umjesto vodikovog peroksida u referentnu kivetu. Smanjenje apsorbancije bilježilo se 3 minute u intervalima od 30 sekundi. Vrijednosti apsorbancije referentne kivete oduzete su od vrijednosti ispitne kivete prije nego su se izračunale jedinice aktivnosti. Aktivnost katalaze je vrijednost iščitana i zabilježena kao kU/gHb.

Određivanje aktivnosti GRx (EC 1.8.1.7)

Reagens je pripremljen miješanjem 18 mL $KH_2PO_4$139 mM pufera, 0,76 mM EDTA pri pH 7,4 i 2 mL 2,5 mM NADPH. Uzorak (20 μL 1: 20 hemolizata i 20 μL KH_2PO_4 pufera), 220

substances (TBARS) employing the method of Jain *et al*. (15).

Uric acid determination

Simple colorimetric method of Buchanan *et al*. (16) was employed.

Determination of GPx (EC 1.11.1.9) activity

GPx activity was measured spectrophotometrically (17) 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. The hemolysate was pretreated with Drabkin's reagent to produce stable cyanmethemoglobin, eliminating methemoglobin-reductase-mediated (or nonenzymatic) oxidation of NADPH. One unit of GPx was considered to be the amount necessary to oxidize 1 μmol NADPH/min. The activity was expressed as U/gHb.

Determination of SOD (EC 1.15.1.1) activity

SOD activity was measured according to the method of Beutler (13). Briefly, the reaction is dependent on the presence of superoxide anions that cause the oxidation of pyrogallol. The inhibition of pyrogallol oxidation by SOD was monitored and the amount of enzyme producing 50% inhibition was defined as one unit of enzyme activity. The assay mixture contained 1 M Tris, 5 mM EDTA buffer, pH 8.0, and 10 mM pyrogallol. The inhibition of pyrogallol oxidation by SOD was monitored at 420 nm, and the enzyme activity was evaluated and expressed as U/gHb.

Determination of catalase (EC 1.11.1.6) activity

Catalase decomposes H_2O_2 and forms water and molecular oxygen. H_2O_2 absorbs maximum light at 240 nm. The absorbance decreases as H_2O_2 is being decomposed by catalase. Determination of catalase activity was assayed by monitoring the rate of H_2O_2 decomposition spectrophotometrically at 240 nm following the procedure of Aebi (18). The assay mixture contained 0.9 mL of 1M Tris, 5 mM EDTA buffer, pH 7.0 and 0.1 mL of the sample. The reaction was started by adding 1.0 mL of 200 mM hydrogen peroxide (H_2O_2) in the test cuvette and by adding the same volume of distilled water instead of hydrogen peroxide in the reference cuvette. The decrease in absorbance was measured with a recorder at an interval of 30 seconds for 3 minutes. The value of absorbance of the reference cuvette was subtracted from that of the test cuvette before calculating the units of activity. The activity of catalase was evaluated and expressed as kU/gHb.

Determination of GRx (EC 1.8.1.7) activity

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

μL reagensa i 5 μL 0, 315 mM flavin-adenin dinukleotida (FAD) i 10 μL KH_2PO_4 pufera dodano je u kivetu i mjerena je apsorbancija na 340 nm tijekom 200 s (korak A). Nakon toga je dodano 30 μL 22mM GSSG i 10 μL KH_2PO_4 pufera kako bi započela reakcija te je apsorbancija mjerena slijedećih 175 s (korak B). Volumen konačne reakcijske smjese bio je 315 μL. Razlika u apsorbanciji po minuti između koraka A i B upotrebljena je za izračun enzimske aktivnosti. Jedinica je μmol NADPH oksidiranog po minuti i aktivnost GRx izražena je kao U/gHb (19).

Statistička analiza

Podaci su izraženi kao srednja vrijednost ± standardna devijacija. Proveden je Studentov t-test za statističku analizu podataka, kako bi se usporedila normotenzivna kontrolna skupina i skupina bolesnica s preeklampsijom. Vrijednosti P za obostranu vjerojatnost (engl. *two-tailed probability*) izračunale su se pomoću programa GraphPad QuickCalcs Software. Razina statističke značajnosti bila je postavljena na $P \leq 0,05$.

Rezultati

Demografski i klinički parametri zdravih normotenzivnih ispitanica i onih s teškim oblikom preeklampsije prikazani su u tablici 1. Iz kliničkih je rezultata vidljivo da je prosječna vrijednost krvnog tlaka kod žena s preeklampsijom bila vrlo visoka, što ukazuje na ozbiljnost preeklampsije.

Promjene pro- i antioksidacijskih neenzimskih metabolita i osmotske rezistencije

Svi oksidacijski parametri bili su znatno povišeni kod žena s preeklampsijom u usporedbi s onima iz kontrolne

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 was monitored at 340 nm 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 was μmol of NADPH oxidized/min and the GRx activity was evaluated and expressed as U/gHb (19).

Statistical analysis

Data were expressed as mean ± standard deviation. Student's t-test was performed for statistical analysis of data to compare normotensive control and preeclamptic patient groups. The two-tailed probability *P*-values were calculated using GraphPad QuickCalcs Software. The t-test statistical significance was set at $P \leq 0.05$.

Results

Demographic and clinical parameters of the normotensive healthy subjects and patients with severe preeclampsia are summarized in Table 1. It is evident from clinical findings that the average blood pressure was very high in preeclamptic women, indicating the severity of preeclampsia.

Alterations in pro-oxidant and antioxidant nonenzymatic metabolites and osmotic fragility profiles

All oxidant parameters were significantly higher in preeclamptic women when compared with controls, with

Tablica 1. Demografski i klinički parametri normotenzivnih ispitanica (kontrolne skupine) i onih oboljelih od teškog oblika preeklampsije

Table 1. Demographic and clinical parameters of normotensive (control group) and severe preeclampsia subjects

Parameter	Control group (N = 25)	Preeclamptic group (N = 27)	P
Age (yrs)	23 ± 4	24 ± 4	0.474
Mean gestational age at sampling (wks)	35 ± 2	35 ± 2	0.529
BMI at sampling (kg/m^2)	22.8 ± 1.8	23.2 ± 1.6	0.400
Systolic BP (mm Hg)	115.4 ± 10.8	188.2 ± 13.4	**< 0.001**
Diastolic BP (mm Hg)	68.8 ± 10.2	110.8 ± 12.4	**< 0.001**
Pulse rate (beats/min)	70.6 ± 2.2	71.2 ± 1.4	0.243
Proteinuria (g/day)	/	1.82 ± 0.98	/
Edema	/	++ in all cases	/

Values are expressed as mean ± SD; BMI - body mass index; BP - blood pressure

skupine, s iznimkom GSH čija je koncentracija bila znatno smanjena, što je ukazivalo na povećan oksidacijski stres u teškom obliku preeklampsije (Tablica 2.). Kod skupine bolesnica s preeklampsijom eritrociti su pretrpjeli jaču lizu od onih prikupljenih od kontrolne skupine prvorodilja. Krivulja osmotske rezistencije kod bolesnica s teškom preeklampsijom pomaknula se udesno u odnosu na krivulju normotenzivne skupine, zbog smanjene osmotske rezistencije eritrocita (P = 0,001). Osmotska rezistencija obiju skupina prikazana je na slici 1.

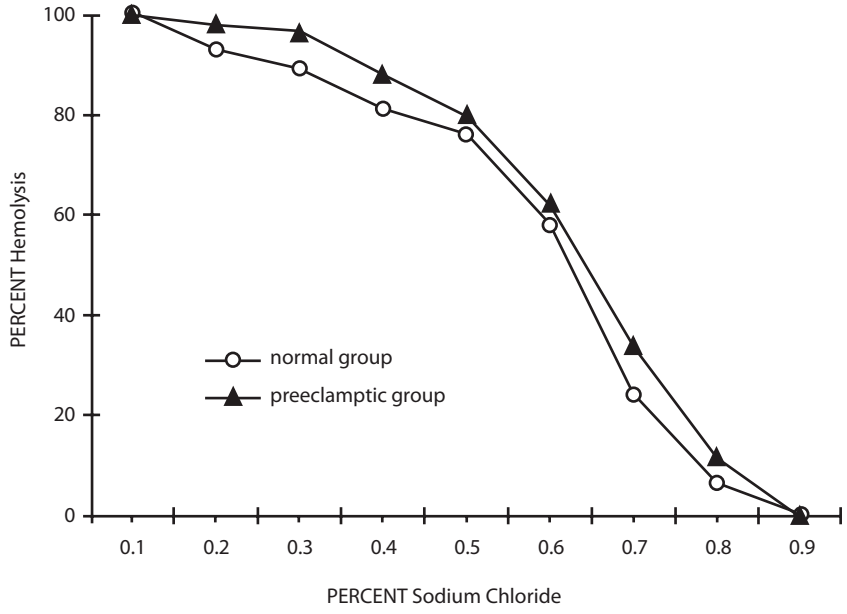
the exception of GSH, which was significantly decreased, pointing to the enhanced oxidative stress in severe preeclampsia (Table 2). The erythrocytes from preeclamptic patients underwent higher lysis than those from normal primiparae. In patients with severe preeclampsia, the osmotic fragility profile showed a shift to the right from the normotensive one due to their increased erythrocyte osmotic fragility (P = 0.001). The osmotic fragility profiles are depicted in Figure 1.

Tablica 2. Oksidacijski i antioksidacijski sadržaji u kontrolnoj skupini i skupini bolesnica s preeklampsijom

Table 2. Oxidant and antioxidant contents in control and severe preeclampsia subjects

Parameter	Control group (N = 25)	Preeclamptic group (N = 27)	% Increase ↑ % Decrease ↓	P
Uric acid (mmol/l)	0.384 ± 0.14	0.524 ± 0.08	36.5% ↑	**< 0.001**
MDA (nmol/gHb)	7.98 ± 2.92	10.64 ± 2.62	33% ↑	**0.001**
GSH (µmol/gHb)	9.24 ± 2.14	7.36 ± 1.86	20% ↓	**0.001**
GSSG (µmol/gHb)	1.21 ± 0.22	1.44 ± 0.26	19% ↑	**0.001**
GSSG/GSH	0.131 ± 0.08	0.196 ± 0.07	49.6% ↑	**0.003**

Values are expressed as mean ± SD; MDA - malondialdehyde, GSSG - oxidized glutathione, GSH - reduced glutathione



Slika 1. Osmotska rezistencija kontrolne skupine i bolesnica s teškom preeklampsijom. Kontrolna skupina: MEF = 0,622 ± 0,027; Bolesnice s preeklampsijom: MEF = 0,647 ± 0,025 (P = 0,001). MEF – osmotska rezistencija eritrocita.

Figure 1. Osmotic fragility profiles of normal healthy control and severe preeclampsia patients. Normal group: MEF = 0.622 ± 0.027; Preeclampsia group: MEF = 0.647 ± 0.025 (P = 0.001). MEF – mean erytrocyte fragility.

Promjene u enzimskom antioksidacijskom statusu

Procijenili smo mjeru enzimske antioksidacijske zaštite kod kontrolne skupine i skupine trudnica s teškom preeklampsijom i pronašli znatne varijacije u njihovim profilima (Tablica 3.). Antioksidacijski enzimi (SOD, GRx, GPx) pokazali su povećanje aktivnosti kod osoba s preeklampsijom u usporedbi sa ženama iz kontrolne skupine, što ukazuje na gubitak njihovog antioksidacijskog kapaciteta. S druge strane, primijetili smo značajno povećanje aktivnosti katalaze, što ukazuje na njenu kompenzirajuću regulacijsku ulogu u odgovoru na povećan oksidacijski stres.

Alterations in the enzymatic antioxidant status

We evaluated the quantum of enzymatic antioxidant defense both in normal and severely preeclamptic women, and found much variation in their profiles (Table 3). The antioxidant enzymes (SOD, GRx and GPx) showed a decrease in their activities in preeclamptic women as compared with normal women, indicating the loss in their antioxidant capacity, whereas a significant increase in catalase activity showed its compensating regulatory role in response to the increased oxidative stress.

Tablica 3. Aktivnosti različitih antioksidacijskih enzima u kontrolnoj skupini i i skupini bolesnica s preeklampsijom

Table 3. Activities of various antioxidant enzymes in control and severe preeclampsia subjects

Parameter	Control group (N = 25)	Preeclamptic group (N = 27)	% Increase ↑ % Decrease ↓	P
Superoxide dismutase (SOD U/gHb)	697.8 ± 86.2	532.4 ± 98.6	23.7% ↓	**< 0.001**
Glutathione peroxidase (GPx U/gHb)	13.8 ± 4.2	11.8 ± 4.6	14.5% ↓	0.109
Glutathione reductase (GRx U/gHb)	9.81 ± 2.96	7.66 ± 3.12	21.9% ↓	**0.014**
Catalase (kU/gHb)	81.4 ± 24.6	103.2 ± 23.4	26.8% ↑	**0.002**

Values are expressed as mean ± SD

Rasprava

U našem smo istraživanju procijenili promjene pro- i antioksidacijske ravnoteže kod teškog oblika preeklampsije, određujući koncentracije neenzimskih hvatača, (npr. reduciranog glutationa), aktivnosti antioksidacijskih enzima i glavnih metabolita peroksidacije lipida. Ocjenjivali smo, također, osmotsku rezistenciju eritrocita, koja je izravno povezana s patofiziološkim promjenama kod preeklampsije. To smo učinili zato što su eritrociti osobito osjetljivi na oksidacijsko oštećenje, budući da djeluju kao nosioci kisika (izloženi su visokoj zasićenosti kisikom), nemaju sposobnost samoobnavljanja, eritrocitna membrana podložna je peroksidaciji lipida, dok je hemoglobin sklon autooksidaciji.

Ovo je istraživanje otkrilo značajno povećanje koncentracije MDA u eritrocitima kod žena s teškim oblikom preeklampsije u usporedbi s kontrolnom skupinom. To bi moglo rezultirati većom mogućnošću endotelnog oštećenja, što bi na kraju dovelo do povišenja dijastoličnog tlaka (20), a to nadalje pogoršava stanje oboljelih od preeklampsije (4). Povišeni ROS pak mogu oksidirati mnoge druge

Discussion

In the present study, we evaluated the alterations in prooxidant and antioxidant balance in severe preeclampsia by determining the levels of nonenzymatic scavengers like reduced glutathione, antioxidant enzymatic activities and major metabolites of lipid peroxidation. We also evaluated erythrocyte osmotic fragility that is directly related to the pathophysiological conditions of preeclampsia. We did so because red blood cells are particularly susceptible to oxidative damage as they act as an oxygen carrier (getting exposed to high oxygen tension); do not have the capacity to repair themselves; their membranes are prone to lipid peroxidation; and hemoglobin is more susceptible to auto-oxidation.

Our present investigation revealed a significant increase in erythrocyte MDA concentration in severely preeclamptic patients in comparison to normal controls. This may result in a greater potential for endothelial damage ultimately leading to elevated diastolic pressure (20), which further aggravates the condition of preeclamptic patients (4). Enhanced ROS in turn can oxidize many other important biomolecules including erythrocyte membrane

bitne biomolekule, uključujući fosfolipide na membrani eritrocita. Kao što smo prije spomenuli, dostupan je niz članaka koji pokazuju povećane koncentracije MDA i TBARS kod preeklampsije. Stoga se naši rezultati slažu s prethodnima (4,20).

Iz naših je rezultata jasno vidljiva uloga reduciranog glutationa u zaštiti makromolekula od oksidacijskog oštećenja, budući da je koncentracija GSH bila znatno viša kod kontrolne skupine nego kod trudnica s preeklampsijom. Reducirani glutation osigurava stanicama otpornost na oksidacijsku reakciju s dovoljno visokom unutarstaničnom koncentracijom GSH. Tijekom oksidacijske reakcije GSH oksidira u GSSG, što za posljedicu ima povećanje koncentracije GSSG. Međutim, ukupna koncentracija GSH i GSSG značajno se smanjila kod oboljelih od preeklamsije, što bi moglo biti uslijed poremećenog stvaranja GSH u eritropoezi ili povećanog izlaska GSSG iz eritrocita u preeklampsiji. Pojačano otpuštanje GSSG je, prema mišljenju Srivastave i Beutlera (21), jedan od razloga zbog kojih eritrociti prenose GSSG pri visokim koncentracijama unutarstaničnog GSSG, a eritrocitna membrana prenosi GSSG, ali ne i GSH.

Odnos GSSG/GSH u eritrocitima može poslužiti kao rani i osjetljiv pokazatelj oksidacijske neravnoteže i važan cilj budućih kliničkih pokusa, kako bi se kontrolirali učinci antioksidacijskog liječenja kod žena s povećanim rizikom od sindroma preeklampsije (22). Naši podaci jasno ukazuju na to da se koncentracija GSH u eritrocitima znatno smanjila u patofiziološkom stanju preeklampsije, s usporednim povišenjem koncentracije MDA i GSSG što se slaže rezultatima što su ih objavili Padmini i Geetha (23) te Yoshio i sur. (24).

Uočili smo da je povećana liza eritocita rezultat oksidacijskog oštećenja eritrocitne membrane, a uzrokuje smanjenu fluidnost membrane i smanjuje njenu sposobnost otpora osmotskim promjenama. Unutarstanični glutation u eritrocitima oksidira se u većoj mjeri kod skupine prvorotkinja s preeklampsijom nego kod normotenzivnih prvorotkinja. Naše je zapažanje sukladno izvješću o preeklampsiji Spicketta i sur. (10). Već smo prije izvještavali o smanjenoj osmotskoj rezistenciji eritrocita kod dijabetičara koji su pokazali normalnu osmotsku rezistenciju uz liječenje inzulinom (25). Glavna odrednica hemolize *in vitro* je volumen stanice u bilo kojem trenutku u odnosu na njenu najveću moguću površinu membrane. Osmotska rezistencija *in vitro* ovisi o: i) otopini za suspenziju čiji se pH i tonicitet kontroliraju testom osmotske rezistencije; ii) ukupnom broju unutarstaničnih osmotski aktivnih čestica koje određuju volumen stanice u bilo kojem vanjskom okruženju; i iii) kritičkom volumenu hemolize kao složenom parametru ovisnom o kvantitativnim i kvalitativnim čimbenicima povezanim s lipidima i proteinima stanične membrane. Stoga, bitna veza u određivanju osmotske rezistencije jest omjer kritičnog volumena hemolize

phospholipids. As mentioned before, there are literature reports on increased levels of MDA or TBARS in preeclampsia. Thus, our findings are consistent with those reported elsewhere (4,20).

The role of reduced glutathione in the protection of macromolecules against oxidative damage was clearly evident from our findings since the level of GSH was significantly higher in normotensive women as compared with preeclamptic women. Reduced glutathione provides resistance to cells against oxidative insult with sufficient intracellular concentration of GSH. During oxidative insult, GSH is oxidized to GSSG, as a consequence of which the level of GSSG increases. However, total level of GSH and GSSG was decreased significantly in preeclamptics, which may be due to defective synthesis of GSH in erythropoiesis or increased export of GSSG from preeclamptic erythrocytes. The enhanced efflux of GSSG seems to be one of the reasons, as according to Srivastava and Beutler (21) human erythrocytes transport GSSG at high levels of intracellular GSSG and red blood cell membranes transport GSSG but not GSH.

The erythrocyte GSSG/GSH ratio may serve as an early and sensitive parameter of the oxidative imbalance and a relevant target for future clinical trials to control the effects of antioxidant treatment in women at an increased risk of the preeclampsia syndrome (22). Our data clearly indicated that red blood cell GSH decreased profoundly in the pathophysiological condition of preeclampsia with a parallel increase in MDA and GSSG concentration which is in agreement with those reported in preeclampsia by Padmini and Geetha (23) and Yoshio *et al*. (24).

We observed that increased lysis resulted from oxidative damage to the erythrocyte membrane, causing a decrease in membrane fluidity and reducing its ability to withstand osmotic changes, and intracellular glutathione was more oxidized in erythrocytes from preeclamptic women as compared to normotensive primiparae. Our observation is in harmony with the report on preeclampsia by Spickett *et al*. (10). We have previously reported increased osmotic fragility of diabetic erythrocytes, which yielded normal osmotic fragility profile on insulin treatment (25). The main determination of *in vitro* hemolysis is the volume of the cell at any given time in relation to its maximal possible membrane surface area. *In vitro* osmotic fragility is dependent on: i) the suspending medium, whose pH and tonicity are controlled in the osmotic fragility test; ii) total number of intracellular osmotically active constituents, which determine cell volume in any given external environment; and iii) the critical hemolytic volume, a complex parameter dependent on quantitative and qualitative factors associated with the membrane lipid and protein. Therefore, the important relationship determining osmotic fragility is the ratio of critical hemolytic volume to the internal osmotic conten-

i unutarstaničnih osmotskih aktivnih čestica eritrocita. Naši rezultati koncentracija GSH, GSSG i MDA u eritrocitima kod kontrolne i ispitne skupine jasno pokazuju znatnu promjenu u njihovom unutarnjem sadržaju. Izvještavali smo (26) o tome da, kada eritrocit izgubi sposobnost zadržavanja svoje koncentracije GSH, aktivira se proteolitički mehanizam na membrani i uzrokuje otpuštanje sijaloglikopeptida i promjene površine koje organima koji uklanjaju stare stanice omogućuju odstraniti takve stanice iz krvotoka.

U našem su se ispitivanju aktivnosti antioksidacijskih enzima SOD i GRx u eritrocitima značajno snizile. SOD je bitan antioksidacijski enzim koji ima antitoksični učinak na superoksid-anion i katalizira reakciju u kojoj se radikali superoksida pretvaraju u H_2O_2 and O_2. SOD snižava koncentraciju superoksid-aniona u stanicama krvnih žila (27), a to je mehanizam koji bi se mogao suprotstaviti razvoju hipertenzije. Naši rezultati su pokazali kako su oba ova enzima zakazala u izvršavanju svojih uloga u potrebnoj mjeri kod preeklampsije, i to zbog njihove smanjene aktivnosti. Glutation-peroksidaza (GPx), enzim induciran oksidacijskim stresom, ima važnu ulogu u mehanizmu uklanjanja peroksila i u održavanju integriteta stanične membrane (28). Njegova aktivnost je smanjena u preeklampsiji, ali ne značajno, što bi moglo značiti da ne pruža zaštitu integriteta eritrocitne membrane. Naši rezultati o aktivnostima SOD, GPx i GRx u preeklampsiji sukladni su s literaturnim izvješćima (1-4). Oprečni izvještaji (5-7) o povećanju njihove aktivnosti mogli bi se protumačiti nedostatkom vitamina E kod ispitanika skupine oboljelih od preeklampsije, o čemu ovisi stupanj težine bolesti. Tijekom normalne trudnoće, koncentracija vitamina E u plazmi progresivno se povećava, možda zbog povećanja koncentracije cirkulirajućih lipoproteina kao prenositelja vitamina E tijekom trudnoće. Kod bolesnica s blagim oblikom preeklampsije koncentracija α-tokoferola u majčinoj krvi nisu smanjene u usporedbi s vrijednostima u normalnoj trudnoći (29,30), ali kod bolesnica s teškom preeklampsijom koncentracija α-tokoferola u plazmi značajno pada u usporedbi s kontrolnom skupinom. Smatra se da je razlog tome što se antioksidansi u većoj mjeri iskorištavaju za suprotstavljanje poremećajima što ih stanica trpi posredstvom slobodnih radikala, a to pak dovodi do smanjenja njihove koncentracije u plazmi (31). Značajno povećanje aktivnosti katalaze u skupini oboljelih od preeklampsije pokazuje obrambeni učinak ovoga enzima, koji štiti stanice od nakupljanja H_2O_2 razgrađujući ga na vodu i kisik, koji onda rabi kao oksidans u kojem djeluje poput peroksidaze (32).

U zaključku, na temelju rezultata koji su pokazali smanjenu aktivnost SOD, GRx i GPx, koji nisu uspjeli kontrolirati povišenu proizvodnju kisikovih slobodnih radikala, postavljamo hipotezu o povećanom oksidacijskom stresu kod preeklampsije. Povećana aktivnost katalaze mogla bi biti kompenzirajući regulator u odgovoru na povećats of the red blood cell. Our results on the red cell contents of GSH, GSSG and MDA in normal and preeclamptic women clearly pointed to a significant change in their internal contents. We have reported (26) that, when the erythrocyte loses the ability to maintain its GSH concentrations, the membrane proteolytic mechanism becomes active, causing sialoglycopeptide release and surface modifications that enable hemocatheretic organs to remove old cells from the blood circulation.

In the present study, the erythrocyte SOD and GRx antioxidant enzyme activities decreased significantly. SOD is an important antioxidant enzyme having an antitoxic effect against superoxide anion and catalyzing the reaction in which superoxide radicals are converted to H_2O_2 and O_2. It decreases superoxide anion concentration in the vascular cell (27), a mechanism that could counteract the development of hypertension. Our results showed the failure of both these enzymes to perform their roles in case of preeclampsia up to the required extent because of their reduced activities. Glutathione peroxidase (GPx), an oxidative stress inducible enzyme, plays a significant role in the peroxyl scavenging mechanism and in maintaining the cell membrane integrity (28). Its activity decreased in preeclampsia, although non-significantly, which could be interpreted as not providing protection to the red cell membrane integrity. Our findings on the activities of SOD, GPx and GRx in preeclampsia are in harmony with the reports of others (1-4). Contrast reports of an increase in their activities (5-7) might be explained on the basis of the lack of vitamin E levels in the study subjects, which depends on the severity of preeclampsia. During normal pregnancy, plasma vitamin E concentrations show progressive elevation, what could be due to the gestational increase in circulating lipoproteins as vitamin E transporters. In patients with mild preeclampsia, maternal blood α-tocopherol concentrations were not decreased as compared with normal pregnancies (29,30), but in patients with severe preeclampsia plasma α-tocopherol was significantly decreased as compared with controls, which is thought to be caused by the fact that antioxidants may be utilized to a greater extent to counteract free radical-mediated cell disturbances, resulting in a reduction in their plasma levels (31). The significant elevation in preeclamptic catalase activity shows the protective effect of this enzyme, which protects the cells from the accumulation of H_2O_2 by dismutating it to form water and oxygen by using it as an oxidant in which it works as a peroxidase (32).

In conclusion, we hypothesize the oxidative stress to be increased in preeclampsia, based on our results showing decreased SOD, GRx and GPx activities, which failed to control higher oxygen free radical production therein. The increased activity of catalase may be a compensatory regulation in response to the increased oxidative stre-

ni oksidacijski stres. Povećana aktivnost katalaze može se protumačiti kao uzaludno suprotstavljanje prevelikom stvaranju reaktivnih kisikovih spojeva (ROS) i kao pomoć kod povećanog oksidacijskog oštećenja u preeklampsiji. Međutim, smanjena osmotska rezistencija jasno upućuje na gubitak integriteta stanične membrane i skraćen životni vijek eritrocita u preeklampsiji. Lipidni peroksidi mogli bi biti dio citotoksičnog mehanizma koji uzrokuje endotelne ozljede i povišen krvni tlak. Konačno, naši rezultati su ukazali na to da prooksidansi prevladavaju nad antioksidansima kod preeklampsije i da se ravnoteža na kraju narušava u korist oksidacijskog stresa koji, doduše, nije bio uzročni čimbenik, nego posljedica razvoja preeklampsije. Daljnja istraživanja učinka antioksidacijske terapije u suzbijanju oksidacijskog stresa mogla bi pomoći u razumijevanju mehanizma razvoja patofiziološkog stanja preeklampsije.

Zahvala

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ss. The increased catalase activity could be interpreted as a futile effort to counteract the overproduction of reactive oxygen species and providing relief to the increased oxidative damage in preeclampsia. However, the increased osmotic fragility clearly indicated the loss in cellular membrane integrity and shortened life span of preeclamptic red blood cells. Lipid peroxides could be a part of the cytotoxic mechanism leading to the endothelial injury and elevated blood pressure. Finally, our findings suggested pro-oxidants to prevail over antioxidants in preeclampsia and the balance was ultimately disturbed in favor of oxidative stress, which was not the causative factor but the consequence of preeclampsia development. Further studies on the effect of antioxidant therapy, to combat the oxidative burden, may be more helpful to understand properly the mechanism of the development of pathophysiological conditions of preeclampsia.

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