Bulgarian Chemical Communications, Volume 51, Special Issue A (pp. 41 - 46) 2019

Nitric oxide radical production increase during normal pregnancy and pregnancy complicated by preterm labor in a Bulgarian women population

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Received February 25, 2019; Accepted April 17, 2019

The physiological normal pregnancy (NP) and pathophysiologic pregnancy, complicated by preterm labor (PTL), were associated with redox imbalances in reactive oxygen/nitrogen species (ROS/RNS) and with increased oxidative/ nitrosative stress damages.

The aim of the study was to investigate and compare oxidative stress processes and nitric oxide (•NO) radical production during normal pregnancy (NP) and in pregnancies complicated by preterm labor (PTL) in a Bulgarian women population. In the current study, 140 patients were included into 3 groups: 1) n=40 non-pregnant volunteers (control group, CG); 2) n=40 healthy normotensive pregnant women (NP); and 3) n=60 women with pregnancies complicated by preterm labor (PTL). The healthy NP and PTL groups were divided into 3 sub-groups by different gestational age. Age, social class, and gestational age were recorded for each group. By using for the first time Electron Paramagnetic Resonance (EPR) spin- trapping technique, real-time changes in •NO levels were investigated in blood isolated from non- pregnant, NP and PTL pregnant women. Plasmatic •NO levels were determined using the spin-adduct formation between Carboxy-Ptio.K and generated •NO radicals in real time. It is important to emphasize that •NO radical production and oxidative/nitrosative stress increases with advancing gestation during NP and PTL groups, compared to CG. Moreover, a positive correlation was found between the NP and PTL patients indicating ongoing pathological oxidative/nitrosative stress processes during pregnancy.

Key words: NP, PTL, EPR, pathogenesis

INTRODUCTION

The physiological normal pregnancy (NP) and pathophysiologic pregnancy, complicated by preterm labor (PTL), were documented to be associated with oxidative stress damages and redox imbalances in reactive oxygen/nitrogen species (ROS/RNS). Increased oxidative/nitrosative stress and destructive effects of free radical formation can be an important reason for pathological processes in pregnancy. Antioxidant/pro-oxidant imbalances have been reported in the maternal-fetal intrauterine compartments and inflammation, under conditions of endothelial dysfunction [1, 2].

Under normal circumstances, the placenta is a site of equilibrium between pro-oxidant/ antioxidant activities in pregnant woman, as the body's antioxidant defense mechanisms prevail and cope with free radical formations [3] and ongoing oxidative stress.

Moreover, in normotensive pregnancy, the placenta delivers oxygen and nutrients to the fetus and thus plays a key role in the fetus's cardiovascular health. Therefore, elevated endothelial nitric oxide (•NO) production contributes to the maintenance of vascular tonus to increase uterine blood flow of the pregnant woman [4, 5, 6]. Endothelial cells produce •NO from L-arginine as the substrate for endothelial NO synthase. As a highly active free radical, •NO has a short half-life time and acts as a vascular relaxation factor originating from the endothelium, with a potent inhibitory effect on smooth muscle contraction [7, 8].

Some researchers reported that elevated reactive nitrogen species (RNS/•NO radicals) can reduce heme-containing proteins. Also RNS are susceptible of forming a hemoglobin (Hb) stable complex that promotes erythrocyte oxidation [9]. Nitrosative stress is characterized by an increase in •NO/RNS levels. The increased •NO levels/ nitrosative stress induces an accelerated state of the immune system activity [10]. Free •NO radicals are a major factor in the feto-placentation process involved in the regulation of placental vascular reactivity, bed resistance, and can react with molecular oxygen and other ROS [10]. Pregnancy progression is accompanied by •NO formation by nitric oxide synthases (NOS, neuronal NOS, endothelial NOS, and inducible NOS) and its conversion into nitrite oxide metabolites, NO_3^-(nitrates) and NO_2^-(nitrites) [7, 10]. Under normal circumstances, •NO release into the placental circulation expands the placental vascular fetus distribution [11]. ROS overproduction leads to

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the •NO radicals formation which rapidly react with superoxide anion ($•O_2^-$) to form peroxynitrite ($ONOO^-$) anions which suppress the endothelial NOS activity [7, 10, 12]. NOS activity can change from increased •NO production to superoxide ($•O_2^-$) radicals [10, 11]. Furthermore, excessive generation of both ROS and RNS in living organisms can cause a decrease in antioxidant defenses and damage to normal cellular responses and cell growth in preterm damage [3, 10].

Preterm labor (PTL) is the main challenge in prenatal health care, leads to high rate of embryo mortality. PTL may result from early maternal risk factors as well as a feeling of tightness of the abdomen, constant low-dull back pain, pelvic or lower abdominal pressure, abdominal cramps, pre-tear of the placental membranes, endothelial dysfunction and systemic inflammation and chronically elevated ROS levels [13, 14]. Oxidative and immune disorders, ROS and RNS overproduction can influence the normal placental functions [13] and lead to increased intrusive stress and •NO radical levels [10]. Different clinical and experimental data elucidated that the •NO radical production and •NO metabolites in PTL patients was inhibited [15, 16], or increased [17-20] in the maternal body, which can easily be measured in plasma, urine, and vaginal secretions [19, 21].

The aim was to study and compare oxidative stress processes in blood of Bulgarian pregnant women with PTL symptoms, and to compare them to women with normal pregnancy and non-pregnant healthy individuals. For this reason, for the first time we studied the plasmatic •NO levels as real-time oxidative/ nitrosative stress parameters, using spin-trapping EPR spectroscopy.

EXPERIMENTAL

Patient and study design

The •NO radicals levels were measured in plasma samples from 140 women, age 17- 40 years old, including n=40 normotensive pregnant women, n=60 pregnant women complicated by PTL, and n=40 healthy non-pregnant volunteers (**Table 1**). Gestational age (GA) was determined by an experienced sonographer, using a transvaginal ultrasound (Aloka, Prosound alpha 6) when the patient's bladder was empty and the date of the last menstrual period was determined. The NP women were divided into 3 groups by gestational age; the first trimester (2 – 12 weeks, n= 13), the second trimester (12.3 – 24 weeks, n= 13), and late third trimester (31.3 – 40 weeks, n= 14). Pregnant women with PTL symptoms have been detected in late preterm labor 32.1-36.6 weeks (n=60). In the NP and PTL groups, participants had no history of type 1 or 2 diabetes, gestational diabetes, high blood pressure, eclampsia, incompetent cervix, uterus anomaly, hypertension (n=3), cardiovascular and infectious diseases (n=3), maternal complications, fetal anomaly or amniotic fluid, pre-eclampsia. PTL women using tocolytic (*corticosteroids*) drugs (n=2) were excluded from the experiment. A non-pregnant group (CG) included healthy (17- 36 years old) women (n= 40), without history of pregnancy or recent/ family disease. All NP and PTL patients (*provided informed written consent under ethical approval No4 2017/2018MF*) were hospitalized between June 2017 and July 2018 at the Clinic of “Obstetrics and Gynecology”, UMHAT "Prof. St. Kirkovich" in Stara Zagora, Bulgaria.

Body weight, blood pressure, and urine protein concentrations *(>170 mg for the last 24 hrs*) were evaluated in all groups. The term-preterm cases (PTL) were diagnosed by strict clinical criteria [22]: 1) Risk factors presence of preterm birth; 2) Cervical status determined by vaginal smear and trans-vaginal echography; 3) Uterine activity monitoring - anamnestic according to the data of the pregnant woman and by cardio-tocography; 4) Traceability for genital bleeding - anamnestic and vaginal obstruction.

Fresh peripheral blood (10 milliliters) of patients was collected directly by venous puncture from the ante-cubital region, in all participants, after fasting. Fasting (>17 hrs) blood samples were collected from 8.00 to 10.00 a.m. Blood samples, containing EDTA anticoagulant was collected into plastic tubes, and centrifuged at 4000 rpm for 10 min at 4°C. 1 ml of plasma samples was separated and stored at –20°C until further assay was done.

•NO radical levels were measured by reduction and spin-adduct formation between Carboxy-Ptio.K and generated radical using the methods of Yoshioka *et al*. [23] and Yokoyama *et al*. [24]. The EPR method was adapted and analyzed with EMX^{micro}, X-band spectrometer/ standard Resonator (Bruker, Germany) at 3505 G centerfield, 6.42 mW microwave power, 5 G modulation amplitude (20-23° C), 75 G sweep width, 2.5×10^2 gain, 40.96 ms time constant, 60.42 s sweep time, 1 scan per sample. The •NO radical was calculated by double integration of the corresponding EPR spectra (*arbitrary units*).

Statistical analysis

Spectral processing was performed using Bruker WIN-EPR and SimFonia software. Statistical analysis was performed using Statistica 8.0, Stasoft, Inc., one-way ANOVA, to determine significant difference among 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.

RESULTS AND DISCUSSION

Maternal adaptation to pregnancy is complex process in which various biological functions are activated intend to fetus protection. Pregnancy, complicated by preterm labor (PTL), is a common perinatal problem that leads to different social, neonatal, physical diseases and fetus mortality [1, 25]. The World Health Organization (WHO) reported, around 15 million premature babies are born in the world every year, and 1 million babies die due to preterm labor difficulties [26]. Elevated uterine contractility, cervical changes, hormonal increase and fetal membranes oxidative processes and uterine decidua, were reported during NP and PTL [1, 26]. Boots et al. demonstrated that nitric oxide and its metabolites produced by neuronal, endothelial, or inducible NOS were an informative PTL marker involved in cervical maturation [27]. Furthermore, indirect •NO generation occurs in the uterus and probably inhibits uterus contractility, i.e. •NO levels could be PTL predictors [28].

Clinical characteristics

Clinical characteristics of the patients included in the investigation are summarized in **Table 1**. We did not observe statistically significant differences in systolic/diastolic blood pressure, heart rate, and pregnancy parity in the three gestational NP groups. The statistically significant differences were observed between NP and PTL groups: for systolic blood pressure (p<0.001, t-test); for diastolic blood pressure (p<0.002, t-test); for maternal pulse pressure (p<0.002, t-test), and pregnancy parity (p<0.002, t-test). The statistically significant differences in age (p=0.035, t-test), body weight (p<0.035, t-test) and registered secondary diseases between NP and PTL groups were not recorded. The PTL group demonstrated statistically significant higher chronic hypertension values (p<0.0041, t-test) and pulse pressure (p<0.002, t-test) compared to NP groups. Statistically significant differences between NP, PTL groups and controls were not observed. There was a statistically significant difference between NP and PTL with regard to the two measured parameters, respectively (p<0.001, LSD).

Ex vivo EPR study the plasmatic •NO radical levels

In our study, the spin-trapping EPR method was used to investigate the changes in real time •NO levels and oxidative/nitrosative damage during normal pregnancy and in pregnancy, complicated by PTL. As a highly sensitive technique, EPR spectroscopy measures the extremely unstable ROS and RNS free radical structures *ex vivo*, such as in human blood and tissue [29]. Nitrosative stress is characterized by an increase in •NO radical levels measured in blood. A typical •NO radical spectrum obtained from plasma gives rise to a characteristic 5-line pattern with 1:2:3:2:1 intensity distribution (*EPR spectrum is not given*).

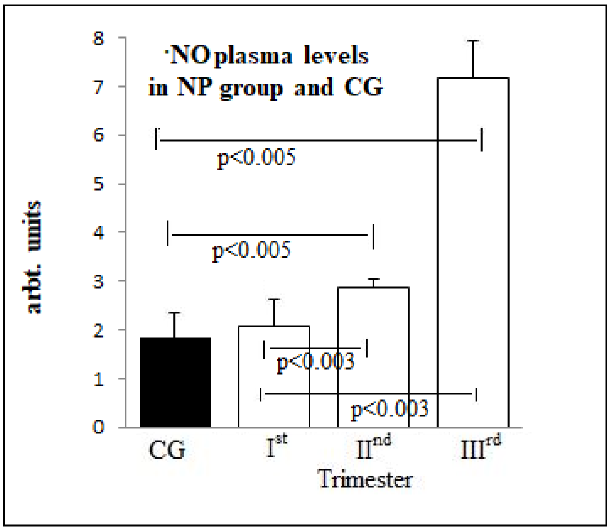

Figure 1. Plasma NO radical activity in different trimesters of pregnancy in NP groups (n=40) (t-test) and controls. There was statistically significant difference between IInd trimester (p<0.005, t- test) and the late IIIrd trimester (p<0.005, t-test), compared to CG; between three NP groups, p<0.03, t-test. Statistically no significant differences in plasma •NO levels between NP in the Ist trimester and CG (2.05 ± 0.8 *a.u*., vs 1.84 ± 0.07 *a.u*., p<0.003, t-test) were recorded

The plasmatic •NO radical levels in NP groups: •NO radicals values in the IInd trimester (2.88±0.5 *a.u*; vs 1.84 ± 0.07 *a.u.*, p<0.005, t- test) and the late IIIrd trimester (7.18±1.48 *a.u*; vs 1.84 ± 0.07 *a.u.*, p<0.005, t-test) were statistically significantly higher, compared to CG. We did not find significant differences in plasma •NO radicals levels between healthy NP in the Ist trimester and CG (2.05 ± 0.8 *a.u*., vs 1.84 ± 0.07 *a.u*., p<0.003, t-test) (**Fig 1**). There were significant differences among the three NP groups, p<0.03, t-test Statistically significant difference $p \leq 0.05$ [ANOVA?]. •NO radicals continued to increase throughout NP, and the maximal peak was at 34.6 gestational weeks. These levels were 5-6.2 times higher compared to the NP group I^{-st} trimester and non-pregnant volunteers.

Our results are in accordance with the investigations of Choi *et al*. [30] and Shaamash *et al*. [31] who established that the •NO levels increased in NP woman, especially after IInd trimester and maximum was registered in the IIIrd trimester of normal pregnancy. Several researchers have shown the elevated •NO levels and NOx metabolites in PTL subjects, and that serum nitric oxide levels in pre-eclamptic patients are increased in comparison to controls and NP [6, 19, 32-34].

Table 1. Clinical characteristics of the NP, PTL and CG groups. Results are expressed as the mean value ± SE (n=140). Data were analyzed by ANOVA with LSD post hoc testing for statistical difference between groups of two. Statistical significance was set at p<0.05. The statistically significant differences were observed between NP and PTL groups: for systolic blood pressure (p<0.001, t-test); for diastolic blood pressure (p<0.002, t-test); for maternal pulse pressure (p<0.002, t-test), and pregnancy parity (p<0.002, t-test).

Characteristics	**Control group (n= 40)**	**Normotensive pregnancy (n=40)**	**Pregnant women complicated by PTL (n=60)**	***p**
Age, years	29.5 ± 3.4	27.8 ± 3.4 Non	31.8 ± 2.6	<0.035
Smokers	Non	172 ± 9.4	Non	-
Height, cm	168 ± 11.4	22%	170 ± 0.8	≤0.01
Schooling	22%	11(25.3)	56%	≤0.061
Family history of diabetes	9 (21,08)		Non	≤0.002
Body mass index, kg/m^2	39.34 ± 7.11	37.5 ± 2.7	41.5 ± 0.17	0.53
Gestational age, weeks/ range	**NA**	35.5 ± 1.12	31.5 ± 1.02	-
Systolic blood pressure (SBP, mmHg)	126.2 ± 7.0	119.2 ± 11	136.2 ± 3.1	≤0.001
Diastolic blood pressure (DBP, mmHg)	76.8 ± 9.1	69.1± 6.0	80.1± 4.0	≤0.002
Mean arterial pressure (MAP, mmHg)	99.712 ± 9.1	93.51 ± 2.2	93.42 ± 1.8	≤0.3
Eclampsia	Non	Non	Non	-
pre-eclampsia	Non	Non	Non	-
incompetent cervix, uterus	Non	Non	Non	-
anomaly,	Non	Non	Non	-
cardiovascular diseases	Non	Non	(n=3)	-
infectious diseases	-	-	(n=3)	-
maternal complications, fetal	Non	Non	Non	-
anomaly	Non	Non	Non	-
amniotic fluid	Non	Non	Non	-
urine protein concentrations	Non	Non	Non	-
pregnancy parity				-
Pulse pressure	NA	40.4± 1.0	48.4± 2.0	
Chronic hypertension	64 ± 7	62.2 ± 8	78.3 ± 9	<0.002
	0.36 %	0.22%	0.12%	≤0.002^a
				≤0.041^a
Data presented as mean ± SD	**NA- not applicable**			**p*- comparison bet. NP and PTL groups**

^{a}p < 0.001 NP vs PTL, computed by LSD post hoc test.

The plasmatic •NO radical levels in PTL groups: The •NO radical levels measured in all PTL groups (**Fig. 2**) were statistically significant compare to the CG: for PTL Ist trimester (p<0.0001, LSD); for IInd trimester (p<0.003, LSD) and the late IIIrd trimester (12.19 ± 2.84 *a.u.* vs 1.84 ± 0.07 *a.u.*, p<0.003, LSD). A statistically significant difference in •NO levels was also observed in PTL IInd trimester and the PTL late IIIrd trimester, compared to PTL Ist trimester (p<0.001, t-test).

In late PTL group •NO levels were increased 9-10.7 times compared to non-pregnant CG (p<0.003, t-test). The •NO levels in NP group were significantly correlated with the PTL group (NP vs PTL r=0.56, p=0.004).

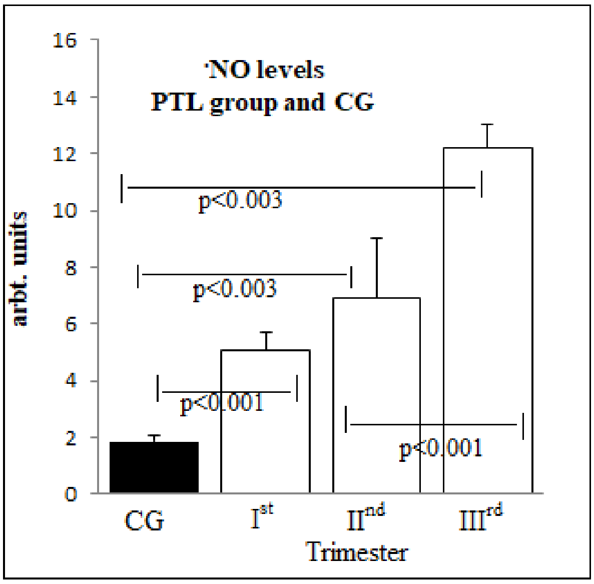

Figure 2. Plasmatic NO levels in different trimesters of PTL pregnancy (n=60); (t-test) and controls. The •NO radical levels measured in all PTL groups (**Fig. 2**) were statistically significant compare to the CG: for Ist trimester (p<0.0001, t-test); for IInd trimester (p<0.003, t-test) and the late IIIrd trimester (p<0.003, t-test). A statistically significant difference in •NO levels was also observed in IInd trimester and late IIIrd trimester vs. Ist trimester (p<0.001, t-test). The •NO levels in NP group were significantly correlated with the PTL group (NP vs PTL r=0.56, p=0.004).

The PTL group had extremely high •NO levels which could be explained by food intake, which contains exogenous NO_3^- (nitrates) and NO_2^- (nitrites) [8] and by NO-decreased placental nutrient transport. The study of Seligman et al. [33] reported that nitrates and nitric oxide (•NO) plasma levels increased during pregnancy, especially in the late gestation. In our study, all women diagnosed with late PTL and late third trimesters NP, hospitalized at the Clinic of "Obstetrics and Gynecology" was subjected to an identical food intake within five days.

Increased •NO levels can be attributed to the increased oxygen (O_2) intake and superoxide ($•O_2^-$) radical synthesis, performed in the mother's body during pregnancy. Nitric oxide radicals are powerful uterine smooth muscle relaxants [30]. In fact, at favorable conditions, •NO radicals, due to their short half-life, rapidly oxidized to metabolites, containing NO_3^- and NO_2^- [34]. Increased nitrosative stress implies an adverse effect on placental hemodynamics [20, 35-36], It is reasonable to assume that increased •NO synthesis is a consequence of uterine hypertrophy, which results in a smooth muscles relaxation and probable PTL complications [37].

In normal pregnancy, all processes in maternal body result in regular uterine contractions, shortening of the cervix, cervical canal enlargement and amniotic fluid bursting [1, 38]. In idiopathic premature labor these mechanisms are earlier activated, ancestral activity is spontaneously accelerated and finished with preterm child birth [38].

According to literature data, •NO radicals participates in genetic mechanisms for embryonic programming, placental development, fetal growth [39-40] and birth. Ventolini *et al*. pointed out that prostaglandins, corticotropin-relizing (CRH), progesterone, estrogens and oxytocin are hormones that promote birth commencement [38]. The normal term birth and preterm labor are associated with a "mother-fetus" redox-imbalance [1, 38]. Monitoring of •NO levels in PTL patients may be diagnostic in interventions that attempt to regulate oxidative/nitrosative stress damage due to maternal-fetal placental blood flow [39] and redox imbalance. Based on our findings and published studies, it is conceivable that increased •NO levels adversely affect the function of mitochondrial electron transport proteins [40-41], leading to an increase in placental oxidative [42] an nitrosative stress.

CONCLUSION

For the first time, using the EPR spin-trapping method, nitric oxide (•NO) radical production during normal pregnancy (NP) and in pregnancy complicated by preterm labor (PTL) in a Bulgarian women population was investigated in real time. Our study demonstrated that •NO radical production and oxidative/nitrosative stress increases with advancing gestation during NP and PTL groups.

Acknowledgments. *The study was supported by PhD programs of Medical Faculty, Trakia University, Bulgaria. Dr. Iliana Koleva- Kerkelia thanks the EPR Center for support of these investigations.*

Conflict of interests. *The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.*

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