A Study of Effect of Experimental Pre-Eclampsia on Plasma Lipocalin-2 Level in Rats

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ABSTRACT

Background: preeclampsia (PE) is a pregnancy related complication defined as a disease that begins in the placenta and ends at the maternal endothelium. It is a multi-stage disease that starts by utero-placental insufficiency and leads to generalized maternal endothelial dysfunction.Lipocalin2 (LCN2) is a 25kDasecretory glycoprotein implicated in many functions such as apoptosis and innate immunity. Also, it has been recognized to have potential effects in obesity, inflammation and insulin resistance in mice and humans**.** Many controversial studies about the changes in the plasma LCN2 levels in PE are reported.

Aim: The current study was designed to perform an animal model of experimental PE in a trial to demonstrate the possible relationship between PE and the circulating LCN2 levels.

Design: forty eight healthy adult female albino rats and eight adult male albino rats were used. The male rats were used for induction of pregnancy. The adult female rats (n=48) were divided into four equal groups: group I (control non-pregnant group), group II (non-pregnant treated with L-NAME), group III (normal pregnant group) and group IV (pregnant treated with L-NAME to induce a model of experimental PE). In all groups, body weight, body mass index (BMI), blood pressure, circulating levels of urea, creatinine, triglycerides (TGs), IL-6, endothelin-1(ET-1), vitamin D (VD), LCN2 and D-dimers in addition to total urinary proteins are measured. Histopathological examination of placental sections was done in group III and group IV.

Results: The results of the present study revealed a significant increase in the body weight, BMI, MAP, total urinary proteins, circulating levels of urea, creatinine, TGs, IL-6, ET-1, LCN2 and D-dimers in both group II and IV. In addition to a significant decrease in VD in the same two groups. In group III, there was a significant increase in body weight, BMI, total urinary proteins and circulating levels of TGS, D-dimers and LCN2. There was a significant decrease in VD and MAP. Moreover, there was a positive significant correlation between LCN2 and all measured parameters except VD in group IV together with a positive significant correlation between LCN2 and MAP, IL-6, ET-1and D-dimers in group II.The results in group IV are supported by the histopathological examination results.

Conclusion: L-NAME can be used for induction of experimental PE and plasma levels of LCN2 can be used as an indicator for the renal complications and coagulopathies in PE. Further studies are needed to ascertain this association.

Keywords: Pre-eclampsia, lipocalin2, L-NAME.

INTRODUCTION

PE is a pregnancy related disorder characterized by hypertension and proteinuria occurring after 20 weeks of gestation. Several promising biomarkers have been proposed, alone or in combination, that may help in predicting women who are at risk of PE [1].

The exact etiology of PE has not been surly identified; so many pathophysiological mechanisms had been suggested. But it is reported that the placenta is the main key to its pathogenesis [2, 3].

Experimental PE is the induction of PE like state in animals to facilitate the studying of different pathophysiological mechanisms of PE and investigating its potential preventive and therapeutic measures [4]. The ideal animal model of PE was suggested to show all the symptoms seen in women with PE that include hypertension, proteinuria, endothelial dysfunction and an imbalance of angiogenic factors [5]. However the placentation differences among mammals and the fact that the PE does not occur naturally in animals other than humans and two species of higher order primates, the patas monkey [6] and baboon twins [7] make this model as a challenge [8].

So, many animal models are suggested including those induced by; reducing the utero-placental perfusion [9, 10], inhibition of nitric oxide (NO) [11, 12, 13], by low dose lipopolysaccharide (LPS) [14, 15] and many other models.

LCN2 is a 25 kilo Daltons (kDa) secretory glycoprotein that belongs to the lipocalin family of proteins. It has been implicated in many functions such as apoptosis and innate immunity. Also, LCN2 has been recognized as an

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adipocyte-derived acute phase protein that is positively correlated with potential effects in obesity, inflammation and insulin resistance in mice and humans [16, 17, 18].

Many controversial studies about the changes in the plasma LCN2 levels in PE are reported. *Arikan et al.* [19] reported that there is a decrease in its levels in pre-eclamptic subjects with a non-significant correlation between LCN2 levels and the BMI, triglyceride, gestational week at delivery, birth weight, systolic and diastolic blood pressure in pre-eclamptic and healthy pregnant women.

However, other investigators found that LCN2 level increases significantly in PE with relation to the severity of the disease [20]. Furthermore, *Kim et al.* [21] found that LCN2 level had been elevated in normotensive pregnant women without any medical or obstetrical problems.

So, this study was designed to perform experimental PE in a trial to clarify the mechanism of it and to demonstrate the changes occurring in the LCN2 plasma levels in rats with experimental PE.

MATERIAL AND METHODS

All the experimental procedures were conducted in accordance with the guiding principles for the care and use of research animals and were approved by the Institutional Review Board of Faculty of Medicine –Zagazig University.

Animals:

Forty eight healthy adult female albino rats and eight adult male albinos (12-16 weeks old, 200-250 g) were purchased from the animal house of Faculty of Veterinary Medicine-Zagazig University. Animals were housed in plastic cages with wood chips as bedding in a controlled environment at 20–24°C and 12 hour light/dark cycles. Rats were fed a standard laboratory diet and water ad libitum. After 3 days of adaptation, the rats were mated overnight with adult male rats. The next day was taken as day 1 of pregnancy if spermatozoa were found in vaginal smears. Weights of pregnant rats were recorded at day 1 and day 15 of gestation to calculate the weight gain. The results were written in a record for each labeled rat [22].

METHODS

Female rats were divided into four equal groups; group I (control non-pregnant group);healthy adult female rats received saline solution (0.5 ml/100 g body weight) subcutaneously daily, group II (non-pregnant treated with L-NAME group);healthy adult female rats injected with sterile solution in the sequential dosage of 10 mg L-NAME (L-nitro-arginine methyl ester) /0.5 ml/100 g body weight subcutaneously daily, group III (normal pregnant group);adult pregnant female rats received saline solution (0.5 ml/100 g body weight) subcutaneously daily starting from day 7 to day 14 of gestation and group IV (pregnant treated with L-NAME group);adult pregnant rats injected with sterile solution in the sequential dosage of 10 mg L-NAME /0.5 ml/100 g body weight every day starting from the day 7 to day 14 of gestation , to make an animal model of preeclampsia [11].

Calculating BMI index

BMI equals body weight (gm) / length^2 (cm^2), this index can be used as an indicator of obesity where the cutoff value of obesity BMI is more than 0.68 gm/cm^2 [23].

Measurement of Blood Pressure: The blood pressure of the overnight fasting pregnant rats was measured using the power lab device (AD Instruments Pty Ltd, Australia) according to *Parasuraman and Raveendran* [24]. The systolic and diastolic BP was recorded on day 15 of gestation for all dams. Three measurements with 30 s intervals were recorded and the average of these readings was calculated followed by calculation of the mean arterial blood pressure (MAP).

Determination of urinary proteins excretion: On day 15 of pregnancy, the rats were placed in metabolic cages for 24-hour urine collection. Urine protein concentrations were determined by the principle of turbidimetry by adding 5% trichloro-acetic acid and using Microlab 300 according to [25].

Specimen collection: On day 15 of pregnancy, rats were anaesthetized between 9:00 a.m. and 10:00 a.m. Maternal blood was collected by cardiac puncture and put in polyethylene tubes pre-rinsed with EDTA. Plasma was prepared by centrifugation for 10 min at 3000 r.p.m and was stored at −80°C until analysis.

Biochemical assay

- *Plasma LCN2 was measured using* enzyme-linked immunosorbent assay kit (Sunredbio Shanghai, 201-11-5109, CHINA) [26].
- *Serum urea level: using* rat kits for urea level estimation (Spinreact, S.A.U. ctra. Santa Coloma, 7e-17176 Santesteve de bas (gi), Spain) [27].
- *Serum crearinine level: using* rat kits for creatinine level estimation (Spinreact, S.A.U. ctra. Santa Coloma, 7e-17176 Santesteve de bas (gi), Spain) [28].
- *Serum TGs levels: using* TGs ESPAS SL kits (Elttech S.A., Sees, France) [29].

- *Serum IL-6 levels: using* double-antibody sandwich ELISA kits (Sigma Chemicals CO., Aldrich, St. Louis, Mo) [30].
- *Serum endothelin-1:* using double-antibody sandwich ELISA kits (Wuhan USCN Business Co., Ltd, CEA482Ra) [31].
- *Serum vitamin D levels: using* double sandwich ELISA kits (Sunlong, Zhejiang (Mainld) China, SL0752Ra) [32, 33].
- *Plasma D-dimers levels: using* double-antibody sandwich ELISA kits (Gen Way Biotech, Inc, ca 40-88-234402, USA) according to ***[34]*.**

Histopathological studies: After the rats were killed, pups were delivered by caesarean section and parts of the placentae were harvested and fixed horizontally in 10% neutral-buffered formaldehyde solution. After dehydration, the samples were embedded in paraffin, and 4-µm sections were cut by a microtome and collected for routine H & E.

Statistical analysis

Data were presented as mean ± S.D Statistical significance was determined by one way analysis of variance (ANOVA) followed by LSD test, P values less than 0.05 were considered to be significant. In statistical analysis, SPSS version 18 program for Windows (SPSS Inc. Chicago, IL, USA) was used [35].

RESULTS

%of change in body weight (BW) and BMI, the biochemical and hemodynamic characteristics of the studied groups are summarized in table 1

AS regards group IV, there was a significant increase in %of change in body weight and BMI, MAP, total urinary proteins, serum levels of urea, creatinine, TGs, IL-6, ET-1, D-dimers, LCN2 and a significant decrease in the vitamin D.

As regards group II, there was a significant increase in the body weight, BMI, MAP, total urinary proteins, serum levels of urea, creatinine, TGs IL-6, ET-1, D-dimers, LCN2 and a significant decrease in the vitamin D.

Table (1): % of change in BW and BMI, biochemical and hemodynamic parameters of the studiedgroups.

	Group I (N= 12)	**Group II (N=12)**	**Group III (N= 12)**	**Group IV (N=12)**	**F**	**p**
% of change in weight (%)	13.91 ± 3.42	37.73 ± 9.41	32.94 ± 3.27	44.27 ± 4.67	61.62	<0.001**
% of change in BMI (%)	14.01 ± 3.33	39.08 ± 6.72	32.65 ± 3.26	44.29 ± 4.69	94.44	<0.001**
MAP: (mmHg)	88.51 ± 4.74	126.07±12.33	68.51 ± 4.39	126.26±8.81	145.67	<0.001**
Urea: (mg/dl)	21.15 ± 5.25	44.85 ± 11.05	26.08 ± 10.29	65.15 ± 13.56	43.81	<0.001**
Creat : (mg/dl)	0.17 ± 0.69	0.34 ± 0.22	0.55 ± 0.33	1.27 ± 0.39	31.53	<0.001**
TG : (mg/dl)	48.75 ± 6.7	157.17±21.35	168.92±29.54	271.08±36.88	145.11	<0.001**
Protein : (mg/24h)	14.39 ± 1.31	53.79 ± 13.65	43.97 ± 13.3	107.74±6.15	180.92	<0.001**
IL6 : (pg/ml)	71.37 ± 9.52	106.72±12.86	67.31 ± 10.98	114.09±12.12	52.6	<0.001**
Endothelin1: (pg/ml)	1.61 ± 0.13	5.36 ± 0.8	1.62 ± 0.16	5.44 ± 0.9	154.04	<0.001**
VitD : (ng/ml)	13.69 ± 1.41	6.79 ± 1.55	7.99 ± 0.8	4.48 ± 0.93	124.53	<0.001**
DDimer : (mg/dl)	138.11 ± 7.9	188.6 ± 4.69	205.45 ± 8.75	255.59 ± 4.16	632.44	<0.001**
Lipocalin2: (ng/ml)	13.4 ± 3.03	47.48 ± 4.44	28.84 ± 4.04	60.64 ± 6.7	229.2	<0.001**

**: highly significant.

The correlation between LCN2 and the measured parameters are summarized in table 2.

In group IV, there was a significant positive correlation between LCN2 levels and body weight, BMI, serum levels of urea, creatinine ,TGs, IL-6 and total urinary proteins with a highly significant positive correlation between LCN2 levels and MAP, ET-1and D- dimers levels. However, no significant correlation was found between LCN2 levels and vitamin D.

While in group II, there was a significant positive correlation between LCN2 levels and iL-6 and ET-1 and a highly significant positive correlation between LCN2 levels and MAP and D- dimers levels.

Table (2): Correlation between LCN2 and other parameters in different studied groups

		Group I LCN2	Group II LCN2	GroupIII LCN2	Group IV LCN2
Final body Weight	r	0.13	0.07	0.05	0.39
	P	0.30	0.83	0.87	0.02*
Final BMI	r	0.17	0.13	0.15	0.29
	P	0.60	0.70	0.63	0.04*
MAP	r	0.20	0.97	0.17	0.93
	P	0.53	<0.001**	0.59	<0.001**
Urea	r	0.27	0.25	0.21	0.45
	P	0.40	0.43	0.32	0.01*
Creatinine	r	0.26	0.35	0.09	0.55
	P	0.13	0.26	0.79	0.007*
TGs	r	0.19	0.06	0.28	0.35
	P	0.37	0.87	0.09	0.03*
Protein	r	0.18	0.32	0.28	0.29
	P	0.22	0.31	0.09	0.04*
IL-6	r	0.16	0.37	0.21	0.47
	P	0.61	0.02*	0.12	0.01*
Endothelin-1	r	0.12	0.34	0.15	0.50
	P	0.17	0.03*	0.15	<0.001**
VD	r	0.06	0.34	0.06	-0.15
	P	0.85	0.28	0.85	0.26
D-Dimer	r	0.03	0.88	0.05	0.89
	P	0.92	<0.001**	0.95	<0.001**

*: significant (P <0.05) **: highly significant (p<0.001) *r: correlation coefficient*

HISTOPATHOLOGICAL RESULTS

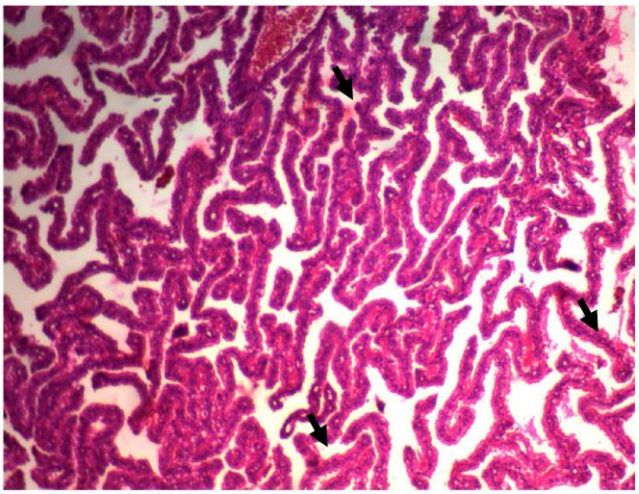

Figure (1): photomicrograph of H & E stained placental section from control pregnant rats (group III). Showing the normal placental tissue of normal pregnant rats exhibiting normal- sized chorionic villi lined by cytotrophoblastic cells ↓ (H &E X 400).

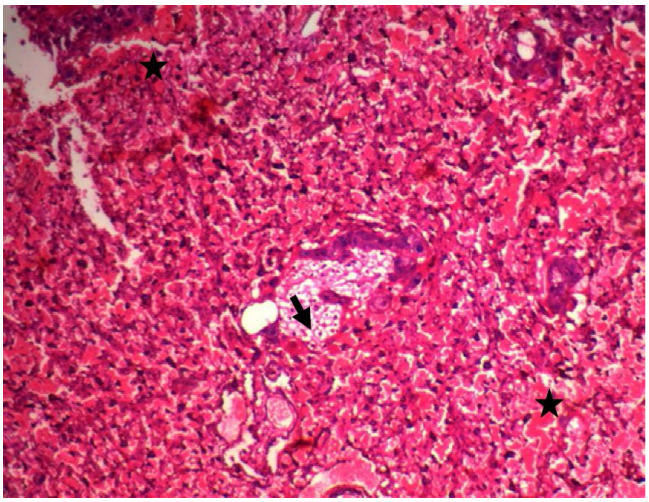

Figure (2): photomicrograph of placental section from group IV showing dilated and congested blood vessel with atherotic wall ↓ surrounded by necrotic and edematous areas with leucocytic infiltration ∗ (H & E X 400).

DISCUSSION

PE is a pregnancy related disorder defined by [36] as a disease that begins in the placenta and ends at the maternal endothelium. It has been accepted that PE is a multi-stage disease that starts by utero-placental insufficiency and leads to generalized maternal endothelial dysfunction **[37, 38]**. *Roberts and Hubel* **[39]** suggested that PE is a two-stage disorder starting by placental under-perfusion or ischemia (Stage I) followed by secretion of many soluble factors into the maternal circulation with subsequent endothelial dysfunction and multiple organ injuries responsible for its clinical manifestations (Stage II) **[39, 40]**.

Experimental PE is the induction of PE-like state in animals to facilitate the studying of different pathophysiological mechanisms of PE and investigating its potential preventive and therapeutic measures **[4]**.Due to different suggested pathophysiological mechanisms of PE and the placentation differences among mammals**[6,** 7], many PE animal models are suggested.

LCN2 is a 25kDasecretory glycoprotein that belongs to the lipocalin family of proteins. It has been implicated in many functions such as apoptosis and innate immunity. Also, it has been recognized as an adipocyte-derived acute phase protein that is positively correlated with potential effects in obesity, inflammation and insulin resistance in mice and humans **[16, 17, 18]**.

Many controversial studies about the changes in the plasma LCN2 levels in PE are reported as they may be increased **[20]** or decreased **[19]**. So, the present study was designed to perform an animal model of experimental PE in a trial to clarify the possible changes occurring in the circulating LCN2 levels in this case and trying to found an explanation to these changes.

The results of the current study showed that the L-NAME injection into pregnant rats (group IV) was found to induce a significant increase in the body weight and BMI which are in agreement with **[41]** and in contrast to *Fernandez Celadilla et al.* **[42]** that reported that there was a lower body weights and lower BMI in the preeclamptic cases.

Moreover, the signs of experimental PE induced by L-NAME were proved in this study by the significant increase in the MAP, total urinary proteins (i.e. proteinuria) and in the serum levels of urea and creatinine which are consistent with other reports of *Adamcova et al.* [43] *& Zhou et al.* **[44]**.

Furthermore, as regarding the possible suggested pathophysiological mechanisms of PE, there was a significant increase in the serum levels of IL-6, ET-1 and significant decrease in the vitamin D levels which are the same results of *Bodnar et al.* [45], *Tabesh et al.* [46] *& Mohaghegh et al.* **[47]** and unlike to *Oken et al.* [48] *& Shand et al.* **[49]** who denied this. Also, the current work also shows a significant increase in serum TGs in group IV. This finding was in line with the study of *Siddiqui et al.* **[50]**.

Interestingly, there was a significant increase in plasma D-dimers (D2D) levels in the same group which are in line with *Lindholm et al.*[51] who found that there was high levels of plasma D2D in cases of severe PE..

In a trial to explain the results of the current work, the used L-NAME was found to be a potent competitive NOS inhibitor with subsequently decreasing NO synthesis. This leads to increasing the adhesion molecules expression with subsequent acceleration of the inflammation in systemic vasculature and placenta (which indicated in our results by increasing IL-6 as an inflammatory marker) and causing endothelial dysfunction with utero-placental perfusion failure **[52, 53]**. This endothelial dysfunction is demonstrated in this study by the significant increase in the arterial blood pressure and the circulating levels of ET-1together with defective renal functions that indicated by increasing the serum levels of urea and creatinine and the total urinary proteins. Moreover, increase in ET-1 may be attributed to the reduction in NO synthesis as NO was known to be a potent inhibitor of ET-1 production **[54]**.

Noteworthy, *Sandrim et al.* **[55]** demonstrated that NO synthesis was inversely related to the serum levels of anti-angiogenic factors including sFlt-1 and sEng. So, inhibition of NO synthesis will be associated with an increase in these anti-angiogenic factors which are strongly incriminated in the pathophysiology of PE **[56, 57]**. This supports our work in using L-NAME for induction of the experimental PE. Also, this increase in antiangiogenic agents that targets VEGF due to NO reduction may be a mechanism for the underlying hypertension **[55]**.

In addition to its NO inhibitory effect, L-NAME was found to decrease prostacyclin and increase the plasma endothelin and thromboxane A2 with more vasoconstriction, oxidative stress and hypertension **[58, 59, 60]**.

As regards to the increase in serum TGs levels in PE, *Mikhail et al.* **[61]** found that increased serum TGs levels leads to its endothelial accumulation with subsequent endothelial dysfunction. This accumulation is likely to be mainly in placental decidua basalis tissue; (the layer of the placenta

that contains the spiral arteries and where the process of atherosis may increase the risk of placental vascular disease), contributing to the endothelial dysfunction in PE, both directly and indirectly through generation of small, dense lipoproteins [50, 61, 62].

Also, we found a significant decrease in vitamin D levels in group IV which was in line with *Arain et al.* [63] who demonstrated that PE was positively associated with VD deficiency. So, the present study may confirm the incrimination of VD deficiency in PE pathophysiology. As *Bednarek Skublewska et al.* [64] found that VD deficiency was found to be associated with increased IL-6 concentrations (which was already increased in the current work) through stress induced kinase and inhibition of other inflammatory cytokines as TNFα pointing out the role of deficient VD in PE through increasing inflammation.

Furthermore, the significant increase in the D-dimers (D2D) in this study in this group (IV) can also be attributed to NO inhibition as normally; NO acts as an anticoagulant factor by inhibiting the adhesion of leukocytes and platelets to the endothelium [65, 66]. Moreover, the increased D2D levels in our work may be explained by the increased IL-6 levels which was found by *James et al.* [67] to increase the endothelial permeability and reduce prostacyclin synthesis (by inhibiting the cyclooxygenase enzyme), resulting in an increased thromboxane A2/ prostacyclin ratio with subsequent promotion of vasospasm, induction of platelet aggregation and endothelial damage ending with thrombosis in microcirculation, end organ degenerative necrosis and placental infarction in severe PE.

In addition to the mentioned data, PE induced by L-NAME was indicated in the current study by the findings of the histopathological examination of placental sections. These findings include dilated congested vascular spaces filled with round cells with central nuclei and clear cytoplasm (atherosis) surrounded by areas of necrosis infiltrated by aggregates of inflammatory cells. These findings were consistent with the findings of *Salafia et al.* [68] *& Powe et al.* [36]. These results reflect the poor trophoblastic invasion of maternal spiral arteries which is the key feature of PE [69]. *Powe et al.* [36] suggested that some of the preeclamptic placental abnormalities may be consequences of the hypertension and endothelial injury that occurs in the PE.

As regards the circulating LCN2 levels, there was a significant increase in them in the L-NAME induced PE group (group IV). These results are in line with those of *Artunc – Ulkumen et al.* [20] who reported that LCN2 levels increased significantly in PE with relation to its severity. In contrast to our results, *Arikan et al.* [19] reported that there is a decrease in LCN2 levels in pre-eclamptic subjects.As a third opinion, *Kim et al.* [21] found that LCN2 levels had been elevated in the normotensive pregnant women without any medical or obstetrical problems.

Concerning the correlation between LCN2 levels and the measured parameters in group IV in the light of our results, there was a significant positive correlation between LCN2 levels and body weight, BMI, serum levels of urea, creatinine and total urinary proteins which were in line with the studies of *Dogan et al.* [70], *Simonazzi et al.* [71] *& La Chesnaye et al.* [72].

A significant positive correlation between LCN2 and TGs is also present like the study of *Mahfouz et al.,* [73]. Moreover, there was a highly significant positive correlation between LCN2 levels and MAP, ET-1and D- dimers levels. Also, *Mahfouz et al.* [73] reported positive correlation between LCN2 levels and MAP and *Liu et al.* [74] found a positive correlation between LCN2 and ET-1(as a marker of endothelial dysfunction). Moreover, *Hemdahl et al.* [75] *& Eilenberg et al.* [76] found a positive correlation between LCN2 and D-dimers.

Also, we found that there is a positive correlation between LCN2 and IL-6. This was in line with *Wallenius et al.* [77] *& Yilmaz et al.* [78] .However, no significant correlation was found between LCN2 levels and vitamin D.

The studies done by *Borzychowski et al.* [79] *& Chaiworapongsa et al.* [80] showed that LCN2 could be used in the prediction of PE before its clinical diagnosis as they found that there was an up regulation of circulating LCN2 levels as a consequence of the generalized endothelial injury associated with PE.

ThepositivecorrelationbetweenLCN2levels and serum levels of urea, creatinine and total urinary protein scan be explained by the studies that demonstrated that the LCN2 is a reliable marker of acute renal injury that known to be a common consequence in PE [81, 82].

As regards the positive correlation between the LCN2 and serum TGs, *Choi et al.* [83] *& Ni et al.* [84] explain this correlation as LCN2 has an atherogenic mechanism which may be related to disruption in the lipid metabolism. This is in line with the results of the current study which showed a significant correlation between LCN2and TGs and between LCN2 and D-dimers as a marker of hypercoagulability.

About the positive correlation between LCN2 and IL-6, *Wallenius et al.* [77] & *Yilmaz et al.* [78] suggested that LCN2 may reflect the inflammatory process occurring in the PE. Moreover, *Hamzic et al.* [85] identified LCN2 as a new factor involved in the pathway of inflammatory IL-6 signaling and suggested that there is a direct relationship between IL-6 and LCN2as LCN2 expression is induced in the endothelial cells under immune stimulation and this induction is inhibited in the absence of IL-6 and increased after recombinant IL-6administration. Also, mice lacking IL-6 were have two-times lower expression of the LCN2 gene [85].

As regards the positive correlation between the LCN2 and ET-1, *Liu et al.* [74] was demonstrated that LCN2 can induce endothelial dysfunction (which indicated in our study by increasing ET-1) by eNOS uncoupling, increasing cyclooxygenase activity and promoting the oxidative stress. An Interesting information is that LCN2 is polyaminated and that its inflammatory and endothelial damaging effects are mediated by its deaminated form [86].

The highly significant positive correlation between LCN2 and D-dimers (which is a marker of hypercoagulability) in this study may indicate the higher risk of coagulopathy complications occurrence. As LCN2 was found to stimulate the action of MMP-9 which is a mediator of vascular remodeling or plaque instability predisposing to atherosclerotic complications [87].

According to the aforementioned data, we can suggest that there is an association between LCN2 plasma levels and PE and these levels may reflect the severity of the disease.

CONCLUSION

L-NAME can be used for induction of experimental PE and plasma levels of LCN2 can be used as an indicator for the renal complications and coagulopathies in PE. So, the diagnosed preeclamptic patients with highly elevated LCN2 levels are advised to be continuously followed up by renal functions and hemostatic parameters.

RECOMMENDATIONS

Further studies are needed to ascertain this association and the possibility of its clinical applying.

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