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CXCL9/CXCL10 angiostasis CXC-chemokines in parallel with the CXCL12 as an angiogenesis CXC-chemokine are variously expressed in pre-eclamptic-women and their neonates

Shokoofeh Darakhshan[a], Gholamhossein Hassanshahi[b,c], Zohreh Mofidifar[a,b], Boshra Soltani[a,b], Mojgan Noroozi Karimabad[b,*]

[a] Department of Pediatrics, Faculty of Medicine, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
[b] Molecular Medicine Research Center, Research Institute of Basic Medical Sciences, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
[c] Dept. of Hematology, Faculty of Biomed, Biomedical Sciences, Kerman University of Medical Sciences, Kerman, Iran

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ABSTRACT

Background: The disorder of pre-eclampsia is described as a complicated gestational state in which some of biomolecules, including cytokines and chemokines are involved. The main purpose of the current study was examining of the circulating levels of CXCL9, CXCL10 as inducible, angiostasis chemokines as well as CXCL12 as an angiogenesis, homeostatic chemokine, in pregnant women with and without pre-eclampsia and their neonates.
Methods: Peripheral blood and cord samples were collected from 53 preeclampsia patients and 53 normal pregnant women without preeclampsia and their related neonates. The differences in serum levels of CXCL9, CXCL10 and CXCL12 and the placental tissue expression of these chemokines were investigated by ELISA and western blot analysis, respectively.
Results: Findings of the present study demonstrated that the levels of CXCL9 chemokine in parallel with CXCL12 as homeostatic chemokine were induced in pre-eclamptic women compared with normal pregnant women while CXCL10 remained unchanged. The CXCL9 and CXCL10 were both decreased in neonates who were delivered by pre-eclamptic women in compare to normal pregnant women. A CXCL12 level was elevated in neonates and has followed a similar fashion as mothers.
Conclusion: According to the results, the CXC chemokines are involved in pathogenesis of pre-eclampsia and play important roles in several processes such as neovascularization, embryonic development and inflammatory responses that are mediated by pre-eclampsia.

1. Introduction

Approximately 5% of pregnancies are associated with pre-eclampsia, which is of the main contributors, involved in morbidity and mortality of mother and baby [1]. Pre-eclampsia was initially defined as a pregnancy-specific syndrome which is, emerging after 20 weeks of gestation. In addition to hypertension, several other clinical manifestations such as proteinuria, and generalized edema are also of the most frequent signs and symptoms associated with per-eclampsia [2–4]. The etiology of per-eclampsia is complicated and need to be elucidated, however, the key role of abnormal placentation in pre-eclampsia) as an initiating factor) is well evidenced [5–7]. Despite, the extensive research efforts have been made the exact etiology and pathogenesis of pre-eclampsia yet to be fully defined. Compelling evidences suggested that the maternal systemic inflammatory responses against pregnancy (via activation of both the innate and adaptive arms of the immune system) are pivotally involved pre-eclampsia pathogenesis [8–10]. Redman and Sargent addressed a generalized maternal inflammatory response in pre-eclamptic women, which is correlated with elevated levels of a wide spectrum of pro-inflammatory chemokine family members, such as TNFα, IL-6 and CXCL8 [11]. Cytokines are a broad and loose category of small proteins (~5 to 20 kDa), playing important parts in cell signaling. Chemokines, interferons, interleukins, lymphokines, and tumor necrosis factors [12,13]. Chemokines are a well-known subgroup of low molecular weight proteins weighing (8–10 kDa) including four major subdivisions of C, CC, CX_3C, and CXC [14]. All of chemokines which have been investigated in present study (CXCL9, CXC10 and CXC12) fit within the CXC subdivision of chemokines.

* Corresponding author.
E-mail addresses: mojgan.noroozi@yahoo.com, mojgan.noroozi@rums.ac.ir (M.N. Karimabad).

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CXCL9 and CXC10 are induced by interferon-γ (IFN-γ). These chemokines play predominant role(s) in appropriate immune responses and are amongst the main chemokines in recruitment of immune cells to the inflamed/infected organs [15,16]. Chemokines are multifunctional with various functions varied from chemotaxis, angiogenesis, angiostasis to homing of stem cell and organogenesis. As an example, selective expression of CXCL12 in hypoxic tissues is associated with migration of adult stem cells recruitment and further tissue regeneration [17–19]. These (IFN- γ) induced chemokines are also fundamentally involved in prevention of neovascularization, anti-angiogenesis [20,21]. The CXCL12 in association with its respective receptor (e.g. CXCR4) was addressed to be expressed by endothelial and hematopoietic progenitor cells [22,23]. A function which is essential in mobilization and homing of hematopoietic stem cells [24]. Another CXC chemokine which is of our interest in current study, the CXCL10, is an inducible CXC chemokine which is induced by several pro-inflammatory molecules, including interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α) [25,26], some of viruses and microbial products like bacterial lipopolysaccharide (LPS), either in a direct fashion or through activation of nuclear factor-kappa B (NF-κB) consensus elements exist in its promoter [27]. In contrast to the CXCL1 and CXCL12 that are known as angiogenic, IFN-γ-inducible chemokines, (CXCL9, CXCL10 and CXCL11) exhibit anti-angiogenic properties. Contrastly, CXCL12 is not inducible but is instead a homeostatic CXC chemokine [28,29]. Given the aforementioned introductory concerns regarding roles played by CXC chemokines in hypoxia, angiogenesis/angiostasis and inflammatory responses, we hypothesized that alteration in pattern of angiogenic (CXCL12) and angiostasis/anti-angiogenic (CXCL9 and CXCL10) CXC chemokines expression may affect pre-eclampsia. Moreover, our recent studies in the field of chemokine expression in pregnancy encouraged us to spread our investigations towards the role of chemokines in pre-eclamptic pregnancy [30]. Therefore, this study was aimed to investigate the expression of these chemokines in pre-eclamptic pregnant women in compare to women with normal pregnancy and their respected neonates (cord blood levels).

2. Methods

2.1. Study subjects

In the current case-control study, 53 pre-eclamptic pregnant women and 53 women with normal pregnancy were enrolled. All of participants had Iranian nationality and shared same geographical region in Iran (Table 1). The criteria for exclusion included multifetal gestation, previous history of chronic hypertension, any types of diabetes mellitus, any sort of infection, and autoimmune diseases such as multiple sclerosis (MS), angiopathy, renal dysfunction, and congenital abnormalities. The active labor or rupture of membranes was not observed in recruited pregnant women. All of investigated women were suffering from severe pre-eclampsia, and the illness was diagnosed based on the criteria mentioned by American College of Obstetricians and Gynecologists [31]. Presence both hypertension and proteinuria beyond 20 weeks (approximately 140 days) further of gestation by expert gynecologists. Pre-eclampsia was identified by increased systolic blood pressure (SBP) (more than 140 mmHg systolic or diastolic blood pressure (DBP) (more than 90 mmHg) for more than two onsets around 6 h apart which was happened following 20 weeks of gestation in a pregnant female who had previously normal blood pressure (Table 1). In addition to hypertension, patients also had proteinuria (more than 0.3 g urinary protein in 4 h without having evidences for urinary tract infection). Pre-eclampsia was considered severe if the below criteria were present: having SBP more than 160 mmHg or DBP more than 110 mmHg, presence of proteinuria in pre-eclamptic women more than 5 g of protein in 24 h (Table 1).

Some of related clinical laboratory parameters such as Blood Urine Nitrogen (BUN), Creatinine, Bilirubin, C-reactive protein (CRP) and Ferritin have also been measured in both pre-eclamptic and non-pre-eclamptic pregnant women (Table 2).

The protocol of the investigation was approved by the ethical committee for the Rafsanjan University of Medical Sciences. All of study subjects were recruited, if signed the written informed consent from, separately. This investigation has also been under taken based on the Declaration of Helsinki.

2.2. Measurement of chemokines by ELISA

Specific and sensitive technique of enzyme-linked immunosorbent assay (ELISA) was performed for assessment of studied CXC chemokines (CXCL9, CXCL10 and CXCL12) in maternal blood and placenta. Immunoassay kits for detection of chemokines were purchased (R&D Systems Minneapolis, MN, USA). As described before [29], in brief the obtained samples were incubated in duplicate wells of the microtiter plates, pre-coated with specific monoclonal antibodies against CXC chemokines. During the incubation period, CXC chemokines are bound by the immobilized antibodies. Following repeated steps of rinsing and aspiration, unbound components have been eliminated and, enzyme-linked polyclonal specific antibodies against chemokines were further added to the wells. Following multiple steps of gentle washing and removing excess and unbound materials and further addition of, substrate solutions to wells, color was developed in proportion to the coupled measures CXCL9, CXCL10 and CXCL12 chemokines during initial phase. The process of color development was inhibited by addition of an acid solution and then related color optical density (OD) was read using a programmable spectrophotometer, (PD-303UV, APEL, JAPAN). The concentrations of CXCL9, CXCL10 and CXCL12 were examined in samples by interpolation from individual standard curves composed of recombinant human CXC chemokines. The sensitivity of used kits was 2 pg/mL and inter and intra-assay assessments of reliability of kits were 14% and 3%, respectively [29,32].

2.3. Western blotting analysis

Protein samples were isolated from freeze-dried, dissected and homogenized placental samples further thawing by lysis buffer (Cell Signaling Technology, Beverly, MA, USA). The resultant protein samples were then subjected to Sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), to semi-quantitatively, quantify CXCL9, CXCL10 and CXCL12, immunoblotting and densitometer methods were under taken. An equal measure of extracted protein (35 g) was loaded into each separate well and resolved on a 10% SDS-PAGE. The resolved proteins were further transferred onto a nitrocellulose membrane and have then been blocked with 3%(w/v) milk in PBS/Tween (10 mM Tris, pH 7.4 containing 140 mM NaCl, 0.1%[v/v] Tween 20) to prevent non-specific binding. The nitrocellulose membranes were incubated overnight at 4 °C in PBS/Tween containing 3% (w/v) milk, in presences of anti-human CXCL9, CXCL10 and CXCL12 (R&D system, UK). The specific monoclonal antibody for β-actin (Sigma, Mo, USA) was used for normalization, as the housekeeping protein. In the other words β-actin was consumed as an internal control to compare the data from different films Subsequently, an anti- mouse horseradish peroxidase conjugated antibody (Sigma, Mo, USA) (diluted, 1:1000) was also used, as the secondary antibody accordingly, and the enhanced chemi-luminescence (ECL) detection system (Amersham Life Science, UK) was employed to define both protein localization as well as amount [33,34].

2.4. Statistical analysis of the data

The Student's “t” test was performed for comparison of the variables. The demographic characteristics of patients and statistical analysis of in densitometry data obtained from western blotting films were compared by Mann–Whitney *U* test using Statistical Package for the Social Sciences Version 18.0 (SPSS Inc., Chicago, IL, USA). Data were

Table 1
Demonstrates some clinical characteristics of the participants.

Variable	Non pre-eclamptic Women (n = 50)		Pre-eclamptic Women (n = 53)	
Age of Mother (year)	33.1 ± 1.8		28.66 ± 1.55.19	
Delivery age (weeks)	38.2 ± 1.2		36.9 ± 1.6	
SBP at sampling (mmHg)	114.2 ± 8.8		184.2 ± 6.7*	
DBP at sampling (mmHg)	69.6 ± 10.1		121 ± 7*	
Neonate birth weight (kg)	3.21 ± 0.27		2.67 ± 0.3*	
Maternal BMI at sampling (kg/m2)	27 ± 0.5		31.5 ± 6*	
Proteinuria mg/dL	NP		48	
Smoking history (%)	NP		8	
Alcohol drinkering (%)	NP		NP	
Average Apgar (score)	9.06 ± 0.03		8.8 ± 0.08	
Blood pressure before pregnancy(mmHg)	P	0	P	7(13.21%)
	NP	53(100%)	NP	46(86.79%)
Method of Childbirth (%)	Caesarean section	13(24.52%)	Caesarean section	35(66.03%)
	Normal childbirth	40(75.47%)	Normal childbirth	18(33.97%)
Respiratory distress (%)	P	0	P	0
	NP	53(100%)	NP	53(100%)
SBP/DBP over 140/90 during pregnancy (mm Hg)	P	0	P	53(100%)
	NP	53(100%)	NP	0
Anomaly (%)	P	0	P	0
	NP	53(100%)	NP	53(100%)
Delivery age (week)	above 37	22(41.50%)	above 37	27(50.95%)
	under 37	31(58.49%)	under 37	26(49.05%)
Weight of neonate of birth (gr)	above 2500gr	38(71.69%)	above 2500gr	21(39.62%)
	under 2500gr	15(28.30%)	under 2500gr	32(60.38%)
Gender of neonate (Sex)	female	25(47.16%)	female	21(39.62%)
	male	28(52.83%)	male	32(60.38%)

Data are expressed as mean ± SEM.
SBP = Systolic Blood Pressure, DBP = Diastolic Blood Pressure, BMI = Body Mass Index, NP = not present, p = present.
* = significant difference with non-pre-eclamptic women.

Table 2
Demonstrates some clinical laboratory characteristics of participants.

Laboratory Parameter	Non pre-eclamptic Women [n = 53] (Ranges)	Pre-eclamptic Women [n = 53] (Ranges)
Serum BUN (mg/dl)	2.74(2.2–3.2)	3.7*(2.6–3.9)
Creatinine (mg/dl)	51(43–54)	67*(57–68)
Bilirubin (mg/dl)	4.4(4–6.4)	6.8*(5.2–75)
CRP (mg/dl)	2.7(1.6–5.7)	7.3*(2.7–8.3)
Ferritin (ng/mL)	167(121.2–187)	214*(136.8–223.1)

Data are expressed as mean ± SEM.
BUN = Blood Urine Nitrogen, CRP = C-reactive protein.
* = significant difference with non pre-eclamptic women.

expressed as mean ± SEM and a $p < 0.05$ was regarded statistically significant.

3. Results

In the present study we enrolled 53 pregnant women suffering from pre-eclampsia, according to both clinical and para-clinical feathers and 53 pregnant women with normal pregnancy. As demonstrated in Table 2, all measured clinical laboratory characteristics were considerably elevated in pre-eclamptic pregnant women in compare to normal pregnant women.

Results of the current study demonstrated that the serum levels of CXCL9 was 277.64 ± 29.7 pg/mL and 203.45 ± 13.58 pg/mL in pre-eclamptic and normal pregnant women, respectively (Fig. 1a).

We observed that serum levels of CXCL10 was 130.35 ± 12.52 pg/mL and 139.5 ± 21.62 pg/mL in pre-eclamptic and normal pregnant women, respectively (Fig. 2a).

Our findings demonstrated that the CXCL12 circulating levels in pre-eclamptic and non pre-eclamptic pregnant women were 115.48 ± 12.4 pg/mL, and 86.68 ± 10.5 pg/mL, respectively (Fig. 3a).

The results of ELISA and western blot analysis showed that CXCL9 was elevated (Fig. 1c), while CXCL10 remand sustainly unchanged (Fig. 2c) We have also found that CXCL12 was elevated in pre-eclamptic pregnant women in comparison to normal pregnant women (Fig. 3c).

In an inverse pattern, the mean level of CXCL9 level was decreased pg/mL in neonates who were delivered by pre-eclamptic women and non pre-eclamptic women. It was 125.43 ± 14.21 pg/mL and 203.45 ± 1.95 in neonates delivered by preeclamptic and non-pre-eclamptic woman, respectively (Fig. 1b).

Our findings also revealed that the mean level of CXCL10 was decreased in neonates who were delivered by pre-eclamptic women (77.93 ± 7.77 pg/mL) incompare to neonated who were delivered by normal pregnant women (139.5 ± 1.62 pg/mL) respectively (Fig. 2b).

Interestingly, present findings also revealed that the mean level of CXCL12 followed a similar fashion as mothers and was elevated in neonates who were delivered by pre-eclamptic 106.14 ± 1.41 pg/mL in compare to non-pre-eclamptic women 86.68 ± 1.27 pg/mL (Fig. 3b).

Although, we were unable to obtain intra uterine tissue samples from either pre-eclamptic or non-preeclamptic pregnant women but were able to collect placental tissues to western blot analysis. We have shown densitometry data of the CXCL9, CXCL10, CXCL12 in pregnant women with and without pre-eclampsia Fig. 4.

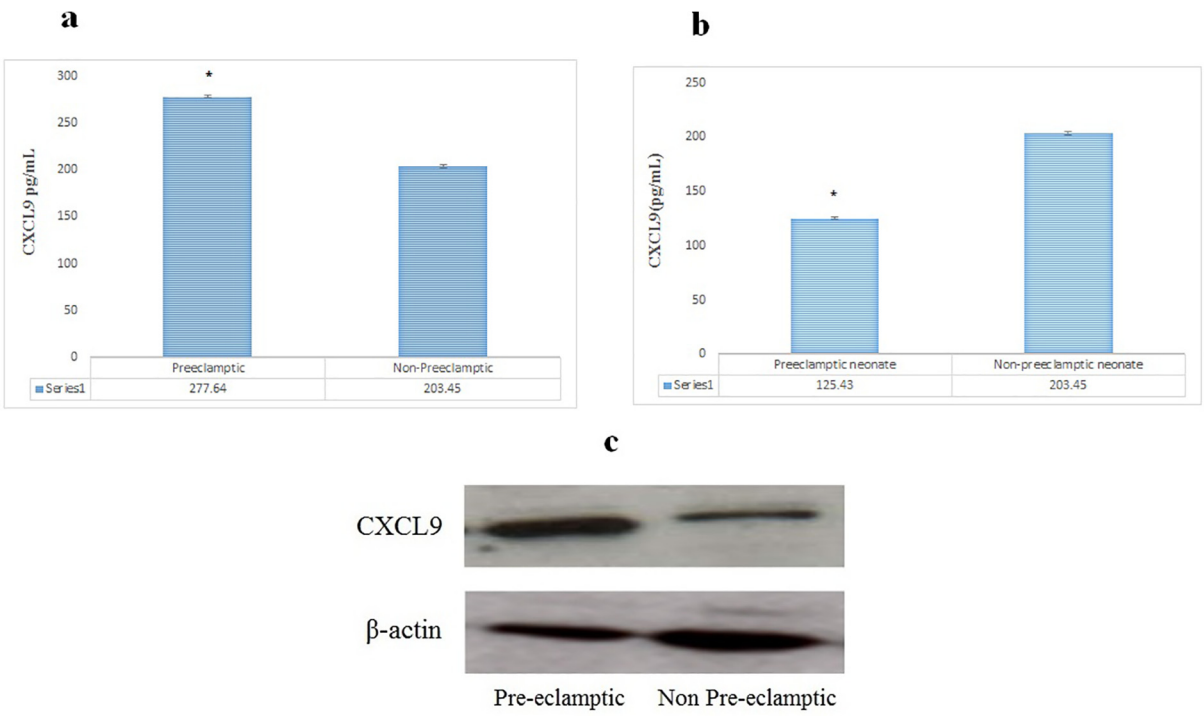

Fig. 1. Demonstrates the maternal and neonatal serum CXCL9 levels in mother/neonate pair of deliveries with and without pre-eclampsia. Profile a: Represents the maternal levels of CXCL9 in pre-eclamptic and non pre-eclamptic pregnant women. Profile b: Represents the CXCL9 neonatal levels in neonates who delivered from pre-eclamptic and non pre-eclamptic pregnant women. Profile c: Shows a representative profile of placental CXCL9 against β-actin in pregnant women with and without pre-eclampsia achieved by western blotting method. * = Significant difference with non-Pre-eclamptic women.

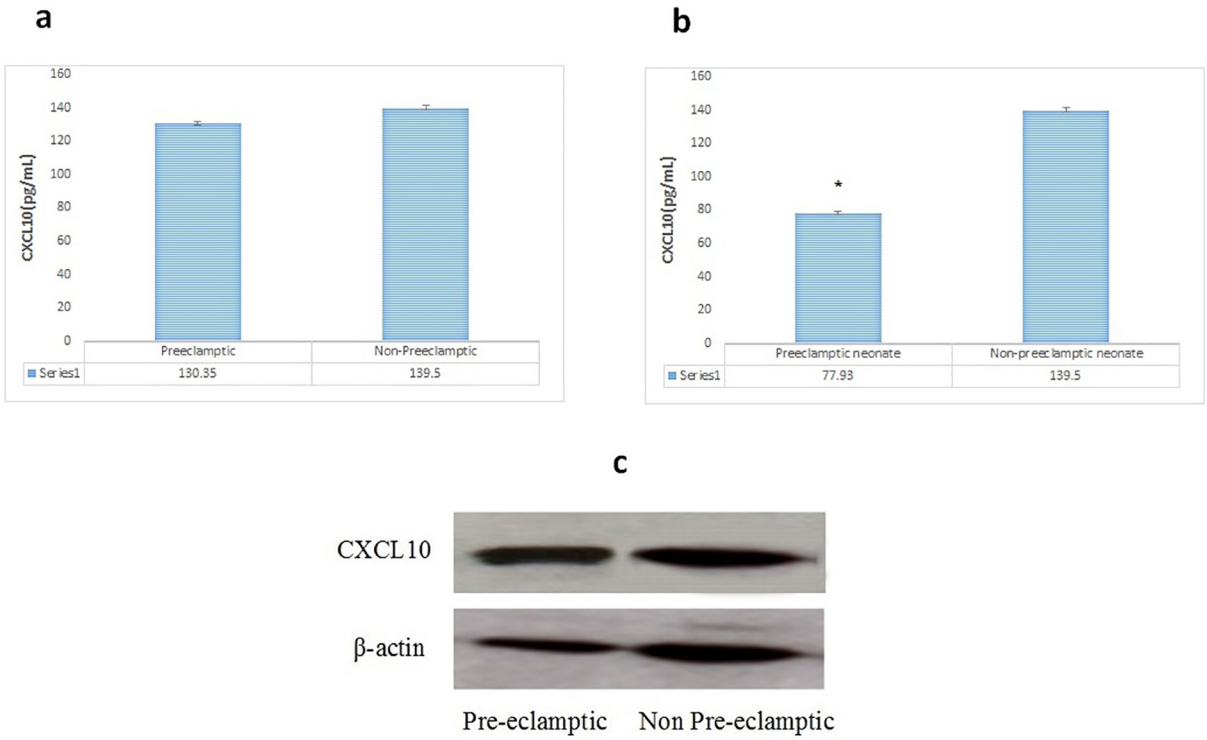

Fig. 2. Demonstrates the maternal and neonatal serum CXCL10 levels in mother/neonate pair of deliveries with and without pre-eclampsia. Profile a: Represents the CXCL10 maternal levels in pre-eclamptic and non pre-eclamptic pregnant women. Profile b: Represents CXCL10 neonates who levels in neonate delivered from pre-eclamptic and non pre-eclamptic pregnant women. Profile c: Shows a representative profile of the placental CXCL10 against β-actin in women with and without pre-eclampsia achieved by western blotting method. * = Significant difference with non-Pre-eclamptic women.

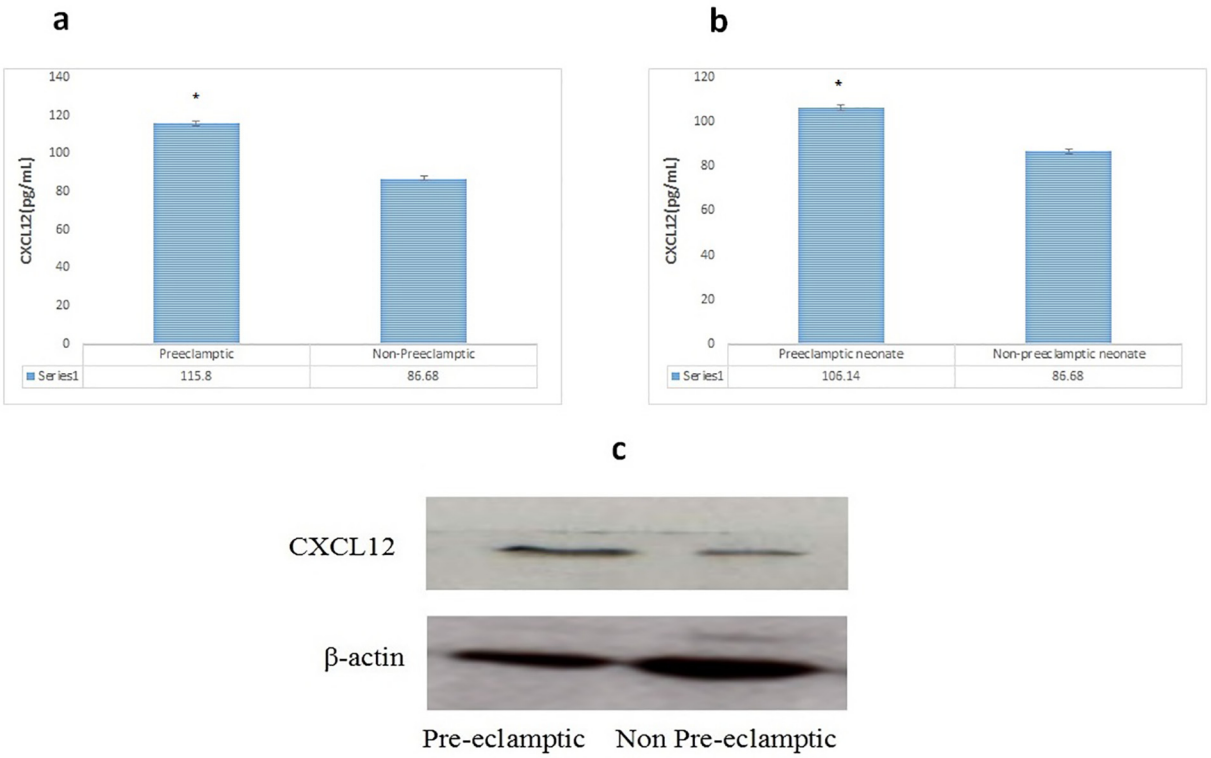

Fig. 3. Demonstrates the maternal and neonatal serum CXCL12 levels in mother/neonate pair of deliveries with and without pre-eclampsia. Profile a: Represents the maternal levels of CXCL12 in pre-eclamptic and non pre-eclamptic pregnant women. Profile b: Represents the CXCL12 neonatal levels in neonates who delivered from pre-eclamptic and non pre-eclamptic pregnant women. Profile c: Shows a representative profile of placental CXCL12 against β-actin in pregnant women with and without pre-eclampsia achieved by western blotting method. * = Significant difference with non-Pre-eclamptic women.

4. Discussion

Several genetic, behavioral and environmental factors are pivotally involved in pathogenesis of pre-eclampsia [35–39]. We designed the present study to measure CXCL9, CXCL10 and CXCL12 in normal and pre-eclamptic women using ELISA and western blotting analysis. Our findings revealed almost a differential patter for investigated chemokines in pre-eclamptic, non-preeclaptic women and their neonates. Elevated level of these CXC chemokines in pre-eclamptic women is probably subsequent to and during onset of pre-eclampsia. We have also indicated the decreased levels of CXC chemokines in neonates (cord blood) who delivered by pre-eclamptic women. However, there is not similar studies within the data base for comparison, Saito and co-workers observed decreased Th2 lymphocytes and increased levels of some upstream inflammation molecules involved in regulation of chemokines expression such as interleukin (IL-2), interferon-γ (IFN-γ) and tumor necrosis factor (TNF-α) in parallel with enhanced number of peripheral blood mononuclear cells (PBMCs) in pre-eclampsia women [31,40].

Taken together with our and others previous studies regarding elevated levels of IFN-γ inducible CXC chemokines, specially CXCL9 and CXCL10, the elevated levels of this chemokine could possibly be due to the over production of TNF-α and IFN-γ (by Th1 cells) in pre-eclampsia, because the clinical state of preeclampsia is regarded as an inflammation disorder [41]. As we have previously reported the increased levels of CXC chemokines further treatment with recombinant IFN-γ and TNF-α in invitro cell cultures or in different disorders associated with inflammatory states [33,41,42] the increased expression of the mediators specific for pro-inflammatory responses including cytokines such as IL-6 and TNF-α and CXC chemokines like CXCL8, CXCL10, as well as CC chemokines like CCL2 is well evidenced in pre-eclampsia. clinical state which an overall pro-inflammatory systemic environment for the disorder [43]. Elevated levels of the pro-inflammatory chemokines presented in studied pre-eclamptic patients is in agreement with previous findings which may suggest might be a compensatory phenomenon [44]. It is now well evidenced that the third normal pregnancy trimester is a state associated with a form of systemic inflammation [45], and this type of inflammation at the feto-maternal interface is almost useful for trophoblast invasion at early stage of pregnancy. Chemokines are regarded as pivotal components which are deserved for these processes [46,47]. It has been demonstrated that cytokines stimulate release of free oxygen radicals and reactive oxygen species (ROS) which these reactive oxygen metabolites interrupt-regulate the expression of genes the expression of essential for pro-inflammatory cytokines and adhesion molecules [48] which all are defined as inducers of chemokine expression[16]. A correlation has been evidenced between pro-inflammatory chemokines and blood pressure and other laboratory parameters, in our studied pre-eclamptic, women which suggesting a critical role for these chemokines in pre-eclampsia and hence and the inflammatory responses in these patients may be associated development of hypertension in pre-eclampsia. On the other side, chemokines and other pro-inflammatory mediators could be regarded as potential regulators for endothelial dysfunction that is considered as a major hallmark for maternal syndrome of pre-eclampsia. Several vital organs including kidneys, liver (especially sinusoids) as well as brain (choroid plexus) are highly affected by pre-eclampsia. However, in the present work, we did not aim to properly check clinical parameters related to the liver function (due to financial issues) but measured kidney function parameters in parallel with homeostatic (CXCL12) and inflammatory chemokine CXCL9 and CXCL10. Evidences have indicated the central role of these inflammatory molecules in mediating kidney damage [49]. Consistently, some other studies have also presented the placenta as one of the rich sources of circulatory pro-inflammatory cytokines in pre-eclamptic women [50] and this involves dysfunction of maternal endothelial cells [51–53]. To the best of authors' knowledge the present study is that has addressed the

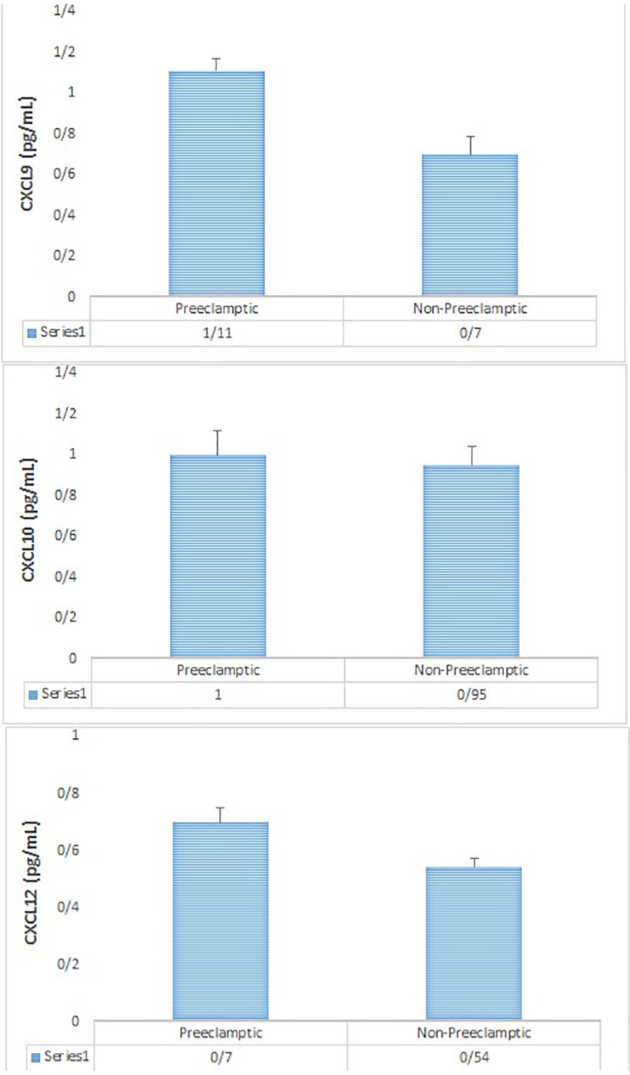

Fig. 4. Shows densitometry data of the CXCL9, CXCL10, CXCL12 in pregnant women with and without pre-eclampsia achieved by western blotting method.

involvement of CXCL10 and CXCL12 in pathogenesis of pre-eclampsia which affect their neonates as well. Overall, these results are novel and, suggesting a role for these pro-inflammatory chemokines in the pathophysiology of preeclampsia. CXCL9 and CXCL10 exhibit potent anti-angiogenic (angiogenesis) properties, while, CXCL12 acts as angiogenesis factor [54]. The nuclear factor-kappa B (NF-κB) pathway activation [40] via ligation of recognition receptors (TLR4 and TLR3) is also able to upregulate gene and protein expression of these chemokines [41]. Interestingly, CXCL9 and CXCL10 but not CXCL12 fit within the 'NF-kB responsive genes [42]. The fundamental bio-functions of CXCL9 and CXCL10 are varied from chemotaxis and endothelial adhesion of activated T cells to chemotaxis and enhancement of natural killer (NK) cell-mediated cytolysis. Although, CXCL9 and CXCL10 are poor neutrophil recruiters but there exist controversial report regarding their effects on monocytes and B cells [34]. The elevated levels of pro-inflammatory chemokines, CXCL9 and CXCL10 in pre-eclampsia is in some points in line with the findings which evidenced that pregnancy is associated with systemic intravascular inflammation [30,43].

Overall, evidences are in favor of the following key points in pre-eclampsia. Elevated maternal serum concentrations of IL-2, TNF-α, IFN-γ [55], and IL-12 and IL-1β as mediators of inflammation, as the Th1 immune response elements [56]. Decreased maternal serum concentrations of IL-10 [57] and IL-4 [55]; as anti-inflammatory response mediators, as the Th2 immune response elements. Thus, the high maternal serum concentrations of CXCL9 and CXCL10 in pre-eclamptic women may exhibit yet another aspect of a pro-inflammatory state or intravascular inflammation or even incorporate to the formation of such state. Given the above evidences concerning anti-angiogeneic nature and properties of CXCL9 and CXCL10 as elements involved in pre-eclampsia as the features of preeclampsia, we propose that changed anti-angiogeneic chemokines in maternal serum may probably contribute to the creation of an anti-angiogeneic state in pre-eclamptic women.

5. Conclusion

According to the results, the CXC chemokines are involved in pathogenesis of pre-eclampsia and play important roles in several processes such as neovascularization, embryonic development and inflammatory responses that are mediated by pre-eclampsia.

Declaration of Competing Interest

None of authors declared conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.preghy.2019.05.001.

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