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Investigation of the plasmatic nitric oxide levels in women with preterm birth and women with symptoms of threatened preterm labor

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Prevention of preterm birth (PTB, birth before 37 gestational weeks) and threatened preterm labor (TPL) is a major undertaking in pregnant women health in prenatal care and identified as one of the main problems associated with redox imbalances in the reactive oxygen/nitrogen species (ROS/RNS) and increased nitrosative stress (NS) damages. The main goal of the herein reported study was to evaluate and compare plasmatic nitric oxide (•NO) radicals as a real time parameter of nitrosative stress in women with PTB and women with TPL symptoms, using spin-trapping EPR spectroscopy. Possible role of •NO radicals as a reliable marker for predicting PTB, TPL and for therapeutic purposes was also discussed. The •NO radical formations were measured in plasma specimens from 243 women divided into 3 groups: 1) n=73 pregnant women complicated by PTB; 2) n=30 pregnant women with symptoms of threatened preterm labor (TPL); and 3) n= 75, controls including singleton pregnant women in term. For the first time, •NO radical production during pregnancy complicated by PTB and TPL in Bulgarian women population was investigated in real time using the EPR spin-trapping method. It is important to emphasize that •NO radical production and oxidative/ nitrosative stress increases with advancing gestation during PTB and decrease in PTL groups. Based on previous studies and on our results, we argue that •NO radicals could be a reliable marker for predicting PTB, TPL and for therapeutic purposes.

Keywords: PTB, PTL, EPR, nitrosative stress

INTRODUCTION

Prevention of preterm birth (PTB) and threatened preterm labor (TPL) is a major undertaking in pregnant women health and in prenatal care. Preterm birth (birth before 37 gestational weeks) is identified as one of the main problems associated with advanced pregnancy and the cause of neonatal mortality. According to a WHO report from 2012, about 15 million premature babies were born each year in the world and in Europe, this number is around 500,000. The 1.1 million premature babies do not survive because of the complications related to premature birth. The premature born-children are with a high rate of mental, physical and neurological complications, [1, 2]. This leads to a number of socio-economic issues and a global problem for humanity. It was found that socio-economically poor families are at higher risk of premature birth [1, 2]. The preterm birth is observed in 12% of pregnancies and is associated with 50% long-term neurological consequences for the fetus [1, 2]. The etiological factors of preterm birth are: maternal stress, infection and inflammation, uterine distension, abnormal amounts of amniotic fluid, cervical insufficiency, and placental dysfunction. Early diagnosis of PTB symptoms is medically difficult. The PTB prediction is carried out considering obstetric history, but these methods are neither sensitive nor specific [3]. In early and middle normal pregnancy, the uterus is usually calm and inactive. As gestational age advances, maternal uterine activity and contractility increase and birth usually begins or is nearing. No precise mechanisms are known related to the initiation of normal, preterm (PTB), and threatened preterm labor (TPL), despite the studies described in the literature [4]. Several hypotheses identify redox imbalance in reactive oxygen/nitrogen (ROS/RNS) species and oxidative damages as a major cause of pathophysiological pregnancy, complicated by PTB and TPL. The increased oxidative stress and the destructive effects of free-radical formation are capable of leading to pathological processes during PTB and TPL. Different studies commented the antioxidant/ pro-oxidant imbalance in the intrauterine compartments and inflammation in the endothelial dysfunction [5, 6].

Nitric oxide is a highly active free radical (•NO) and a biological mediator, with short half-life time, that was synthesized by an enzyme group known as NO synthase (NOS). •NO is a vascular relaxation factor originating from the endothelium, with a potent inhibitory effect on smooth muscle contraction.

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Cells produce •NO radicals of three isoforms (endothelial NOS, inducible NOS and neural NOS) which have been identified and they catalyze the conversion of L-arginine to endothelial NO synthase and citrulline [7-9]. •NO metabolites, NO_3^-(nitrates) and NO_2^-(nitrites) are a factor in the feto-placentation process involved in the placental vascular reactivity regulation, bed resistance. Therefore, locally synthesized •NO can participate in physiological reproductive activities, including egg maturation, fertilization and embryonic progression, and can react with molecular oxygen and other ROS species. Numerous investigations reported results of •NO measured in maternal plasma, urine, and vaginal secretions [9, 10], and also of NOS activity in human pregnant myometrium, villous trophoblastic and fetal membranes [4, 11]. Studies on the pregnant women uterus and myometrium contractility demonstrated a declining level of NOS expression after birth, labor [4, 12, 13] and PTB complicated pregnancies.

However, there are conflicting data regarding the role of •NO radicals/ NOS system activity in control and prevention of the PTB and TPL during pregnancy. The clinical study of Rowlands *et al*., 1996 [14] demonstrated that •NO/ NO donors delay the preterm labor/ birth, which determines the •NO role in tocolysis and in PTB and TBP prevention [4]. Other clinical investigation describes that both endogenous and exogenous NO donors participate in uterine contractility to both ET-1 and AVP peptides, resulting in a local imbalance between narrowing and relaxing mediators [15]. Moreover, •NO radicals react with superoxide anion ($\bullet O_2$) radicals to formation of peroxynitrite (ONOO-) anions that suppress endothelial NOS activity [16, 17] and chronically elevated oxidative stress levels and immune disorders. Extreme ROS/ RNS generation leads to decreased antioxidant enzyme protection, impaired normal cellular responses and cellular growth in preterm injury [18]. Different experimental studies comment that RNS/•NO metabolites affect the normal function of the placenta [18] in PTB patients and at different preterm phases were able to be inhibited or increased [18-21]. Despite the serious number of experimental studies, the exact role of •NO radicals/ NOS system activity in control and prevention of the PTB and TPL during pregnancy has not yet been fully clarified.

The main goal of the herein reported study was to evaluate and compare plasmatic nitric oxide (•NO) radicals as a real time parameter of nitrosative stress (NS) in women with preterm birth and women with symptoms of threatening preterm labor, using spin-trapping EPR spectroscopy. Possible role of •NO radicals as a reliable marker for predicting PTB, TPL and for therapeutic purposes was also discussed.

EXPERIMENTAL

Ethics Statement

This work was conducted according to the Declaration of Helsinki, and approved by the Ethics Board, Clinic of "Obstetrics and Gynecology", UMHAT "Prof. St. Kirkovich" in Stara Zagora, Bulgaria. Written informed consent (*2017/2019 MF, TrU, Stara Zagora*) was obtained from the patients after hospitalization between June 2017 and September 2019.

Study design and subjects

The •NO radical formation was measured in plasma specimens from 243 women, 17-41 years old, including n=73 pregnant women complicated by PTB, n=30 pregnant women with symptoms of threatened preterm labor (TPL) and control group (CG; n= 75) including healthy singleton pregnant women in term (Table 1).

Gestational age was determined by an experienced sonographer, using transabdominal ultrasound (*Aloka, Prosound alpha 6*) when the patient's bladder was empty and the date of the last menstrual period was determined. Pregnant women with PTB symptoms have been detected in late preterm birth 32.1-36.2 weeks (n=73). The pregnant women with threatened preterm labor have been detected in late preterm birth 32.3-36.1 weeks (n=30). In the PTB and TPL groups, participants had no history of type 1 or 2 diabetes, gestational diabetes, high blood pressure, incompetent cervix, uterus anomaly, hypertension (n=5), cardiovascular and infectious diseases (n=6), maternal complications, fetal anomaly or amniotic fluid, pre-eclampsia.

The venous blood samples of patients with PTB and TPL were taken before the start of tocolytic therapy and before the onset of corticosteroid prophylaxis of neonatal respiratory distress (RDS). PTB and TPL women - smokers (n=14); patients (n= 6) with acute or chronic infection, and women with fetal asphyxia, fetal growth restriction, and placental problems were excluded from the experiment. A singleton pregnant group including healthy (17- 38 years old, n= 75) women delivered at term (after 38.2 weeks' gestation), without history of other pregnancies or family diseases was used as (CG).

Table 1.

Characteristics	CG (n= 75)	PTB (n=73)	TPL (n=30)	*p	**p
Age, years	28.8 ± 3.4	33.8 ± 1.4	34.8 ± 3.5	0.039	0.041
Family history of diabetes	15(27.3)	None	None	≤0.002	≤0.003
Birth weight, g	3,216.93± 50.91	1,670.45± 46.14	1,245.9 ± 96.14	≤0.002	≤0.003
Body mass index, kg/m^2	38.9 ± 2.7	16.7 ± 0.94	17.6 ± 0.99	0.53	0.55
Gestational age, weeks/ range	39.2 ± 2.52	36.1 ± 1.19	30.3± 1.78	- -	
Systolic blood pressure (SBP, mmHg)	119.8 ± 10.4	136.2 ± 3.1	138.7 ± 2.41	≤0.002	≤0.001
Diastolic blood pressure (DBP, mmHg)	69.1± 6.0	80.1± 4.0	83.3± 4.0	≤0.003	≤0.002
Mean arterial pressure (MAP, mmHg)	93.51 ± 2.2	93.42 ± 1.9	95.66 ± 2.1	≤0.002	≤0.003
Eclampsia	None	None	None	-	
pre-eclampsia	None	None	None	-	
incompetent cervix,	None	None	None	-	
uterus anomaly,	None	None	None	-	
cardiovascular diseases	None	None	(n=7)	-	
infectious diseases	-	-	(n=7)	-	
maternal complications,	None	None	None	-	
fetal anomaly	None	None	None	-	
amniotic fluid	None	None	None	-	
urine protein	None	None	None	-	
pregnancy parity	NA			-	
Pulse pressure	62.2 ± 8	79.7 ± 9.12	80.9 ± 8.34		
Chronic hypertension				<0.002	≤0.003
				≤0.002[a]	≤0.003[b]
Data presented as mean ± SD	0.36 %	0.16%	0.14%		
				≤0.051[a]	≤0.053[b]
	NA- not applicable				
				p*- comparison between CG and PTB groups	
				p-** comparison beween CG and TPL groups	

[a] p < 0.002 CG *vs* PTB, computed by LSD post hoc test (pulse / chronic)
[b] p < 0.003 CG *vs* TPL, computed by LSD post hoc test (pulse / chronic)
NA PTB *vs* TPL, computed by LSD post hoc test (birth weight, g)

Body weight, blood pressure, and urine protein concentrations (>170 mg for the last 24 h) were evaluated in the groups. The diagnosis of PTB and TPL cases was made by strict clinical criteria [22]: 1) Risk factors presence of preterm birth; 2) Cervical status determined by vaginal smear and trans-vaginal echography; 3) painful uterian contractions, documented for 1-1.5 h and regular contractions resulting in cervical changes in dilatation and effacement; 4) Uterine activity monitoring - anamnestic according to the data of the pregnant women and by cardio-tocography; 5) Traceability for genital bleeding - anamnestic and vaginal obstruction.

Blood collection and PTB and PTL registration

Venous peripheral blood (5 ml) was collected directly by venous puncture from the ante-cubital region, in the participants, when they were in the active phase of PTB and TPL at mean gestational ages of 36.1 and 30.3 weeks, respectively. The blood collected from the CG1 patients was at a mean gestational age of 39.2 weeks, at the day of birth. The blood samples from the four tested groups, containing EDTA anticoagulant, was collected into plastic tubes, and centrifuged at 4000 rpm for 10 min at 4°C. 1.3 ml of plasma samples

was separated and stored at –20°C until further assay was done.

The PTB and TPL were registered before the end of 37 gestational weeks. In the PTB and TPL patients was observed the appearance of regular contractions in every 5 min, each contraction lasting for about 42 s, and vaginal examination of the cervix was revealing that the cervix was centered, anterior, thin, short (1 cm long, and the cervical os dilated to 12 cm), and soft. In the absence of maternal contraindications, an attempt was made to stop the uterine contractions through tocolytic therapy in order to protect the fetus or at least to "take the time" to perform corticosteroid RDS prophylaxis - before the end of 34 gestational weeks. The births between 34 and 37 gestational weeks are associated with fewer complications for the newborn. In this regard, the preterm births are more problematic at <34 week gestation. In general, immaturity of the lungs, liver, digestive system, and immunity of the fetus lead to increased morbidity and mortality among premature infants [23]. The prematurity is the basis of 75% of perinatal infant mortality and of later psychiatric disorders (*27% of newborns weighing less than 1500 g die, while 50% of children die when weighing less than 1000 g).* A possible intrauterine retardation of the fetus further complicates the prognosis. Every earned day that prolongs pregnancy, brings 2% percent higher chance of fetus survival.

In vivo EPR study on the plasmatic •NO radical metabolism

We measured •NO radical metabolism in all tested groups by reduction and spin-adduct formation between Carboxy-Ptio.K and generated radical using the Yoshioka *et al.* [24] and Yokoyama *et al.* [25] methods. The EPR analysis was adapted for EMX^{micro}, X-band spectrometer/ standard resonator (Bruker, Germany; at 3505 G centerfield, 6.42 mW microwave power, 5 G modulation amplitude, 75 G sweep width, 2.5×10^2 gain, 40.96 ms time constant, 60.42 s sweep time, 1 scan per sample; 20-25 ºC) and results were calculated by double integration of the corresponding EPR spectra (arbitrary units).

Statistical analysis

Data analyses were performed using the Statistica 8.0, Stasoft, Inc., one-way ANOVA, to determine significant difference between data groups. To define witch groups are different from each other we have used LSD post hoc test. The results were expressed as means ± standard error (SE). A $p< 0.05$ value was considered statistically significant. The EPR spectral processing was performed using Bruker WIN-EPR and SimFonia software.

RESULTS AND DISCUSSION

Preterm birth and threatened preterm labor (PTB/ TPL) is an important perinatological problem that leads to various neonatal disorders, physical ailments and even to infant mortality [26]. Numerous studies related to increased risk of PTB/ TPL focused on the expression of fetal fibronectin in the cervicovaginal fluid, short cervix, infection in vaginal microflora and in gestational tissue [27]. On the other hand, other experiments were focused on the molecular mechanisms [27] and transvaginal sonography screening as predictive tests for spontaneous preterm birth (PTB) in symptomatic singleton pregnancy with threatened preterm labor (PTL) [28].

A lot of studies have also indicated that neuronal NOS, endothelial NOS, inducible NOS metabolites and indirect •NO radical production are informative PTB and TPL markers or predictors [4, 29, 30].

In our study, we emphasize on the importance of recorded •NO radicals real time formation in the maternal body, and thus emphasize on the potential functional and clinical significance of presented data for the prevention of preterm birth and threatened preterm labor.

Clinical characteristics

Clinical characteristics of 243 women included in the study are summarized in Table 1. The mean gestational age for PTB (33.2 ± 1.19 weeks, p=0.004, *t*-test) patients, and for TPL (32.8 ± 1.34 weeks p=0.004, t-test) patients, was significantly lower than the mean gestational age for the CG (38.1 ± 2.52 weeks) group of women in term, respectively (p<0.003).

Statistically significant differences were observed between CG and both PTB and TPL groups: for systolic blood pressure (p<0.002, *t*-test; p<0.001, *t*-test); for diastolic blood pressure (p<0.003, *t*-test; p<0.002, *t*-test); for maternal pulse pressure (p<0.002, *t*-test; p<0.003, *t*-test), and pregnancy parity (p<0.003, *t*-test; p<0.003, *t*-test). The statistically significant differences in age (p=0.039, *t*-test; p=0.041, *t*-test), body mass index (p<0.002, *t*-test; p<0.001, *t*-test) and registered secondary diseases between CG and both PTB and TPL groups were not recorded.

We did not observe statistically significant differences in systolic/ diastolic blood pressure, heart rate, and pregnancy parity between the PTB and TPL groups (p=0.003, *t*-test).

However, both PTB and TPB groups demonstrate elevated chronic hypertension values (p<0.0051, *t*-test; p<0.003, *t*-test) and pulse pressure (p<0.0034, *t*-test; p<0.002, *t*-test), statistically significant compared to CG groups. No statistically significant differences between PTB, TPL groups and controls were registered. There was a statistically significant difference between CG and PTB groups, and CG and TPL groups to the two measured factors, respectively (p<0.002, LSD; p<0.003, LSD). The birth weight of the babies in the TPL group (n=7; 1,245.93 ± 963.14 g; p<0.002, *t*-test) and in the PTB group (1,670.45 ± 467.14 g; p<0.002, *t*-test) was statistically significantly lower than those of the CG group (3,216.93 ± 500.91 g), respectively. No statistically significant differences between PTB and TPL groups in this measured factor were registered.

In vivo EPR analysis of the plasmatic •NO radicals

The EPR spectroscopy [31] is characterized by high sensitivity. It is easily scavenged and measures the levels of extremely unstable ROS and RNS free radicals, both in *in vitro* and *in vivo* systems - human blood and tissue. In the present study, the spin-trapping EPR method was used for investigation of the changes in the •NO radical levels during preterm birth and threatened preterm labor. The increase in the level of •NO radicals, measured in blood was characterized as a nitrosative stress (NS) factor. Typical •NO radical spectrum obtained in plasma gives rise to a characteristic 5-line with 1:2:3:2:1 intensity pattern.

The plasma •NO radical levels are illustrated in Figure 1.

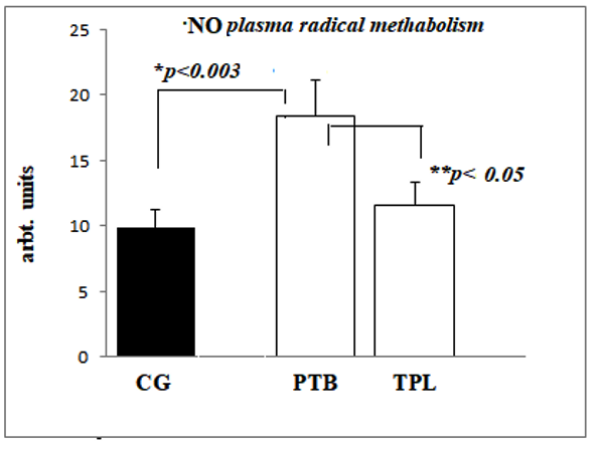

Figure 1. Plasma NO radical metabolism of pregnancy in CG group (n=75, birth in term), PTB group (n=73), (t-test) and TPL group (n=30), (t-test). *considered statistically significant PTB groups *vs* CG (p<0.003, t-test); **considered statistically significant PTB *vs* TPL groups (p<0.05).

The plasma •NO radical levels measured in the PTB group (18.34 ± 3.24 a.u., p<0.003, *t*-test) (Fig. 1) were significantly (two times) higher, compared to the pregnant in term (9.83 ± 2.11 a.u.). Our study was in support of others, showing that hypertensive disorders increased •NO radical levels, and NOx metabolites in patients with PTB increased and are associated with an increased risk of PTB or TPL-complicating pregnancy [18, 32, 33]. Increased endogenous metabolism of •NO radicals during preterm birth, a period of intense activity in pregnancy, could be associated with increased oxygen (O_2) intake, superoxide ($•O_2^-$) radicals synthesis, oxidative stress and increased free radicals generation [34, 35]. Nitric oxide radicals are powerful uterine smooth muscle relaxants that oxidize to NO metabolites containing NO_3^- and NO_2^- [33]. The NO metabolites are capable of relaxing the myometrium during pregnancy [35]. In addition, myometrial contractility (*pregnant, preterm or laboring*) could be enhanced by competing inhibitors of NO synthesis, such as NG-nitro-L-arginine and n-nitro-L-arginine methyl esters [35, 36]. In other studies, significantly increased NO metabolites production in PTB women [20, 32, 37] has been reported in inflammatory processes because of increased macrophage activity, activation of proinflammatory cytokines [4,27] and acidity.

Our results for measurement of the plasmatic •NO radical levels in the spontaneous TPL group (11.58 ± 1.39 a.u., p<0.002, *t*-test) (Fig. 2) were statistically significantly lower compared to the PTB group and almost comparable to the controls at term (9.83 ± 2.11 a.u.; p<0.05, *t*-test). These findings of a decreased •NO radical production in threatened preterm labor patients were in accordance with previous observations of Diejomaoha *et al*., and Ledingham [4, 34]. We suppose that decreased •NO radical production during threatened preterm labor could be explained with the decline of the •NO metabolism, possibly due to excessive oxidative/nitrosative stress. Probably, statistically significantly •NO decrease in TPL group, is recorded in processes that endeavored to convert plasmatic NO_2 to NO_3, to regulate oxidative/ nitrosative stress levels and redox - an imbalance in maternal-fetal placental blood flow [38]. Presumably, significantly reduced •NO levels in the women with threatened preterm labor stimulates uterine contraction, preterm ripening of the cervix and this is associated with a local, not systemic NO pathway activation [39]. The study of the levels of •NO metabolites in women with preterm birth and women with

symptoms of threatening preterm labor is forthcoming.

CONCLUSION

For the first time, nitric oxide (•NO) radical production during pregnancy complicated by preterm birth (PTB) and threatened preterm labor in Bulgarian women population was investigated in real time using the EPR spin-trapping method. It is important to emphasize that •NO radical production and oxidative/ nitrosative stress increases with advancing gestation during PTB and decrease in PTL groups. Based on previous studies, and on our results, we argue that •NO radicals could be a reliable marker for predicting PTB, TPL and for therapeutic purposes.

REFERENCES

1. R. L. Goldenberg, J. F. Culhane, J. D. Iams, R. Romero, *Lancet*, **371**, 75 (2008).
2. M. T. Parsons, W. N. Spellacy, Preterm labour; in: J. R. Scott, P. J. Di Saia, C. B. Hammond, W. N. Spellacy (eds.) Danforth's Obstetrics and Gynecology, Philadelphia, Lippincott, Williams & Wilkins, 1999, p. 256.
3. M. K. Mwaniki, M. Atieno, J. E. Lawn, C. R. Newton, *Lancet*, **379**, 445 (2012).
4. M. F. E. Diejomaoha, A. E. Omua, S. Taher, N. Al-Busiri, T. Fatinikun, S. Fernandes, S. Al-Othman, *Gynecol. Obstet. Invest*., **56**, 197 (2003).
5. R. Menon, *Front Immunol*., **5** (2014).
6. A. Cinkaya, H. L. Keskin, U. Buyukkagnici, T. Gungor, E. A. Keskin, A. F. Avsar, U. Bilge, *J. Obstetrics and Gynaecol*., **36** (2010).
7. K. Matsubara, T. Higaki, Y. Matsubara, A. Nawa, *Int. J. Mol. Sci*., 16 (2015).
8. J. Hodžić, S. Izetbegović, B. Muračević, R. Iriškić, H. Š. Jovic, *Med. Glas (Zenica)*, 14 (2017).
9. S. Chadha, V. Jain, I. Gupta, M. Khullar, *J. Obstet. Gynaecol. Res*., **33**, 710 (2007).
10. D. Mannaerts, E. Faes, J. Gielis, E. Van Craenenbroeck, P. Cos, M. Spaanderman, W. Gyselaers, J. Cornette, Y. Jacquemyn, *BMC Pregnancy and Childbirth*, **18** (2018).
11. J. L. Di Iulio, N. M. Gude, R. G. King, S. P. Brennecke, *Reprod. Fertil. Dev*., **7**, 1505 (1995).
12. R. K. Bansal, P. C. Goldsmith, H. Youping, C. J. Zaloudek, J. L. Ecker, R. K. Riemer, *J. Clin. Invest*., **99**, 2502 (1997).
13. E. Ekerhovd, B. Weidegård, M. Brännström, A. Norström, *Obstet. Gynecol.*, **93**, 987 (1999).
14. S. Rowlands, B. Trudinger, S. Visva-Lingam, *Aust. N.Z. J. Obstet. Gynecol*., **36**, 377 (1996).
15. B. Modzelewska, M. Jóźwik, M. Jóźwik, M. Tylicka, T. Kleszczewski, *Pharmacolog. Rep.*, **71** (5), 892 (2019).
16. K. Matsubara, T. Higaki, Y. Matsubara, A. Nawa, *Int. J. Mol. Sci*., **16** (2015).
17. S. Habib, A. Ali, *Indian J Clinical Biochem*., **26** (2011).
18. I. M. Koleva, Y. D. Karamalakova, G. D. Nikolova, V. G. Gadjeva. *Bulg. Chem.Commun.,* **51(**A), 41 (2019).
19. G. Luzi, G. Caserta, G. Iammarino, G. Clerici, G. C. Di Renz, *Ultrasound in Obstetrics and Gynecol.*, **14** (1999).
20. Z. Shahshahan, M. Nourbakhsh, F. E. Jazi, *J. Res. Med. Sci*., **21** (2016).
21. R. E. Behrman, A. S. Butler, in: Preterm Birth: Causes, Consequences, and Prevention, National Academic Press (US), 2007.
22. L. F. Wong, J. Wilkes, K. Korgenski, M. W. Varner, T. A. Manucka, *BJOG*, **123** (2016).
23. J. Shulhan, B. Dicken, L. Hartling, B. M. Larsen, *Advances in Nutrition (Bethesda, Md.)*, **8**, 80 (2017).
24. T. Yoshioka, N. Iwamoto, K. Ito, *J. Am. Soc. Nephrol.*, **71** (1996).
25. K. Yokoyama, K. Hashiba, H. Wakabayashi, K. Hashimoto, K. Satoh, T. Kurihara, N. Motohashi, H. Sakagami, *Anticancer Res*., **24** (2004).
26. H. Blencowe, A. C. Lee, S. Cousens, A. Bahalim, R. Narwal, N. Zhong, et al., *Pediatr. Res*., **74**, 17 (2013).
27. G. P. Stafford, J. L. Parker, E. Amabebe, J. Kistler, S. Reynolds, V. Stern, M. Paley, D. O. Anumba, *Frontiers in Physiol*., **8,** 615 (2017).
28. V. Berghella, M. Palacio, A. Ness, Z. Alfirevic, K. H. Nicolaides, G. Saccone, *Ultrasound in Obstetrics & Gynecol.*, **49,** 322 (2017).
29. U. von Mandach, D. Lauth, R. Huch, *J. Matern. Fetal Neonatal Med*., **13**, 22 (2003).
30. A. Boots, L. Sanchez-Ramos, D.M. Bowers, A.M. Kaunitz, J. Zamora, P. Schlattmann, *Am. J. Obstet. Gynecol.*, **210** (2014).
31. R. Zhou, Q. Xlong, Y. You, D. Qiu, K. Zhang, S. Liu. *Sichuan Da Xue Xue Bao Yi Xue Ban*, **34** (2003).
32. L. Nanetti, F. Raffaelli, A. Giulietti, G. Sforza, S. R. Giannubilo, A. Ciavattini, et al., *J. Matern. Fetal Neonatal Med*., **28** (2015).
33. D. M. Haas, D. M. Caldwell, P. Kirkpatrick, J. J. McIntosh, N. J. Welton, *BMJ*, **345** (2012).
34. M. A. Ledingham, *British J. Obstetrics and Gynecol*., **107**, 581 (2000).
35. S. R. Bartlett, P. R. Bennett, J. S. Campa, W. J. Dennes, D. M. Slater, G. E. Mann, L. Poston, R. Poston, *The Journ. Physiol*, **521(**3), 705 (1999).
36. J. E. Norman, *Phamacol. Ther.*, **70**, 91 (1996).
37. S. M. Sladek, R. R. Magness, K. P. Conrad, *Am. J. Physiol*., **272**, 441 (1997).
38. G. Ventolini, *OA Med. Hypothesis*, **1**, 2 (2013).
39. F. Facchinetti, P. Venturini, I. Blasi, L. Giannella, *BJOG: An Internat. J. Obstetrics & Gynaecol.*, **112**, 23 (2005).