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The Upregulated Exosome Protein ET-1 Induces the Increased Level of Ang-II and Contributes to the Progression of Pre-Eclampsia

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ABSTRACT

Pre-eclampsia (PE) is one of the leading complications of pregnancy that contributes to the increasing morbidity and mortality. However, the effects of exosomes secretion and expression of ET-1 and Ang-II on PE progression has not been fully investigated. The PE (n=100) and normal (n=100) women with singleton pregnancy and in the second or third trimester of pregnancy were included in the present study. The healthy group included healthy women (n=100) aged 28 to 40. The blood samples were collected, and exosomes were isolated using commercial kit. The exosomes were observed by transmission electron microscope. Western blotting was done to detect the protein expression. Transmission electron microscope analysis revealed that exosomes could be significantly observed in the PE group. The expression of surface markers that included CD63 and CD81 were dramatically upregulated in PE group. The protein level of ET-1 and Ang-II were significantly increased in PE group, and the upregulated ET-1 and Ang-II expression were highly correlated with PE progression. The present study demonstrated that the exosome protein ET-1 upregulated the expression of Ang-II and contributed to the progression of PE.

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INTRODUCTION

Pre-eclampsia (PE) has become one of the leading complications of pregnancy in the past few decades, which has affected around 5% of pregnancies and even caused over 50,000 maternal deaths worldwide (Phipps *et al*., 2019). It was reported that PE largely contributed to the increasing maternal and fetal morbidity and mortality, and enhanced the rate of cardiovascular disease in the pregnancies (Kuklina *et al*., 2009). Women suffered with PE had around 10 to 20-fold increased risk of severe complications, including abruptio placentae and aspiration pneumonia. PE was reported to be marked by the presence of onset hypertension and proteinuria that occurred in the second or third trimester of pregnancy (Bartsch *et al*., 2016).

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Recently, potential mechanisms that included angiogenic, structural and metabolic pathways have been studied in PE, such as spiral artery remodeling and placental oxygenation (Karumanchi, 2016). Though various biomarkers have been used in the early diagnosis, they have been demonstrated to be unsuccessful in the different stages of the syndrome (Gilani *et al*., 2016).

Extracellular vesicles have been identified as a new mechanism that mediating cellular crosstalk and interactions (Ruiz-González *et al*., 2015). Bioactive molecules (DNAs, RNAs, lipids and proteins) and chemical compounds could be delivered to adjacent or distant target cells by extracellular vesicles and played vital roles in regulating cell biological functions (Zhao *et al*., 2018). Exosomes, as one of the extracellular vesicles (with a diameter of about 40 ~ 200 nm) released after the fusion of intracellular multi vesicle bodies and cell membrane, has been reported to be involved in various biological pathways (Rezaie *et al*., 2018). Exosomes were synthesized from different parts of the female tissues, including oviductal epithelium, endometrium and the placenta (Kropp and Khatib, 2015). Production and release of placental-derived exosomes were reported to be induced during complications of pregnancy (Salomon and Rice, 2017). Whether identifying exosomes during pregnancy

would contribute to the clinical diagnosis of PE remained unclear.

Endothelin 1 (ET-1), belonged to the predominant member of ETs family that was compromised of 21-amino-acid peptides, could be synthesized, secreted by endothelial cells, and induced vasoconstriction. Stimulus that included norepinephrine, thrombin, and angiotensinogen-II (Ang-II) were reported to be induced by the secretion of ET-1 (Saleh *et al*., 2016). PE was often accompanied by immune injury, which released various cytokines and aggravated the oxidative stress injury of vascular endothelial cells (Saleh *et al*., 2016). Therefore, in the present study, we detected the secretion of exosomes and ET-1 and Ang-II in the pregnancies with PE to elucidate their relationships.

MATERIALS AND METHODS

The retrospective case-control study was implemented in the current research. The inclusion criteria for the clinical trial were as follows: The ages between 28 and 40, singleton pregnancy and the women (n=100) in the second or third trimester of pregnancy attending the obstetric outpatient clinic and diagnosed with PE. The patients with fetal anomalies, serious mental illness, severely ill status, multiple gestations, and diabetes were excluded from this study. The normal group included women (n=100) in the second or third trimester of pregnancy with uncomplicated pregnancy while attending the obstetric outpatient clinic. The healthy group included healthy women (n=100) aged 28 to 40. The blood samples were collected from all the participants. Written informed consent was obtained from all participants and this study was approved by the Ethics Committee of Fuyang Minsheng Hospital.

The exosomes were isolated from the blood samples of the participants using ExoQuick-TC kit (System Biosciences, CA, USA) as per the manufacturer's instructions (Wu *et al*., 2021). Briefly, the blood samples were centrifugated at 3000 g for 10 min and the serum samples were collected. Subsequently, the serum samples were incubated with ExoQuick-TC solution for 12 h at 4 °C, after which the samples were centrifugated at 1500 g for 30 min. The extracted exosomes were further purified using immune-affinity Exo-Flow Exosome Capture kit (System Biosciences, CA, USA) according to the protocol. The expression of exosomes surface markers that included CD63 and CD81 were detected by western blotting.

The purified exosomes were incubated in 2.5% glutaraldehyde for 2 h, fixed with a 200 mesh Formvar and stained with 2% uranyl acetate for 1 h. Then the prepared samples were observed under an HT7700 transmission electron microscope (Hitachi, Tokyo, Japan).

The samples were collected and treated with lysis buffer, after which supernatant was collected by centrifugation and the concentration of protein were determined by BCA Assay Kit (Beyotime Biotechnology, Shanghai, China). Proteins were separated by polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore, Massachusetts, USA). Subsequently, 5% skim milk was used for blocking, after which proteins were incubated with primary antibodies (Abcam, USA) including anti-CD63 (ab134045, 1:1000), anti-CD81 (ab79559, 1:1000), anti-ET-1 (ab2786, 1:1000) and anti-Ang-II (ab124734, 1:1000). The samples were then reacted with horseradish peroxidase (HRP)-conjugated IgG secondary antibody (ab6759, 1:1000) for 2 h at room temperature. The bands on the membranes were observed and analyzed using ImageJ software.

Data were presented as the mean ± SD. All statistical analyses were conducted using GraphPad Prism 8 (La Jolla, CA, USA). Student's t test was performed for comparison between two groups. One-way ANOVA followed by Tukey's post hoc test was conducted for comparison among three or more groups. Multiple linear regression analysis was implemented for evaluating the relationship between protein expression and disease. P-values < 0.05 were statistically significant.

RESULTS

Figure 1 shows that the exosomes are not observed in the blood samples of healthy women or pregnant women with uncomplicated pregnancy, whereas exosomes could be significantly observed in the PE group. Moreover, the expression of surface markers that included CD63 and CD81 were dramatically upregulated in PE group compared with normal group and healthy group (Fig. 2). The western blotting results further suggested that the protein level of ET-1 (Fig. 3) and Ang-II (Fig. 4) were significantly increased in PE group compared with normal group and healthy group. Moreover, the relation between ET-1 expression and PE, as well as the relation between Ang-II expression and PE were analyzed by logistic regression model (Tables I and II). Results demonstrated that upregulated ET-1 and Ang-II expression are highly correlated with PE progression ($P<0.001$).

DISCUSSION

PE could be characterized by onset hypertension and proteinuria in the women with ≥ 20 weeks of gestation, which affected around 3-5% of the pregnancies and contributed to the increased morbidity and mortality

during the perinatal period (Hutcheon *et al*., 2011). Placental ischemia has been regarded as one of the central hypotheses of PE, because of that placental infarction was commonly observed in patients with PE (Chaiworapongsa *et al*., 2014). The hypoxia was also a potential mechanism for induction of PE, in which the protein expression of HIF-1α and HIF-2α was significantly upregulated in women with PE (Rajakumar *et al*., 2004). Moreover, the activation or dysfunction of endothelia cells were observed in the progression of PE. It was reported that the levels of E-selectin and vascular cell adhesion protein 1 were dramatically upregulated in patients with PE than the normal pregnancies (Chaiworapongsa *et al*., 2002). In the present study, we detected the exosomes expression in women suffered with PE, aiming at providing new biomarkers for the early diagnosis and identification of PE.

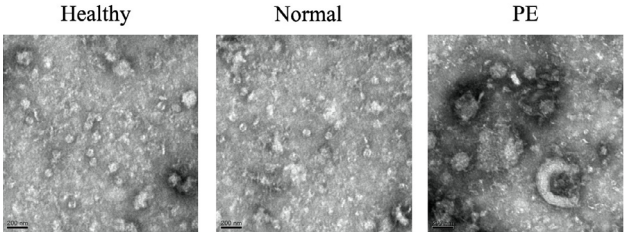

Fig. 1. The exosomes observed by transmission electron microscope. Scale bar = 200 μm.

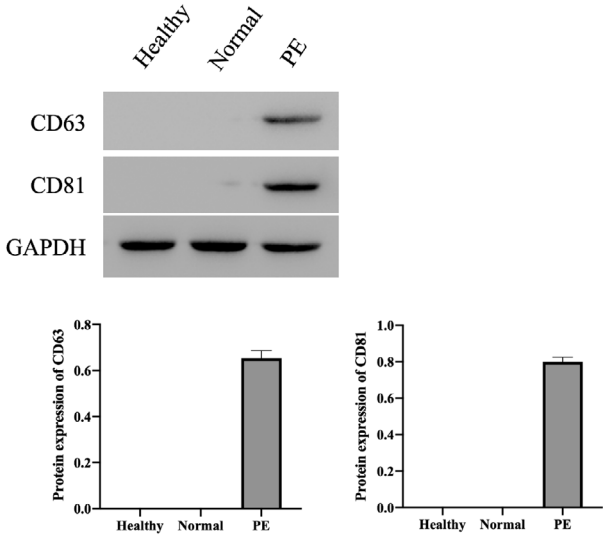

Fig. 2. The protein expression of CD63 and CD 81 detected by western blotting. The relative expression was calculated via normalization to the GAPDH.

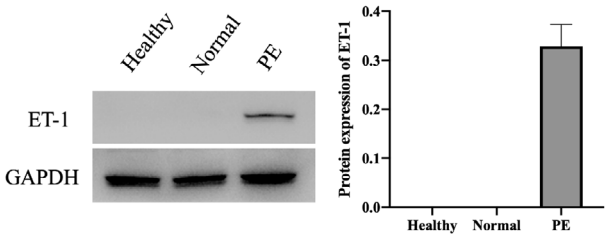

Fig. 3. The protein expression of ET-1 detected by western blotting. The relative expression was calculated via normalization to the GAPDH.

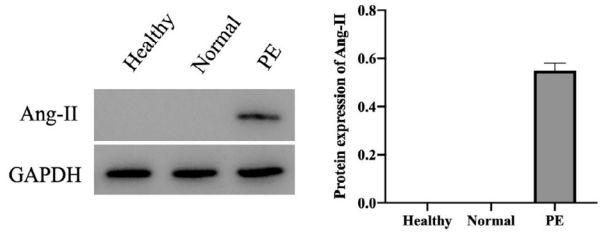

Fig. 4. The protein expression of Ang-II detected by western blotting. The relative expression was calculated via normalization to the GAPDH.

Table I. The average expression level of ET-1 and ANG-II.

	Normal	PE	P-value
ET-1 (pg/mL)	48.98±4.93	67.90±10.74	<0.001
ANG-II (pg/mL)	22.72±4.76	37.60±7.34	<0.001

Table II. The analytical results of logistic regression models.

	Wals	Odds ratio	95%CI	P
ET-1 (pg/mL)	12.610	1.517	1.205~1.929	<0.001
ANG II (pg/mL)	18.298	1.569	1.276~1.910	<0.001

Exosomes are the most broadly studied group among the extracellular vesicles that included exosomes, micro vesicles, and apoptotic vesicles (Zhang and Yu, 2019). The vital regulatory roles of exosomes have been investigated in intracellular communications and tumor progression. Raimondo *et al*. (2015) found that the level of cytokine TGFβ1 was significantly upregulated in the exosomes derived from chronic myeloid leukemia cells, which subsequently bonded with the TGFβ1 receptor on the leukemia cells, resulted in the activation of ERK/AKT pathway and suppressed the apoptosis of tumor cells. Moreover, the exosomes from mesenchymal stem cells

had the ability to modulate pulmonary hypertensive effects based on their miRNAs including miR-34a, miR-122, miR-124, and miR-127 (Aliotta *et al*., 2016). Ellis *et al*. (2019) demonstrated that the expression and phosphorylation of 6-phosphofructo-2-kinase/ fructose-2, 6-bisphosphatase isoforms was increased in urinary exosomes in patients with PE, indicating the presence of renal glycolysis during the progression of disease. We also found that the expression of exosomes was upregulated in the patients with PE.

ET-1, one of the members of the endothelin that served as effective vasoconstrictor peptides, was reported to affect oxidative stress and cell cycle of the human umbilical vein endothelial through regulating the ERβ/FOXN1 signaling pathway and the progression of atherosclerosis (Wang *et al*., 2020).

Jenkins *et al*. (2020) also reported that ET-1 could regulate plasma lipid profiles and insulin signaling that related with pathophysiology of obesity. A previous study reported that ET-1 might play a critical role in the pathophysiology of PE, and the expression levels of ET-1 was correlated with the severity of symptoms (Bakrania *et al*., 2017). High maternal ET-1 production was also proved to be correlated with preeclampsia-like phenotypes during pregnancy, resulting in the trophoblast invasion in the initial stage and maternal peripheral vasculature during late gestation (Li *et al*., 2018). The urinary angiotensinogen levels were also found to be correlated positively with blood pressure and proteinuria in pregnancies with PE but not correlated with age or body weight (Yilmaz *et al*., 2015). Consistently, we demonstrated that the expression of ET-1 and Ang-II was upregulated in PE patients, while logistic regression models indicated the positive correlations of ET-1 and Ang-II and the PE symptoms.

CONCLUSION

This study demonstrated the exosomes, and its exosome protein ET-1 might induce the increased level of Ang-II, which mediated the progression of PE, providing solid references for the molecular mechanism for investigations on PE biological processes. These data indicated that the detection of exosomes and targeting ET-1 might be potential therapeutic target for the clinical treatment of PE. However, further investigations on the other potential molecules in exosomes and their down streaming pathways are still needed to comprehensively understand the molecular mechanisms of PE progression.

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Ethics statement

Written informed consent was gained from all participants and this study was approved by the Ethics Committee of Fuyang Minsheng Hospital.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Aliotta, J.M., Pereira, M., Wen, S., Dooner, M.S., Del Tatto, M., Papa, E., Goldberg, L.R., Baird, G.L., Ventetuolo, C.E., Quesenberry, P.J. and Klinger, J.R., 2016. Exosomes induce and reverse monocrotaline-induced pulmonary hypertension in mice. *Cardiovasc Res*., **110**: 319-330. https://doi.org/10.1093/cvr/cvw054

Bakrania, B., Duncan, J., Warrington, J.P., and Granger, J.P., 2017. The endothelin type a receptor as a potential therapeutic target in preeclampsia. *Int. J. mol. Sci*., **18**: 522. https://doi.org/10.3390/ijms18030522

Bartsch, E., Medcalf, K.E., Park, A.L., and Ray, J.G., 2016. Clinical risk factors for pre-eclampsia determined in early pregnancy: Systematic review and meta-analysis of large cohort studies. *Br. med. J*., **353**: i1753. https://doi.org/10.1136/bmj.i1753

Chaiworapongsa, T., Chaemsaithong, P., Yeo, L., and Romero, R., 2014. Pre-eclampsia part 1: Current understanding of its pathophysiology. *Nat. Rev. Nephrol*., **10**: 466-480. https://doi.org/10.1038/nrneph.2014.102

Chaiworapongsa, T., Romero, R., Yoshimatsu, J., Espinoza, J., Kim, Y.M., Park, K., Kalache, K., Edwin, S., Bujold, E., and Gomez, R., 2002. Soluble adhesion molecule profile in normal pregnancy and pre-eclampsia. *J. Matern. Fetal. Neonatal Med*., **12**: 19-27. https://doi.org/10.1080/jmf.12.1.19.27

Ellis, R., Katerelos, M., Choy, S.W., Cook, N., Lee, M., Paizis, K., Pell, G., Walker, S., Power, D.A., and Mount, P.F., 2019. Increased expression and phosphorylation of 6-phosphofructo-2-kinase/ fructose-2,6-bisphosphatase isoforms in urinary exosomes in pre-eclampsia. *J. Transl. Med.,* **17**: 60

https://doi.org/10.1186/s12967-019-1806-6.

Gilani, S.I., Weissgerber, T.L., Garovic, V.D., and Jayachandran, M., 2016. Preeclampsia and extracellular vesicles. *Curr. Hypertens Rep*., **18**: 68. https://doi.org/10.1007/s11906-016-0678-x

Hutcheon, J.A., S. Lisonkova and K.S. Joseph, 2011. Epidemiology of pre-eclampsia and the other hypertensive disorders of pregnancy. *Best Pract. Res. Clin. Obstet. Gynaecol*., **25**: 391-403. https://doi.org/10.1016/j.bpobgyn.2011.01.006

Jenkins, H.N., Rivera-Gonzalez, O., Gibert, Y., and Speed, J.S., 2020. Endothelin 1 in the pathophysiology of obesity and insulin resistance. *Obes. Rev*., **21**: e13086. https://doi.org/10.1111/obr.13086

Karumanchi, S.A., 2016. Angiogenic factors in preeclampsia: From diagnosis to therapy. *Hypertension*, **67**: 1072-1079. https://doi.org/10.1161/HYPERTENSIONAHA.116.06421

Kropp, J. and Khatib, H., 2015. Characterization of microrna in bovine in vitro culture media associated with embryo quality and development. *J. Dairy Sci*., **98**: 6552-6563. https://doi.org/10.3168/jds.2015-9510

Kuklina, E.V., Ayala, C. and Callaghan, W.M., 2009. Hypertensive disorders and severe obstetric morbidity in the united states. *Obstet. Gynecol*., **113**: 1299-1306. https://doi.org/10.1097/AOG.0b013e3181a45b25

Li, F., Kakoki, M., Smid, M., Boggess, K., Wilder, J., Hiller, S., Bounajim, C., Parnell, S.E., Sulik, K.K., Smithies, O., and Maeda-Smithies, N., 2018. Causative effects of genetically determined high maternal/fetal endothelin-1 on preeclampsia-like conditions in mice. *Hypertension*, **71**: 894-903. https://doi.org/10.1161/HYPERTENSIONAHA.117.10849

Phipps, E.A., Thadhani, R., Benzing, T. and Karumanchi, S.A., 2019. Pre-eclampsia: Pathogenesis, novel diagnostics and therapies. *Nat. Rev. Nephrol*., **15**: 275-289. https://doi.org/10.1038/s41581-019-0119-6

Raimondo, S., Saieva, L., Corrado, C., Fontana, S., Flugy, A., Rizzo, A., De Leo, G., and Alessandro, R., 2015. Chronic myeloid leukemia-derived exosomes promote tumor growth through an autocrine mechanism. *Cell Commun. Signal*, **13**: 8. https://doi.org/10.1186/s12964-015-0086-x

Rajakumar, A., Brandon, H.M., Daftary, A., Ness, R., and Conrad, K.P., 2004. Evidence for the functional activity of hypoxia-inducible transcription factors overexpressed in preeclamptic placentae. *Placenta*, **25**: 763-769. https://doi.org/10.1016/j.placenta.2004.02.011

Rezaie, J., Ajezi, S., AvciÇ, B., Karimipour, M., Geranmayeh, M.H., Nourazarian, A., Sokullu, E., Rezabakhsh, A., and Rahbarghazi, R., 2018. Exosomes and their application in biomedical field: Difficulties and advantages. *Mol. Neurobiol*., **55**: 3372-3393. https://doi.org/10.1007/s12035-017-0582-7

Ruiz-González, I., Xu, J., Wang, X., Burghardt, R.C., Dunlap, K.A., and Bazer, F.W., 2015. Exosomes, endogenous retroviruses and toll-like receptors: Pregnancy recognition in ewes. *Reproduction*, **149**: 281-291. https://doi.org/10.1530/REP-14-0538

Saleh, L., Verdonk, K., Visser, W., van den Meiracker, A.H., and Danser, A.H., 2016. The emerging role of endothelin-1 in the pathogenesis of pre-eclampsia. *Ther. Adv. Cardiovasc. Dis*., **10**: 282-293. https://doi.org/10.1177/1753944715624853

Salomon, C., and Rice, G.E., 2017. Role of exosomes in placental homeostasis and pregnancy disorders. *Prog. Mol. Biol. Transl. Sci*., **145**: 163-179. https://doi.org/10.1016/bs.pmbts.2016.12.006

Wang, Y., Ruan, Y. and Wu, S., 2020. Et-1 regulates the human umbilical vein endothelial cell cycle by adjusting the erβ/foxn1 signaling pathway. *Ann. Transl. Med*., **8**: 1499. https://doi.org/10.21037/atm-20-6560

Wu, S.C., Kuo, P.J., Rau, C.S., Wu, Y.C., Wu, C.J., Lu, T.H., Lin, C.W., Tsai, C.W., and Hsieh, C.H., 2021. Subpopulations of exosomes purified via different exosomal markers carry different microrna contents. *Int. J. med. Sci*., **18**: 1058-1066. https://doi.org/10.7150/ijms.52768

Yilmaz, Z., Yildirim, T., Yilmaz, R., Aybal-Kutlugun, A., Altun, B., Kucukozkan, T., and Erdem, Y., 2015. Association between urinary angiotensinogen, hypertension and proteinuria in pregnant women with preeclampsia. *J. Renin Angiotensin Aldosterone Syst.*, **16**: 514-520. https://doi.org/10.1177/1470320313510585

Zhang, L. and Yu, D., 2019. Exosomes in cancer development, metastasis, and immunity. *Biochim. biophys. Acta Rev. Cancer,* **1871**: 455-468. https://doi.org/10.1016/j.bbcan.2019.04.004

Zhao, H., Achreja, A., Iessi, E., Logozzi, M., Mizzoni, D., Di Raimo, R., Nagrath, D. and Fais, S., 2018. The key role of extracellular vesicles in the metastatic process. *Biochim. biophys. Acta Rev. Cancer*, **1869**: 64-77. https://doi.org/10.1016/j.bbcan.2017.11.005