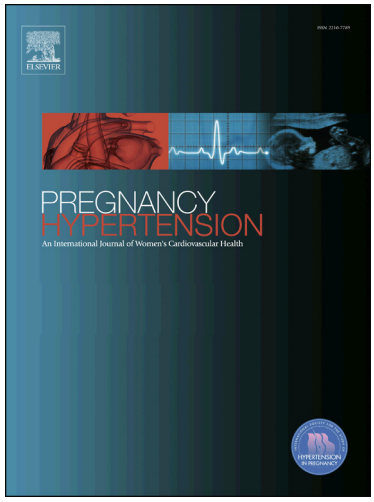

Urinary congophilia in preeclampsia: Experience from a rural tertiary-care hospital in India

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Urinary congophilia in preeclampsia: Experience from a rural tertiary-care hospital in India

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Highlights:

- Urinary congophilia was elevated in preeclamptic pregnant women
- Urinary congophilia was not affected by gestational age of onset.
- Urinary congophilia may be used for predicting the risk of preeclampsia.

Abbreviations

CRDB : Congo Red Dot Blot

CRR : Congo Red Retention

PE : Preeclampsia

IUGR : Intrauterine growth restriction

IUD : Intrauterine death

HELLP : Hemolysis Elevated Liver Low Platelet Count

Abstract

Objectives: To evaluate the presence of urinary congophilia among Indian patients with preeclampsia.

Study design: A prospective case control study in which congophilia of urine samples from preeclamptic pregnant women (n = 62) and normotensive pregnant women (n = 65) was compared by using Congo Red Dot Blot assay.

Main outcome measures: Presence of urinary congophilia.

Results: Mean percentage of Congo Red Retention was 37.9 ± 4.1 in the normotensive pregnant group and 77.9 ± 11.5 in the preeclamptic pregnant group ($P < 0.001$). The mean percentage of Congo Red Retention in both early-onset (70.5 ± 9.0) and late-onset (82.7 ± 10.3) groups were significantly higher than in normotensive controls ($P < 0.001$). The mean percentage of Congo Red Retention in mild (61.2 ± 3.2) and severe (82.4 ± 8.4) types of preeclampsia were also as significantly higher than in normotensive controls ($P < 0.001$). The mean percentage of Congo Red Retention in preeclampsia superimposed by eclampsia (89.4 ± 2.0) and preeclampsia complicated by intrauterine growth restriction and intrauterine death (74.6 ± 5.8) were significantly higher than in normotensive controls ($P < 0.001$).

Conclusions: The results of this study confirms the presence of urinary congophilia in Indian pregnant women with preeclampsia. Furthermore, our study shows that urinary congophilia is not affected by clinical variables like gestational age of onset, severity, superimposition by eclampsia and complication by intrauterine growth restriction and intrauterine death. Urinary congophilia can be used to differentially identify preeclamptic pregnant women from normotensive pregnant women.

Keywords: Preeclampsia; Misfolded proteins; Urinary Congophilia

Introduction:

Mature proteins are folded into a specific three dimensional confirmation. Pathophysiological conditions in some diseases can disturb the ordered folding and lead to the formation of structurally abnormal misfolded proteins. Protein misfolding may contribute to disease pathogenesis by either reducing the biological activity of the protein or due to toxicity of the misfolded proteins [1,2]. Misfolded proteins are well known for their role in the formation of amyloid plaques seen in neurodegenerative disorders like Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis, Spongiform Encephalopathy and Familial Amyloidotic Polyneuropathy [3]. An azo dye called Congo Red exhibits a special affinity for misfolded proteins (a property referred to as congophilia) and is used as gold standard to identify amyloid fibrils[4,5]. Recent studies have shown that misfolded proteins are abundantly present in the urine of pregnant women who develop preeclampsia [6,7].

In addition to its importance in understanding the pathophysiology of preeclampsia, misfolded proteins are indicated to hold diagnostic and prognostic value [6]. Pilot studies have indicated that misfolded proteins appear in urine well before the onset of clinical symptoms [6]. This aspect along with the ease of determining the urinary misfolded proteins holds promise for early diagnosis and risk prediction of preeclampsia particularly in resource limited settings like in developing and underdeveloped nations. Urinary misfolded proteins can be detected by a simple paper-based dot-blot technique using congo red staining; the method is referred to as Congo Red Dot Blot (CRDB) [6]. Congo red is a synthetic diazo dye with specific affinity for β-sheets of amyloid fibrils of misfolded proteins [8-10]. This special affinity of misfolded proteins to congo red dye is known as congophilia. In addition to the simplicity of congophilia based dot blot technique, the non-invasiveness of the technique is useful in adapting the CRBD assay for self-collected urine samples.

In order to establish the clinical utility of CRDB assay, extensive data from diverse geo-ethnic populations is necessary. At present, there are only two studies on the presence of misfolded protein in the urine of preeclamptic women. Both of these studies were carried out in western population mainly involving pregnant women of Caucasian descent [6,7]. There are no studies from developing societies where this assay promises the highest benefit to maternal health management. In this study, we have evaluated the presence of misfolded proteins in the urine of

preeclamptic women in a tertiary care hospital located in a rural and economically backward area in India.

Materials and Methods

Study design: We conducted a case control study comprising of pregnant women with and without preeclampsia. The study was approved by the Institutional Ethics Committee of Sri Devaraj Urs Medical College, Kolar, Karnataka, India. Informed consent was obtained in writing before recruiting the patients. Patients were recruited from the Department of Obstetrics and Gynecology of R. L. Jalappa Hospital and Research Centre attached to Sri Devaraj Urs Medical College between November 2016 – October 2017. A total of 127 pregnant women were recruited of whom 62 were affected with preeclampsia while the remaining 65 pregnant women were normotensive and presented no complications till delivery. Midstream urine sample was collected from all the study participants and congophilia was measured by CRDB assay.

Patient selection: Women were diagnosed with preeclampsia based on the following criteria: i) new onset hypertension (two readings of systolic blood pressure ≥ 140 mm Hg or diastolic blood pressure ≥ 90 mm Hg measured 4 hours apart while the patient is on bed rest ii) ≥ 20 weeks of gestation iii) new onset proteinuria (>300 mg protein for 24 hours of urine 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 disease) nausea, severe headache and convulsions [11]. Inclusion criteria were: (i) pregnant women with preeclampsia, (ii) superimposed eclampsia, (iii) singleton and multiple gestation and, (iv) primigravida and multigravida condition. Exclusion criteria were: (i) pregnant women with chronic hypertension and (ii) co-morbidities such as diabetes mellitus, epilepsy, respiratory diseases, and heart diseases.

Sample collection and storage: Midstream urine samples were collected in sterile urine container (Himedia, Mumbai. India) and centrifuged at 2,500 rpm for 5 min. The supernatant was stored at -80^0 C till analysis.

Congo Red Dot Blot assay: Total protein content of the urine sample was estimated by Bradford method using Bovine Serum Albumin as the standard [12]. Protein concentration of all the urine samples were normalized to 15 µg/ml by concentration or dilution depending on the starting level. Concentration of the urine samples were carried out by using dialysis method [13]. CRDB assay was performed according to the method of Buhimschi *et al* [6]. 5 µl of congo red in water (5 µg/ml) was added to 100 µl of normalized urine sample and vortexed for 1 h at room temperature. 5 µl of vortexed mix was spotted on a strip of nitrocellulose membrane (Himedia, Mumbai, India) in duplicates. The spot was air dried for 15 min, and washed with Milli-Q water for 3 min. Image of the membrane strip was captured using Gel Doc Molecular imager (Bio-Rad, Hercules, USA). The membrane 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 again captured as before. Colour intensity of the spot was measured using Image Lab software which was available in the Gel Doc instrument. Congo Red Retention (CRR) was calculated using the formulae given below and expressed in percentage.

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

Statistical analysis: Statistical analysis was performed with the use of IBM SPSS Statistics for Windows, Version 20.0. The quantitative data was presented using mean, standard deviation and confidence interval and qualitative data by percentages. The data was checked for normality using Shapiro-Wilk test. ANOVA was used to compare the difference in the means across the groups. Post-hoc test was used for pairwise comparison. *P* value <0.05 was considered as statistically significant.

Results:

The baseline characteristics and clinical outcome profile of the participants in the study groups is depicted in Table No 1. The preeclamptic group includes clinical subgroups with respect to severity, gestational age of onset and fetal complications in the form of intrauterine growth restriction (IUGR) and intrauterine death (IUD). The CRR values were found to show normal distribution. Post-hoc analysis indicated that there was > 90% power to detect at least 15 % difference in CRR value.

. The mean CRR (%) of preeclamptic pregnant women was 77.9 ± 11.5 which is 2.1 times higher than that of mean CRR value of normotensive pregnant women which was 37.9 ± 4.1. The difference in the mean CRR of the two groups was found to be statistically significant (P < 0.001). We also analyzed the data after stratifying the preeclamptic patients into clinical subgroups. The mean CRR (%) value of preeclamptic women with early-onset (70.5 ± 9.0) and late-onset (82.7 ± 10.3) was 1.9 and 2.2 times higher relative to that of the mean CRR of normotensive pregnant women. The mean CRR of the two groups were significantly different from that of the normotensive control groups (P < 0.001). The mean CRR (%) of mild subtype (61.2 ± 3.2) and severe subtype (82.4 ± 8.4) of preeclampsia was 1.6 and 2.2 times higher than that of normotensive pregnant women. The mean CRR of the mild and severe groups were significantly different from that of the normotensive control groups (P < 0.001).

We also analyzed the data after stratifying w.r.t., the presence of co-morbidities. The mean CRR (%) of preeclamptic pregnant women superimposed with eclampsia (89.4 ± 2.0) was 2.4 times higher than that of normotensive pregnant women (P < 0.001). The mean CRR (%) of preeclamptic subgroup with fetal complications i.e., IUGR and IUD (74.6 ± 5.8) was 2.0 times higher than that of normotensive pregnant women (P < 0.001).

Discussion:

This study confirms the presence of urinary congophilia in Indian women with preeclampsia. Hitherto, the data on urinary congophilia was restricted to western populations. Availability of urine samples from preeclamptic women of different clinical subgroups is main strength of this study. There were sufficient number of preeclamptic women (P > 0.05) with early-onset (40.3 %) and late-onset (59.7 %) thus permitting us to evaluate the impact of gestational age of onset on the level of urinary congophilia. There was adequate power (> 90%) both at the level of the overall group and subgroups.

Our findings that, there is significant difference in the level of urinary congophilia between early-onset and late-onset of preeclampsia provides useful insight into the source of misfolded proteins. An expanding body evidences indicate that early-onset and late-onset preeclampsia are distinct entities with respect to the underlying pathophysiology [14-22]. Placental factors are indicated to be predominantly associated with early-onset while maternal

factors are mostly associated with late-onset preeclampsia. Maternal factors include pro-inflammatory cytokines and anti-angiogenic factors that induces the activation of maternal endothelial cells through diverse pathways [23-25]. Recent studies have shown that the activation of Unfolded Response Pathway in the placenta is different between early-onset and late-onset preeclampsia [26]. Compared to the placenta from early-onset preeclampsia, the nature of Unfolded Response Pathway was found to be similar in both late-onset and normotensive placentae. Our findings with significant difference in urinary congophilia between early-onset and late-onset preeclampsia indicates that placenta may not be the main source of misfolded proteins in the urine. In the studies by Buhimschi and co-workers, major plasma proteins like serum albumin, alpha-1 antitrypsin, ceruloplasmin, and IgG-kappa chain were identified in the misfolded protein fraction of urine [6]. In addition, presence of misfolded protein has been demonstrated in the plasma of preeclamptic women [27]. Thus urinary misfolded proteins in preeclamptic women appear to be derived mostly from the plasma. Furthermore, a growing body of evidences indicate that endothelial dysfunction, a cardinal pathological feature in preeclampsia, is a consequence of chronic activation of Unfolded Response Pathway [28]. In this light, we are compelled to assume that misfolded proteins in the urine of preeclamptic women reflects maternal and not placental etiologic factor. Furthermore, the maternal factor that leads to urinary misfolded proteins appears to be shared by both early-onset and late-onset preeclampsia and thus represents a core pathophysiological event.

Studies by McCarthy et al found that urinary congophilia was present in women with chronic kidney disease. This issue questions the clinical utility of urinary congophilia in the management of preeclampsia. At present, there is a paucity of data on the prevalence of chronic kidney disease among pregnant women in India. However, in the general Indian population, the mean age for CKD is in the range of 35.6 – 45.2 years [29]. In contrast, the mean age at first birth (given the predominance of preeclampsia in primigravida) in the Indian subcontinent is about 22.7 years [30]. It appears that the age spectrum for the risk of preeclampsia and CKD are separated by at least 10 years in the Indian population. Thus, CKD may not be a major confounding factor in the clinical utility of urinary congophilia. Furthermore, we argue that the utility of urinary congophilia is mainly as a test for screening pregnant women for the risk of preeclampsia rather than as a diagnostic test for women with symptoms indicative of preeclampsia. Screening tests are offered to large group of patients while diagnostic tests are

offered to specific patients with reasonable clinical indications. The cost of the screening test is under pressure since it has to cover large group of patients of whom only a small fraction eventually derive its benefit. CRDB holds promise as a screening test as the cost of the assay consumables and the amount of labour involved are negligible.

This study confirms that urinary congophilia level is elevated in preeclamptic women compared to normotensive pregnant women in the Indian population. The level of congophilia varies across the clinical subgroups of preeclampsia but the average level remains significantly higher than the normotensive pregnant women. We are motivated to explore the utility of CRDB assay in the prediction of preeclampsia well in advance of the onset of clinical symptoms.

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Disclosure of interests: The authors declare that they have no conflict of interests.

References:

1. T.K. Chaudhuri, S Paul, Protein-misfolding diseases and chaperone-based therapeutic approaches, FEBS J. 273(2006) 1331-1349.
2. F. Chiti, C.M. Dobson, Protein misfolding, functional amyloid, and human disease, Annu. Rev. Biochem. 75(2006) 333-66.
3. P. Sweeneney, H. Park, M Baumann et al, Protein misfolding in neurodegenerative diseases: implications and strategies, Transl. Neurodegener. 6(2017) 2-8.
4. H. Inouye, D.A. Kirschner, Alzheimer's β-amyloid: Insights into fibril formation and structure from Cong red binding, Subcell. Biochem. 38(2005) 203-24.
5. A.K. Buell, C.M. Dobson, T.P.J. Knowles, M.E. Welland, Interactions between amyloidophilic dyes and their relevance to studies of amyloid inhibitors, Biophys. J. 99(2010) 3492-349.
6. I.A. Buhimschi, U.A. Nayeri, G. Zhao et al., Protein misfolding, congophilia, oligomerization, and defective amyloid processing in preeclampsia, Sci. Transl. Med. 6(2014) 1-14.
7. F.P. McCarthy, A. Adetoba, C. Gill et al., Urinary congophilia in women with hypertensive disorders of pregnancy and preexisting proteinuria or hypertension, Am. J. Obstet. Gynecol. 215(2016) 1-7.

8. T.T. Ashburn, H. Han, B.F. Mc Guinness et al., Amyloid probes based on Congo Red distinguish between fibrils comprising different peptides. Chem. Biol. 3(1996) 351-358.
9. B. Stopa, B. Piekarska, L. Konieczny et al., The structure and protein binding of amyloid-specific dye reagents. Acta. Biochim. Pol. 50(2003) 1213–1227.
10. W.E. Klunk, J.W. Pettegrew, D.J. Abraham, Quantitative evaluation of Congo red binding to amyloid like proteins with B pleated sheet conformation, J. Histochem. Cytochem. 37(1989) 1273-1281.
11. Task Force on Hypertension in Pregnancy, Classification of hypertensive disorders, in: Hypertension in Pregnancy, American College of Obstetricians and Gynecologists, Washington DC 2013, pp. 13-15.
12. M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72(1976) 248-254.
13. H. Mcfarlane, A simple rapid method of concentrating urine for protein electrophoresis, Clin. Chim. Acta. 9(1964) 376-380.
14. D. Raymond, E. Peterson, A critical review of early-onset and late-onset preeclampsia, Obstet. Gynecol. Surv. 66(2011) 497-506.
15. B.Vasapollo, G.P. Novelli, H. Valensise, Total Vascular Resistance and Left Ventricular Morphology as Screening Tools for Complications in Pregnacy, Hypertension. 51 (2008) 1020-1026.
16. A. Aksornphusitaphong, V. Phupong, Risk factors of early and late onset pre-eclampsia, J. Obstet. Gynaecol. Res. 39(2013) 627–631.
17. S. Sulistyowati, Early and late onset preeclampsia: What did really matter ?, J. Gynecol. Women's Health. 5(2017) 001-003.
18. P.V. Dadelszen, L.A. magee, J.M. Roberts, Sub classification of preeclampsia. Pregnancy Hypertens. 22(2003) 143-148.
19. E.M. Herzog, A.J. Eggink, A. Reijnierse et al., Impact of early and late onset preeclampsia on features of placental and newborn vascular health, Placenta. 49(2017) 72-79.

20. C.W. Redman. Early and late preeclampsia: Two sides of the same coin. Pregnancy Hypertens. 7(2017) 58-64.
21. S. Lisonkova, K.S. Joseph. Incidence of preeclampsia: risk factors and outcomes associated with early- versus late-onset disease, Am. J. Obstet. Gynecol. 209(2013)1-12.
22. F. Cripsi, B.V. Alcaraz, E. Gratacos, Patterns of maternal vascular remodeling and responsiveness in early-versus late-onset preeclampsia, Am. J. Obstet. Gynecol. 209(2013) 14.
23. P. Chatterjee, V.L. Chiasson, K.R. Bounds, B.M. Mitchell, Regulation of Anti-inflammatory cytokines Interlukin-4 and Interleukin-10 during pregnancy, Front. Immunol. 5(2014) 1-6.
24. R. Raghupathy, Cytokines as key players in the pathophysiology, Med. Princ. Pract. 22(2013) 8-19.
25. B.D. La Marka, M.J. Ryan, J.S. Gilbert et al, Inflammatory cytokines in the pathophysiology of during preeclampsia, Curr. Hypertens. Rep. 9(2007) 480-485.
26. H.W. Yung, D. Atkinson, T. Campion-Smith et al, Differential activation of placental unfolded protein response pathways implies heterogeneity in causation of early and late onset preeclampsia, J. Pathol. 234(2014) 262-276.
27. I. Buhimschi, G. Zhao, G. Saade, Evidence for alpha-1-antitrypsin (A1AT) polymerization in preeclampsia: A novel mechanism for endothelial cell injury, Am. J. Obstet. Gyn. 195(2006) S150.
28. S. Lenna, R. Han, M.Trojanowska, Endoplasmic reticulum stress and endothelial dysfunction, IUBMB Life. 66(2014) 530-537.
29. P. Varma, Prevalence of chronic kidney disease in India - Where are we heading ?, Indian J. Nephrol. 25(2015) 133-135.
30. J. Bongaarts, A.K. Blanc, Estimating the current mean age of mothers at the birth of their first child from household surveys, Popul. Health Metr. 13(2015) 1-6.

Table No. 1: Baseline parameters and clinical outcome of the study participants

Parameter	Preeclamptic pregnant women (n = 62)		Normotensive pregnant women (n = 65)
Age (years)	23.9 ± 3.6		24.2 ± 3.1
Gravida • Primigravida • Multigravida	 • 33 (53.2 %) • 29 (46.8 %)		 • 26 (40 %) • 39 (60 %)
Severity • Mild • Severe	 • 13 (20.9 %) • 49 (79.1 %)		NA
Gestational age of onset • Early onset (< 34 weeks) • Late onset (≥ 34 weeks)	 • 25 (40.3 %) • 37 (59.7 %)		NA
Blood pressure (mm Hg)	Mild PE	Severe PE	
• Systolic Blood Pressure	144.6 ± 5.9	172.3 ± 13.5	116.9 ± 4.9
• Diastolic Blood Pressure	93.5 ± 3.8	113.5 ± 4.4	75.5 ± 5.4
Proteinuria • 1+ • 2+ • 3+	 • 37 (59.6 %) • 15 (24.1 %) • 10 (16.1 %)		NA
Co-morbidity • Eclampsia • IUGR • IUD • HELLP syndrome	 • 23 (37.0 %) • 7(11.3 %) • 3(4.8 %) • 1 (1.6 %)		NA

NA = Not Applicable

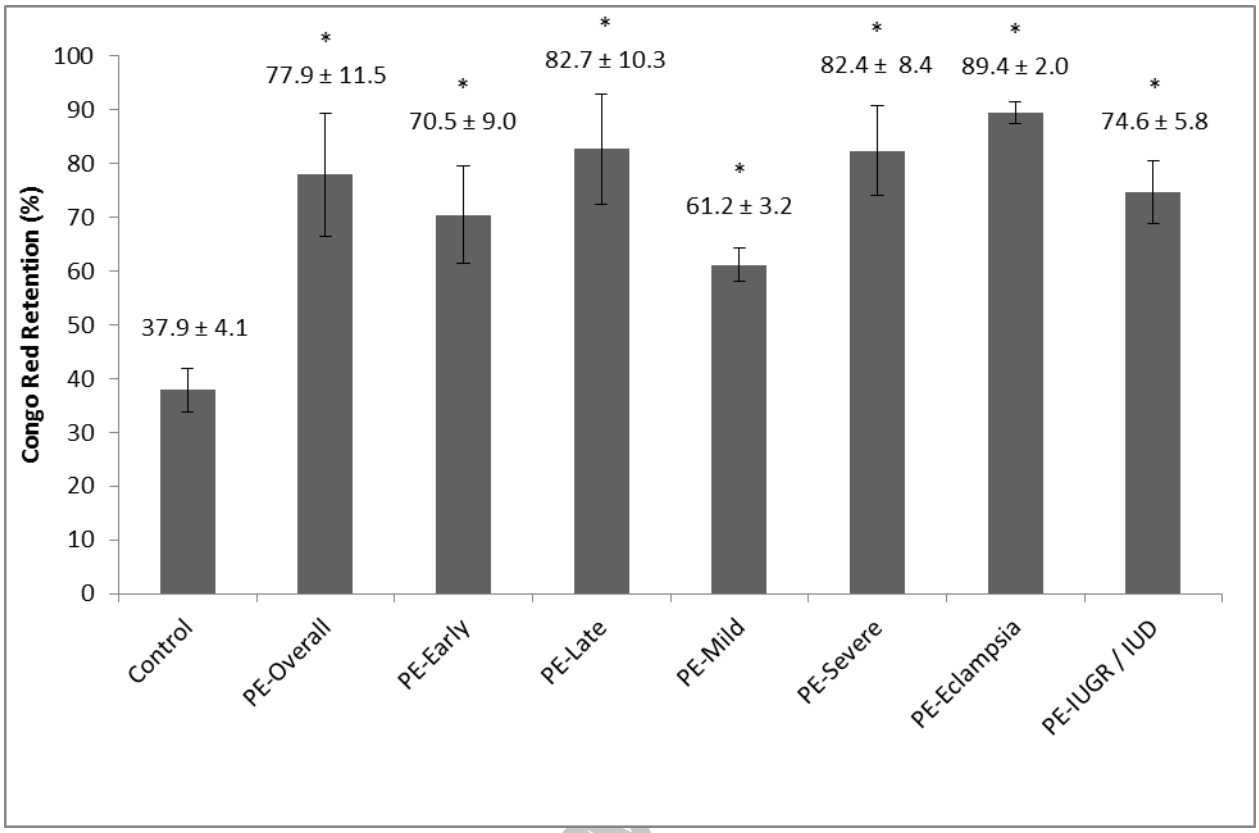

Figure No. 1: Profile of mean Congo Red Retention (%) values in the study groups.

(* *P* < 0.001; w.r.t. normotensive control group)

(PE: Preeclmapsia)

Highlights:

- Urinary congophilia was elevated in preeclamptic pregnant women
- Urinary congophilia was not affected by gestational age of onset.
- Urinary congophilia may be used for predicting the risk of preeclampsia.