Hypertension in Pregnancy

ISSN: 1064-1955 (Print) 1525-6065 (Online) Journal homepage: https://www.tandfonline.com/loi/ihip20

Urinary protein carbonyl levels and its correlation with protein misfolding in preeclampsia

Chandrakala Nagarajappa, Sheela Shikaripur Rangappa, Ravishankar Suryanarayana & Sharath Balakrishna

To cite this article: Chandrakala Nagarajappa, Sheela Shikaripur Rangappa, Ravishankar Suryanarayana & Sharath Balakrishna (2019): Urinary protein carbonyl levels and its correlation with protein misfolding in preeclampsia, Hypertension in Pregnancy

To link to this article: https://doi.org/10.1080/10641955.2019.1590720

Published online: 26 Mar 2019.

HYPERTENSION IN PREGNANCY
https://doi.org/10.1080/10641955.2019.1590720

Taylor & Francis
Taylor & Francis Group

Urinary protein carbonyl levels and its correlation with protein misfolding in preeclampsia

Chandrakala Nagarajappa[a], Sheela Shikaripur Rangappa[b], Ravishankar Suryanarayana[c], and Sharath Balakrishna[a]

[a]Department of Cell Biology and Molecular Genetics, Sri Devaraj Urs Academy of Higher Education and Research, Kolar, India; [b]Department of Obstetrics and Gynecology, Sri Devaraj Urs Medical College, Kolar, India; [c]Department of Community Medicine, Sri Devaraj Urs Medical College, Kolar, India

ABSTRACT

Objective: To evaluate the association of protein carbonylation with preeclampsia and its correlation with urinary protein misfolding.

Method: Protein carbonyl and misfolded protein levels were measured in the midstream urine sample (58 preeclamptic and 44 normotensive pregnancy) by ELISA and Congo Red Dot assay respectively.

Results: Significant difference was observed in the levels of protein carbonyls (P = 0.002) and misfolded proteins (P = 0.001). Correlation between protein carbonyl and misfolded proteins levels was significant but weak (r = 0.3; P = 0.018).

Conclusion: Urinary protein carbonyl level is elevated in preeclampsia but plays a minor role in proteins misfolding.

ARTICLE HISTORY
Received 18 December 2018
Accepted 1 March 2019

KEYWORDS
Preeclampsia; protein carbonyl; misfolded proteins

Introduction

Preeclampsia is a common complication of pregnancy that is characterized by *de novo* hypertension and proteinuria. Preeclampsia is a major public health burden that affects 5–8% of pregnancies globally (1). Also, it is one of the leading cause of perinatal morbidity and mortality. Impaired placental vascularization and endothelial damage are the etiological hallmarks of preeclampsia. Endothelial damage has been documented in the vessels of the glomerulus, umbilical cord, and placenta (2). Also, markers of endothelial activation like adhesion molecules, cytokines, and pro-coagulant and anti-angiogenic factors (sFLT-1 and sENG) are elevated in the plasma of preeclamptic women (2). Endothelial dysfunction is assumed to be responsible for the proteinuria in preeclampsia (2).

Oxidative stress plays an important role in endothelial damage. Involvement of oxidative stress in preeclampsia is indicated by the elevated levels of oxidative stress markers in the placenta, maternal, and fetal plasma. Commonly observed markers of oxidative stress are protein carbonyl (due to protein oxidation), malonaldehyde (due to lipid peroxidation), and 8-hydroxy-2′-deoxyguanosine (due to DNA oxidative) (3–5). Furthermore, a reduction in the total antioxidant status has been demonstrated in the maternal plasma and placenta (6).

Urine is an important specimen in preeclampsia since it is diagnostically altered in most patients due to proteinuria. Recent studies have shown that the urine of preeclamptic women contains misfolded proteins (7–10). This observation extends the spectrum of abnormalities in the urine of preeclamptic women. Though the presence of misfolded proteins has been confirmed by subsequent studies, the cause of misfolding is still not clear. Protein oxidation may be linked to the misfolding of urinary proteins in preeclamptic women. Oxidative damage is an established factor in protein misfolding (11). The aim of this study was to evaluate the urinary levels of protein carbonyl in preeclamptic pregnant women and its correlation with urinary protein misfolding.

Materials and methods

Study design

We conducted a case–control study by including 44 normotensive and 58 preeclamptic pregnant women, respectively. Subjects were enrolled between November 2016 – October 2017 from the Department of Obstetrics and Gynecology of R. L. Jalappa Hospital and Research Centre attached to Sri Devaraj Urs Medical College. The study was approved by the

CONTACT Sharath Balakrishna sharath@sduu.ac.in Department of Cell Biology and Molecular Genetics, Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar 563103, India

Institutional Ethics Committee of Sri Devaraj Urs Medical College, Kolar, Karnataka, India. Written informed consent was obtained prior to the recruitment of the subjects. Midstream urine sample was collected from the study participants and used to evaluate the levels of protein carbonyl and misfolded proteins.

Patient selection

Preeclampsia was diagnosed in the pregnant women on the basis of the following criteria: (i) *de novo* hypertension (systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg measured 4 h apart twice while the patient is on bed rest, (ii) ≥20 weeks of gestation, (iii) new onset proteinuria (≥300 mg protein in 24-h urine sample or +1 on dipstick), (iv) in the absence of proteinuria, other symptoms like Hemolysis Elevated Liver Low Platelet counts syndrome, edema, thrombocytopenia, impaired liver function, new-onset cerebral or visual disturbances and renal insufficiency (in the absence of other renal diseases) nausea, severe headache, and convulsions (12). Inclusion criteria for the cases were: (i) pregnant women diagnosed with preeclampsia, (ii) superimposed eclampsia, (iii) singleton and multiple gestation, and (iv) primigravida and multigravida condition. Exclusion criteria for the cases were: (i) pregnant women with chronic hypertension and (ii) co-morbidities such as diabetes mellitus, epilepsy, respiratory diseases, and heart diseases. Pregnant women were defined as "normotensive" if they did not develop any complications till the time of delivery.

Sample collection and storage

Midstream urine sample was collected in a sterile urine container (Himedia, Mumbai, India) and centrifuged at 2,500 rpm for 5 min at room temperature. The supernatant was preserved at – 80°C till further analysis.

Estimation of protein concentration

Total protein content of the midstream urine sample was determined by the Bradford method using standard Bovine Serum Albumin (13). Urine samples were normalized to the required protein concentration by dilution or concentration by dialysis method (14).

Estimation of urinary protein carbonyl

Protein concentration of the urine samples was normalized to 10 µg/ml. Protein carbonyl levels of the normalized sample were determined by the ELISA method using a commercial kit (Cell BioLabs, San Diego, CA).

Estimation of urinary misfolded proteins

Protein concentration of the urine samples was normalized to 15 µg/ml. Misfolded protein levels were determined by Congo Red Dot assay (8). One hundred µl of the normalized urine sample was mixed with 5 µl of Congo Red (5 µg/ml in water) and vortexed for 1 h at room temperature. Five µl of the mix was spotted in duplicates on a strip of supported nitrocellulose membrane (Himedia, Mumbai, India), air dried for 15 min, and then washed with Milli-Q water for 3 min. Image of the nitrocellulose membrane strip was recorded using Gel Doc Molecular imager (Bio-Rad, Hercules, USA). Nitrocellulose membrane strip was then sequentially washed with 50% methanol for 3 min, 70% methanol for 1 min and 90% methanol for 10 min. The image of the washed spot was recorded as before. The color intensity of the spot was measured by using Image Lab software (Bio-Rad, Hercules, USA). Percentage of Congo Red Retention (CRR) was determined using the formula given below.

$$CRR\ (\%) = \left(\frac{Spot\ intensity\ after\ wash}{Spot\ intensity\ before\ wash}\right) 100$$

Statistical analysis

Statistical analysis was carried out using SPSS Statistics V22.0 (International Business Machine Corporation, Armonk, New York). Quantitative variables were represented as mean, standard deviation and confidence interval. Qualitative variables were represented as percentages. Shapiro–Wilk test was performed with Q–Q plots and normality plots. Mean was determined if the data showed normal distribution; otherwise, the median was calculated. Means of the two groups was compared using student t-test while medians of the two groups were compared using the Mann–Whitney U test. Pearson's correlation coefficient was used to assess the correlation between the variables. P value <0.05 was considered to be statistically significant.

Results

The clinical parameters of the study participants are summarized in Table 1. A total of 102 pregnant women were included in the study. Of these, 58 women were preeclamptic and 44 women were normotensive pregnant. The

Table 1. Clinical parameters of the study groups.

Parameter	Preeclamptic pregnant women (n = 58)	Normotensive pregnant women (n = 44)
Age (years)	23.9 ± 3.5	24.6 ± 3.3
Gravida	• 32 (55.2%)	• 19 (43.2%)
• Primigravida	• 26 (44.8%)	• 25 (56.8%)
• Multigravida		
Severity	• 13 (22.4%)	NA
• Mild	• 45 (77.6%)	
• Severe		
Gestational age of onset	• 25 (43.1%)	NA
• Early onset (< 34 weeks)	• 33 (56.9%)	
• Late onset (≥ 34 weeks)		
Blood pressure (mm Hg)	• 166 ± 17.1	• 117.0 ± 4.6
• Systolic Blood Pressure	• 108.9 ± 9.4	• 75.9 ± 4.9
• Diastolic Blood Pressure		
Dipstick proteinuria	• 36 (62.1%)	NA
• 1+	• 14 (24.1%)	
• 2+	• 08 (13.8%)	
• 3+		
Co-morbidities	• 19 (32.8%)	NA
• Eclampsia	• 7(12.1%)	
• IUGR	• 3(5.2%)	
• Stillbirth	• 1 (1.7%)	
• HELLP syndrome		

NA = Not Applicable

protein concentration of the urine samples was normalized to ensure that the differences observed in the study parameters reflected baseline modifications and were not due to proteinuria.

Firstly, we determined the urinary levels of protein carbonyl both in preeclamptic and normotensive pregnant women (Figure 1). The protein carbonyl levels did not show normal distribution. Therefore, we calculated the median and interquartile range (IQR) for both the patient groups. The median protein carbonyl levels in the urine were 1.1 nmol/mg (IQR = 0.4–2.1 nmol/mg) in the preeclamptic women and 0.58 nmol/mg (IQR = 0.19–1.08 nmol/mg) in the normotensive women. The urinary protein carbonyl levels in the preeclamptic pregnant women were 1.96 times higher than in the normotensive pregnant women. The difference in the urinary protein carbonyl levels between the two groups was statistically significant (P=0.002; Mann–Whitney U test; 2-tailed).

Secondly, we determined the urinary levels of misfolded protein by measuring the percentage of Congo Red Retention (Figure 2). Misfolded protein levels in both preeclamptic and normotensive pregnant women followed the normal distribution. Therefore, we calculated the mean and standard deviation for each patient group. The mean Congo Red Retention of the urine was 77.2 ± 11.5% in the preeclamptic women and 38.0 ± 4.3% in the normotensive women. The mean Congo Red Retention of urine from preeclamptic pregnant women was 2.03 times higher than that of normotensive pregnant women. The difference in the means of Congo Red Retention between the two groups was statistically significant (P=0.001; student t-test).

Thirdly, we carried out Pearson's correlation test to determine the correlation between protein carbonyl levels and Congo Red Retention of the urine from preeclamptic

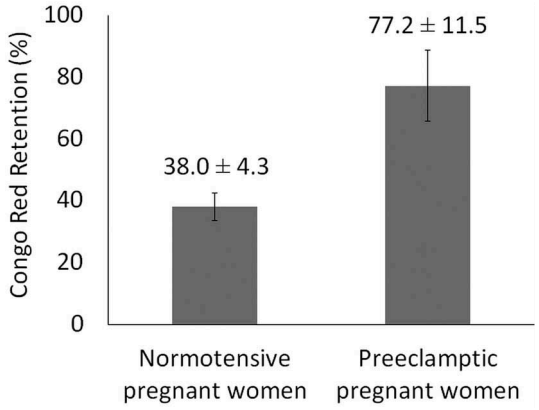

Figure 2. Comparison of urinary misfolded protein levels in preeclamptic and normotensive pregnant women.

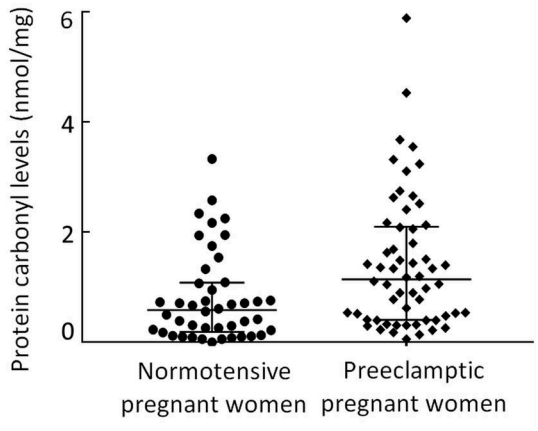

Figure 1. Comparison of urinary protein carbonyl levels in preeclamptic and normotensive pregnant women.

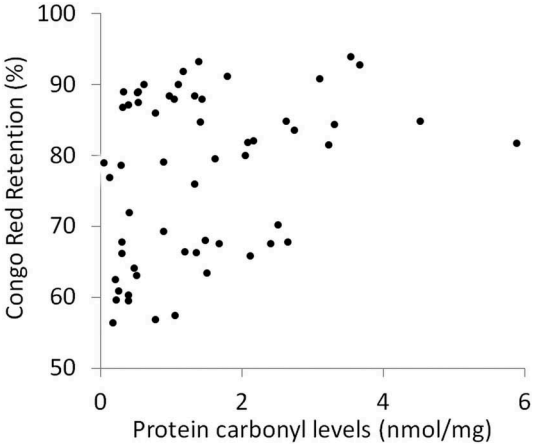

Figure 3. Correlation between urinary protein carbonyl levels and Congo red retention in preeclamptic pregnant women.

pregnant women (Figure 3). The correlation between the two parameters was statistically significant (P = 0.018) but weak (r = 0.3). We also analyzed early-onset and late-onset subgroups for correlation separately. Positive correlation was observed with early-onset but not with late-onset. However, the results were not significant (early-onset: r = 0.22; P = 0.27; late-onset: r = −0.02; P = 0.92). This appears to be due to fractioning of the sample size and the consequent loss of normal distribution in the data (early-onset: skewness = 0.08 $_{misfolding}$, 2.63 $_{carbonyl}$; late-onset: skewness = −1.15 $_{misfolding}$, 0.71 $_{carbonyl}$).

Discussion

In this study, we have evaluated the association of urinary protein carbonyl levels with preeclampsia. To the best of our knowledge, this is the first study to determine the urinary levels of protein carbonyl with preeclampsia. Furthermore, we have examined the association of protein carbonyl with urinary protein misfolding. The main findings of this study are: (i) urinary protein carbonyl levels are elevated in preeclampsia and (ii) elevation in protein carbonyl levels showed a significant but weak correlation with protein misfolding. These results show that urinary proteins in preeclampsia are subjected to oxidative damage and this may be one of the causes of misfolding.

Elevated levels of protein carbonyl have been demonstrated in the plasma of the preeclamptic women. It is seen that the protein carbonyl levels in the maternal plasma are higher in pregnant women than in non-pregnant women (3). Furthermore, the protein carbonyl levels are significantly higher in preeclamptic women than in normotensive women. The association of plasma carbonyl levels with preeclampsia has been confirmed by several studies (6,15–17). The elevation in protein carbonyl levels was in the range of 1.6–2.7 fold.

Elevated levels of protein carbonyl have also been documented in the cord blood of preeclamptic pregnancies (16,18–20). The relative increase in the protein carbonyl levels between preeclamptic and normotensive pregnant women was 1.6–2.0 times. Association of elevated protein carbonyl levels with preeclampsia has also been verified in the placenta (21). Our results extend the spectrum of protein oxidation in preeclampsia to urine. This observation is significant in view of the diagnostic value of urine in preeclampsia.

Recent studies have shown that preeclampsia is an amyloidosis, that is, a disorder of protein conformation. Misfolded proteins have been demonstrated in the maternal plasma, maternal urine, and placenta (7–10). In addition, unfolded protein response that counters accumulation of misfolded proteins has been shown to be elevated in preeclamptic placenta (22). Misfolded proteins are known to bind with high affinity to dyes like Congo Red, Thioflavin T, and Curcumin. The affinity for Congo Red has been exploited to develop a non-invasive test for preeclampsia using urine sample (8). The association of preeclampsia with urinary misfolded proteins is now well established. However, the cause of protein misfolding in preeclamptic women is not known. The major causes of protein misfolding are disruption of protein stability and excessive protein synthesis (23,24). Oxidative damage has been shown to result in protein misfolding (11). Our observation of a weak correlation between protein carbonyl and Congo Red Retention indicates that protein oxidation plays a minor role in the misfolding of urinary proteins in preeclampsia. Plasma proteins like albumin, alpha-1-antitrypsin, IgG κ-free light chain, ceruloplasmin, and interferon inducible protein 6–16 have been observed in the misfolded protein fraction of urine from preeclamptic women (8). Hence, the congophilia in the present study may reflect plasma proteins misfolded as a result of the elevated systemic oxidative stress during preeclampsia. Also, urinary congophilia has been demonstrated even in chronic kidney disease (9). Therefore, kidney lesions, which are common to both preeclampsia and chronic kidney disease, may have also contributed to congophilia. If placenta was the source of congophilia, then the correlation between protein carbonyl and congophilia would have been stronger with early-onset than with late-onset preeclampsia. Because, placental endoplasmic reticulum stress is comparatively higher in early-onset preeclampsia (22). Our results appear to indicate this possibility, but the correlation lacks statistical significance possibly due to insufficient samples at the level of subgroups.

To conclude, this study adds protein oxidation to the spectrum of abnormalities in the urine of preeclamptic women. Protein oxidation along with proteinuria and misfolding may serve as a comprehensive panel to screen and diagnose preeclampsia.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

No specific funding was available for this study.

References

[1] Gathiram P, Moodley J. Pre-eclampsia: its pathogenesis and pathophysiology. Cardiovasc J Afr. 2016;27:71–78.

[2] Aouache R, Biquard L, Vaiman D, et al. Oxidative stress in preeclampsia and placental diseases. Int J Mol Sci. 2018;19:1–29.
[3] Zusterzeel PL, Mulder TP, Peters WH, et al. Plasma protein carbonyls in nonpregnant, healthy pregnant and preeclamptic women. Free Radic Res. 2000;33:471–476.
[4] Atiba AS, Abbiyesuku FM, Oparinde DP, et al. Plasma malondialdehyde (MDA): an indication of liver damage in with pre-clampsia. Ethiop J Health Sci. 2016;26:479–486.
[5] Wiktor H, Kankofer M, Schmerold I, et al. Oxidative DNA damage in placentas from normal and preeclamptic pregnancies. Virchows Arch. 2004;445:74–78.
[6] Serdar Z, Gur E, Colakoethullary M, et al. Lipid and protein oxidation and antioxidant function in women with mild and severe preeclampsia. Arch Gynecol Obstet. 2003;268:19–25.
[7] Buhimschi IA, Zhao G, Funai EF, et al. Proteomic profiling of urine identifies specific fragments of SERPINA1 and albumin as biomarkers of preeclampsia. Am J Obstet Gynecol. 2008;199:1–16.
[8] Buhimschi IA, Nayeri UA, Zhao G, et al. Protein misfolding, congophilia, oligomerization, and defective amyloid processing in preeclampsia. Sci Transl Med. 2014;6:1–14.
[9] McCarthy FP, Adetoba A, Gill C, et al. Urinary congophilia in women with hypertensive disorders of pregnancy and preexisting proteinuria or hypertension. Am J Obstet Gynecol. 2016;215:1–7.
[10] Nagarajappa C, Rangappa SR, Suryanarayana R, et al. Urinary congophilia in preeclampsia: experience from a rural tertiary-care hospital in India. Pregnancy Hypertens. 2018;13:83–86.
[11] Gregerson N, Bross P. Protein misfolding and cellular stress: an overview. Protein Methods Mol Biol. 2010;648:3–23.
[12] Roberts JM, August PABakris G et al. Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. Obstet Gynecol. 2013;122 (5):1122–1131. doi:10.1097/01.AOG.0000437382.03963.88.
[13] Bradford MM. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–254.
[14] Mcfarlane H. A simple rapid method of concentrating urine for protein electrophoresis. Clin Chim Acta. 1964;9:376–380.
[15] Tsukimori K, Yoshitomi T, Morokuma S, et al. Serum uric acid levels correlate with plasma hydrogen peroxide and protein carbonyl levels in preeclampsia. Am J Hypertens. 2008;21:1343–1346.
[16] Bharadwaj S, Bhat VB, Vickneswaran V, et al. Oxidative stress in preeclamptic mother – newborn dyads and its correlation with early neonatal outcome – a case control study. J Matern Fetal Neonatal Med. 2018;31:1548–1553.
[17] Bernardi F, Guolo F, Bortolin T, et al. Oxidative stress and inflammatory markers in normal pregnancy and preeclampsia. J Obstet Gynaecol Res. 2008;34:948–951.
[18] Bernardi FC, Felisberto F, Vuolo F, et al. Oxidative damage, inflammation and toll-like receptor 4 pathway are increased in preeclamptic patients: a case control study. Oxid Med Cell Longev. 2012;1–6. DOI:10.1155/2012/636419.
[19] Howlader MZ, Parveen S, Tamanna S, et al. Oxidative stress and antioxidant status in neonates born to preeclamptic mother. J Trop Pediatr. 2009;55:363–367.
[20] Negi R, Pande D, Karki K, et al. Association of oxidative DNA damage, protein oxidation and antioxidant function with oxidative stress induced cellular injury in preeclamptic/eclamptic mothers during fetal circulation. Chem Biol Interact. 2014;208:77–83.
[21] Zusterzeel PL, Rutten H, Roelofs HM, et al. Protein carbonyl in deciduas and placenta of preeclamptic women as markers for oxidative stress. Placenta. 2001;22:213–219.
[22] Yung HW, Atkinson D, Campion-Smith T, et al. Differentiation activation of placental unfolded protein response pathways implies heterogeneity in causation of early – and late –onset preeclampsia. J Pathol. 2014;234:262–276.
[23] Santucci R, Sinibaldi F, Fiorucci L. Protein folding, unfolding and misfolding: role played by intermediate states. Mini Rev Med Chem. 2008;8:57–62.
[24] Hartl F. Protein misfolding diseases. Annu Rev Biochem. 2017;86:21–26.