Bulgarian Chemical Communications, Volume 56, Special Issue D1 (pp. 132-137)2024 DOI: 10.34049/bcc.56.D.S1P51

Effect of endogenic and exogenic oxidative stress changes during spontaneous preterm birth

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Received: November 3, 2023; Revised: April 11, 2024

This study aimed to assess the effect of endogenic and exogenic oxidative stress (OS) changes in the maternal response and establish the relationship to plasma non-infectious OS-associated inflammation in spontaneous preterm birth (SPTB), (in declared active labor with an intact amniotic sac and no recorded inflammation) beginning at $32–34^{+6}$ gestational weeks. Results were compared with women who gave birth at term (TB) in the active phase. To assess changes in OS, the reactive oxygen-nitrogen (RONS) species, as nitric oxide (•NO), superoxide ($•O_2^-$) radicals, albumin SH-malformation (5-MSL), and ROS peroxidation were investigated by electron paramagnetic resonance (X-band-EPR-Emxmicro spectrometer, Bruker) spectroscopy. The antioxidant enzymes (GSH, GPs1, and TAC) were evaluated by ELISA kits. The present study shows significant associations between the endogenic and exogenic non-infectious OS elevation due to the RONS up-regulation and the secondary OS generation associated with a lack of antioxidant protection in SPTB.

Keywords: inflammation, oxidative stress, birth, free radicals

INTRODUCTION

Spontaneous preterm birth (SPTB) is defined as a birth before <37 gestational weeks (GW), representing a significant contribution to perinatal morbidity and mortality in newborns [1-3]. Clinical studies confirmed that this group includes spontaneous births (mid-second trimester) and resembles miscarriages due to similarities with the main risk factors characteristic for SPTB: *increased uterus contractility, structural changes in the cervix, changes in the fetal membrane, decidual processes of the uterus and hormonal changes* [4]. SPTB onset results in a preterm infant with developmental complications, respiratory distress, pneumonia, bronchopulmonary dysplasia, neurological complications, arthritis, necrotizing enterocolitis, etc. [5-7]. The SPTB reasons are still not fully understood. The ascending bacterial infection of the amniotic membranes, amniotic fluid, placenta, umbilical cord, and the fetus is the main etiological cause of SPTB. Further, the fetus can independently modulate the exact moment of preterm/SPTB initiation, as a result of collapsed feto-maternal communication [7, 8]. Reduced local immunity in the vaginal cavity and cervical canal is thought to lead to intrauterine bacterial infection, an additional indicator of infection in the fetus, increasing fetal plaque, and a strong risk factor for spontaneous hemorrhage and SPTB activation [9]. SPTB can be considered as a „syndrome“ due to multiple pathological processes, such as inflammatory cytokines overexpression in the chorion, amnion, and trophoblasts [10]; high mobility proteins modulation, enhanced placental inflammatory responses and SPTB activation [11]. The stable vaginal microbiota during pregnancy at term (TB) is replaced by altered estrogen levels, carbohydrate structures, and glycogen induction in the vaginal epithelium, i.e. possible factors causing vaginal injuries and acute intra-amniotic infection in spontaneous birth. The only proven mechanism in SPTB cases is induced fetoplacental inflammation associated with earlier gestational age, damage to gestational tissues and maternal circulation; an expression of the defense mechanism after cellular stress and activation of an intense immune response [2, 3, 12].

In contrast, multiple clinical studies associated “non-infectious” OS, endogenic and exogenic antioxidant disturbances, and accumulated reactive oxygen-nitrogen species (RONS) in the intrauterine space, plasma, and fetal aging as additional/ secondary promotors in SPTB activation. Specifically, OS-induced placental RONS (i.e., superoxide ($•O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl (•OH), nitric oxide (•NO) radicals) cause a cascade of irreversible cellular dysfunctionality and premature cervical ripening [13, 14] by acute redox modifications. Tantengco *et al*., and Moore *et al*., [13, 14] comment on the OS/ RONS association in direct contribution to SPTB. More specifically, increased OS provokes modulation of proteins, lipids and DNA oxidation through the altered activity of ion channels in cell membranes. Additional SPTB prerequisite is RONS-

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induced permanent structural damage to carbonylated proteins and activation of collagen degradation in the amniotic membrane [15]. The interrelationship between OS and increased uterine contractility was established in lysine/arginine/proline glycoxidation; C-reactive protein (CRP) elevation in combination with specific pro-inflammatory and immune proteins degradation [16], the typical SPTB complications. Retrospectively, locally induced OS has been predicted to stimulate changes in immune and acute-phase-inflammatory proteins, prostaglandins, and matrix metalloproteinases leading to increased uterine contractility, subsequent intra-amniotic infection, and SPTB [16, 17]. Simultaneously, early fetoplacental development initiated a cascade of "non-infectious" OS accompanied by increased lipid peroxidation (MDA) and RONS, intracellular protein dynamics, leptin up-regulations, and inability of the pregnant mother to respond to the decrease in enzymatic/ non-enzymatic antioxidants [2, 3, 18]. The endo- and exogenous antioxidants incapacity toward protected cellular response against increased OS and RONS [2, 3] leads to non-infectious cellular atrophy due solely to OS-induced senescence, which in turn causes sterile inflammation, generating an immunocompromised intrauterine environment and SPTB induction.

To better understand endogenic and exogenic non-infectious OS changes in SPTB, for the first time, we investigated the following RONS species: nitric oxide (•NO), superoxide ($\bullet O_2^-$) radicals, 5-MSL disorders, ROS peroxidation and compared to antioxidant enzymes (GSH, GPs1, and TAC) in pregnant women from Southern Bulgaria, during $32–34^{+6}$ GW.

EXPERIMENTAL

The study was conducted in the period June 2019 - June 2022, on pregnant women from Southern Bulgaria; and approved by the Institutional Review Boards under all applicable state regulations at the Obstetrics and Gynecology Clinic, Stara Zagora. All 440 patients underwent a routine gynecological examination by the same medical specialist and provided written informed consent. *The exclusion criteria*: ruptured amniotic sac; systemic infection (respiratory, renal, and cardiovascular); nonsmokers; cesarean delivery; previous pregnancy; fetal anomaly; fetal mortality; organ transplant; chronic steroid use; multiple gestations; vaginal intercourse; congenital or chromosomal abnormalities; gestational/ pregestational diabetes; preeclampsia. *The inclusion criteria*: SPTB and TB women (18–42.5 maternal ages) were divided into two groups (Table 1).

Table 1. Obstetric and clinical characteristics of SPTB (n=220) and TB (n=220) participants. To define the difference between both groups the Student t-test was used. The results are presented as mean ± S.E. $p < 0.05$; b -values are means and c values are percentages; 1*NSVD, normal spontaneous vaginal delivery; **NA, not applicable.

Characteristics	SPTB (n=220) $32\text{-}34^{+6}$ g.w.	TB (n=220) $37\text{-}39^{+6}$ g.w.	p
Age (years)	31 ± 4.1	26.6 ± 5.8	0.042 [a, b]
Gestational age, weeks	32.6± 4.11	38.3 ± 1.5	≤0.004 [a, b]
Body mass index (BMI), kg/m^2	19.6± 0.84	39.4 ±2.9	≤0.051 [a, b]
Eclampsia/ pre-eclampsia	None	None	≤0.00
Infectious Diseases History	None	None	≤0.00
Parity	None	None	≤0.00
Gravidity	1	None	≤0.00
Abortion	None	None	-
previous SPTB	**NA	**NA	-
Family history of diabetes	0.00(0-5)	0.00(0-8)	-
Pelvic score	7-11 (7%)	9-13 (8%)	≤0.002 [a, c]
Bishop score (median)	12 (1–18)	-	≤0.00
Papiernik score (CRAP)	>10, 110 (75.2%)	-	≤0.0065 [a,c]
*NSVD	220	220	0.99 [a,]
Cesarean section	0	0	

(I) SPTB (n = 220); 32–34^{+6} GW; entered with declared active birth with preserved amniotic sac integrity and gave birth within 24 hours. The fetus gestational week was calculated from the date of the last regular menstrual period (LMP) and was confirmed by a transabdominal ultrasound AFI examination (Aloka pro sound alpha 6–IPF-1503).

(II) TB (n = 220), singleton pregnancy at 37^{+1}-39^{+6} GW; meeting the above-described criteria; with birth in its active phase at the hospitalization and preserved the amniotic sac integrity; no inflammation registered.

Both groups received supplemental amounts of folic acid (≤300 mg/day), vitamins (D, B6) (≤350 mg/day), and magnesium (≤360 mg/day), during pregnancy.

Venous blood samples (10 mL/plasma) taken from *v. cubitalis* were collected during birth and examined up to 2 h, after 3500 rpm centrifugation at 4°C/15 min. All patients were with 7-13 Pelvic score; and underwent ultrasound fetal biometry (Aloka Prosound alpha 6-IPF-1503) to assess the expected newborn weight, the placenta location, and the amniotic fluid amount.

3-Nitrotyrosine peroxidation (3-NT, mM, at 450 nm/620 nm, 20 nM detection limit) [19]; glutathione (GSH; nmol/ml), glutathione peroxidase (GPx1; ng/ml); total antioxidant capacity (TAC, 0.004 mM detection limit) were assessed by commercially available assay kits (BioVision Inc., CA, USA) according to the manufacturer's instructions.

The albumin oxidation was evaluated using spin-conjugation with 10 mM 5-MSL dissolved in saline (pH=7.4). The mixture was added to 70 mL of plasma, centrifuged at 4000 rpm/10 min, at 4°C; by EPR characteristics: 3505 G centerfield, 6.42 MW power, 5 G amplitude, 1-5 scans [20].

ROS production was examined by a mixture of 900 µl phenyl N-tert-butylnitrone (PBN) (50 mM) in dimethyl sulfoxide (DMSO) to 100 mL plasma, centrifuged at 4000 rpm (10 min), at 4°C [21].

•NO radicals generation: The mixture of 50 µM carboxy-PTIO.K solution, 30 µl plasma, 50 mM Tris (pH 7.5) and DMSO at a ratio 9:1, was centrifuged at 4000 rpm, 10 min, 4°C. The spin adduct between carboxy-PTIO and the •NO radicals was measured, by [22, 23]. $\bullet O_2^-$concentration: 30 µl plasma was activated by 30 µl (30 µM) 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) (1:1) on an ice bath, after 5 min incubation [24, 25].

All RONS formation was read in EPR-Emxmicro spectrometer (Bruker) triplicate, in arbitrary units with characteristics: 3505 G centerfield, 6.42 MW power, 5 G amplitude, 1-5 scans.

Statistical analysis. The clinical data were analyzed using Statistica (Version 10.0 software, StaSoft, Inc., USA), and performed using one-way ANOVA. Student's t-test or Mann-Whitney–U test was used to compare the baseline characteristics between groups. EPR spectral processing was performed using Win-EPR and Sim-Fonia software after consecutive replicates. The $p \leq 0.05$ was considered statistically significant. The primary analysis was performed on GraphPad Prism 9 including log-odds (log2) data of the study components and comparison with birth outcomes (SPTB *vs*. term birth). For the sensitivity of analyses two-tailed Student's t-test was used, and resulting p-values were adjusted by false discovery rates calculated using the Benjamini-Hochberg (BH) method with a threshold ≤ 0.1. Only a p-value ≤ 0.05 and absolute changes ≥ 1.5 were considered significant.

RESULTS AND DISCUSSION

Inflammation and OS are the primary and secondary pathophysiological initiators of changes in the gestational membrane structures and activation of myometrium contractions and cervical maturation, i.e. directly contribute to SPTB activation [2, 3, 16, 17]. The homeostatically - induced endogenous and exogenous OS and RONS activate redox-sensitive transcription factors that are responsible for "non-infectious" inflammation. Furthermore, behavioral risk factors increase RONS and OS, consuming the antioxidant enzymes. Induced "hidden" inflammation activates spontaneous birth [2, 3, 26, 27].

The obstetric and socio-clinical data are reported in Table 1. The average gestational age in SPTB was significantly lower vs. TB ($p \leq 0.004$), and 100% of women in both groups delivered naturally. We observed statistically significant differences between BMI ($p \leq 0.005$) and in the mean age of patients with premature uterine contractions ending in SPTB ($p \leq 0.042$), for the two groups, respectively. We also found a significant association between SPTB and TB in all three measured factors, respectively ($^a p < 0.002$; $^a p < 0.002$; $^a p < 0.005$).

The peroxynitrite ($ONOO^-$)-mediated tyrosine nitration has pathological significance in OS monitoring. The one-electron 3-NT oxidation is supported by oxidants such as •OH, •NO, alkyl, carbonyl ($\bullet CO_3^-$), lipid (LO•), peroxyl (LOO•) radicals and originates in two ways: 1) by $ONOO^-$ generation; and 2) by heme peroxidases, catalyzed processes and activities of myoglobin, hemoglobin, catalase, copper/zinc superoxide dismutase. Placental •NO and $\bullet O_2$ result in increased $ONOO^-$ formation due to reduced antioxidant activation [28, 29]. A higher 3-NT (Figure 1a) concentration was observed in the SPTB group (40.89 ± 30.22 nM *vs*. 61.4 ± 44.7 nM; $p < 0.02$, t-test; $p \leq 0.0021$).

The registered 3-NT-dependent OS was almost two-fould in the SPTB group with a positive correlation registered to •NO radicals. Increased protein nitrosation and the endogenous antioxidants inability to process generated •NO and $ONOO^-$ and to balance amino acid changes in the fetoplacental system in SPTB, is consistent with other studies [27, 30].

5-MSL conjugates (Patent: BG/U/2022/5487) readily to maternal plasma *via* its conjugation to weakened SH-albumin sites and has been used to assess oxidatively induced conformational changes in amyloid albumin aggregates leading to structural disruption [20] and likely SPTB induction. The 5-MSL albumin expression (Figure 1b), was statistically significantly 2.3-fold lower in the SPTB, compared to TB groups (3.97 ± 0.037 a.u. *vs*. 1.11 ± 0.027 a.u.; $p < 0.003$, t-test; $p \leq 0.0001$). Plasma albumin as transporter scavenging OS and RONS, promoted spontaneous birth [31, 32]. We observed a

three-fold decrease in albumin (>67%) and SH- disrupted albumin sites, e.g., peroxyl RS• / RSOO• overproduction, additional initiators of lipid peroxidation, and characteristic hypoalbuminemia, in SPTB women. In agreement with our results, a significant association was observed between reduced albumins during OS induction [33].

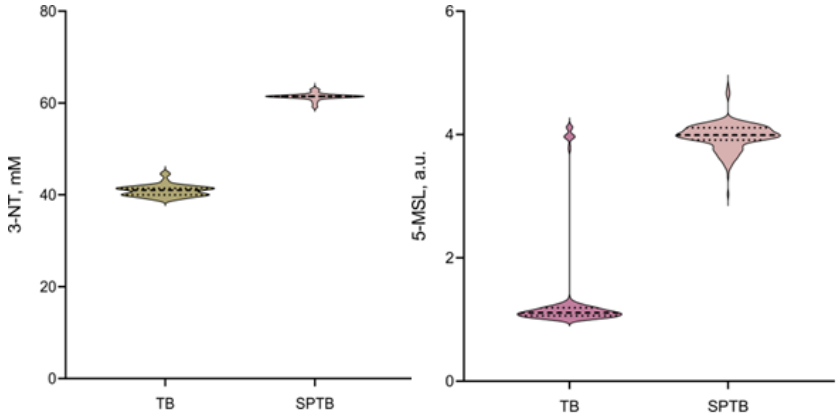

Figure 1. Violin plots of plasma 3-NT (a) and 5-MSL-conjugated albumin levels (b) as a modified protein peroxidation marker followed in patients with SPTB and TB. All data are presented as MFI. To define difference per groups the log2 and two-sided Student t-tests were used; a p-value ≤ 0.05 and changes ≥ 1.5 were considered significant.

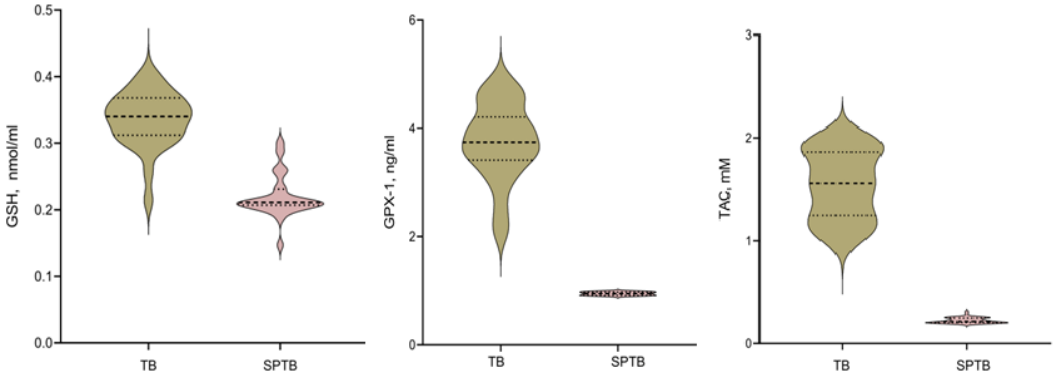

Figure 2. Violin plots of plasma antioxidant enzymes GSH (a), GPx1 (b), TAC (c), in SPTB and TB. To define which groups are different from each other the log2 and two-sided Student t-tests were used; a p-value ≤ 0.05 and changes ≥ 1.5 were considered significant.

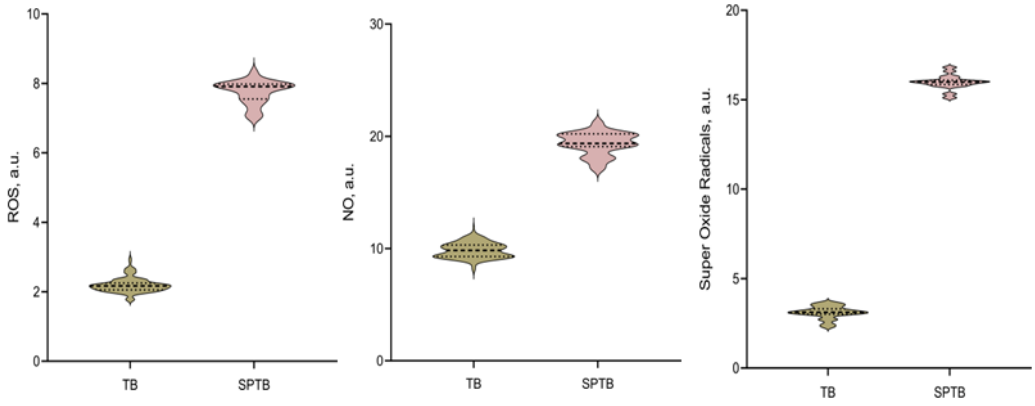

Figure 3. Violin plots of plasma ROS (a), •NO (b) and $•O_2^-$(c) concentrations in SPTB vs TB. To define which groups are different from each other the log2 and two-sided Student t-tests were used; a p-value ≤ 0.05 and changes ≥ 1.5 were considered significant.

Maternal OS competitively consumes endo-exogenous antioxidants, through RONS expression, altering placental and intrauterine redox status, and contributes to SPTB induction [2, 3]. The RONS-mediated collagen damage in the maternal gestational tissue increases uterine centrality and rupture of the chorio-amnionitis sac, which is a sure SPTB sign [34]. Plasmatic GSH (0.219±0.007 nmol/ml *vs*. 0.335±0.02 nmol/ml, p<0.003, t-test; p≤0.145), GPx1(0.94±0.07 ng/ml *vs*. 3.74±0.01 ng/ml, p<0.02, t-test; p≤ 0.0411) and TAC (0.227±0.07 mM *vs*. 1.54±0.06 mM, p<0.05, t-test; p≤ 0.0297) showed statistically significant decreased SPTB, 1.7-fold, 2.9-fold, 4.2-fold, respectively (Figure 2a, 2b, 2c). We further hypothesized that RONS activated lipid peroxidation, altered cellular function through tyrosine and glutathione nitration, in the SPTB cases. GPx1 promotes ROS detoxification of labile lipid hydroperoxides and utilizes reduced non-enzymatic GSH [2, 3, 34]. In two consecutive studies, GSH and GPx1 decreased expression in maternal blood was reported in the delivery of preterm infants (>37 GW), a likely consequence of increased OS. This is consistent with our study, where maternal GPx1 concentrations were dramatically decreased in SPTB, i.e. cellular redox-buffering antioxidant GSH may be associated with SPTB. Interestingly, sharp decreases in the non-enzymatic antioxidants are directly related to OS, reduced gestational weeks, and likely contribute to SPTB pathogenesis [35, 36]. Dysregulation in the endogenic-exogenic maternal antioxidants leads to intracellular TAC decrease [37] in SPTB.

With respect to OS mediation as a possible SPTB factor, the local formation of ROS, •NO and $\bullet O_2^-$ was monitored. The plasmatic ROS production was almost three-fold, statistically significantly increased in SPTB (7.75±0.2 *vs*. 2.19±0.4, p<0.001, t-test; p≤ 0.0001) *vs*. TB. Also, an endogenous metabolic 2.1-fold increase in plasmatic •NO, (19.34±3.51 *vs*. 9.83±2.11, p< 0.003, t-test; p≤ 0.0001); and a 3.9-fold increase in $\bullet O_2^-$ maternal circulation (15.9±2.49 *vs*. 3.11±0.41, p< 0.003, t-test; p≤ 0.0002) was recorded in SPTB *vs*. TB (Figure 3a, 3b, 3c). The 2.89-fold increase in ROS production found in late SPTB cases vs. TB is consistent with other studies [2, 3, 18, 19]. Notably, lipid peroxidation predominance plays a known role in cervical ripening <34 GW and the OS-directed immune response in spontaneous labor [38]. OS-induced late inflammation can be explained, at least in part, by the significant increase in the amplitudes of •NO and $\bullet O_2^-$ radicals and compared to their circulation in cellular endogenous enzymes and TAC, <34 GW. In addition, maternal antioxidants were unable to normalize metabolic changes and deactivate H_2O_2, i.e., OS enhances the non-immune response and SPTB activation. Specifically, dysregulated plasmatic •NO/ $\bullet O_2^-$ suggests the generation of additional OS in the uterine tissue and cervix associated with oxygen species and nitric oxide synthase (NOS) and spontaneous early/late preterm birth [3, 38, 39]. In addition, the inability of GSH, GRx1 and GSH-reductases to donate free –SH/thiols to detoxify •NO/$\bullet O_2^-$ into protein, lipid and amino acid catabolites leads to local placental OS [3, 38-40].

CONCLUSIONS

The present study has several advantages: First, the investigations contribute to building upon the assumption that OS is the causal pathway to spontaneous preterm birth, in pregnant women. Second, the significant RONS elevation increased proteins and glycation products by decreasing the specific endo/exogenous enzymes and TAC in the SPTB group. Third, plasma RONS accumulations disclose additional OS mechanisms in uterine tissue and cervix associated with increased lipid peroxidation, hypoalbuminemia, and NOS dysregulation elevating the SPTB chances. Fourth, the SPTB and TB group in the study were from the same population, and their baseline characteristics were basically similar, probably mediating spontaneous birth progression, at 32–34^{+6} GW. Fifth, importantly, this research considering OS as the important SPTB causes is conducted for the first time on Bulgarian women.

PATENTS

The patent resulting from the work reported in this manuscript is with the incoming number of the patent application: BG/U/2022/5487

Acknowledgement: *This research was funded by the postdoctoral project CM-No 206/07.04.2022, Project No 5/2023/ TrU and Ministry of Education and Science BG-RRP-2.004-0006 "Development of research and innovation at Trakia University in service of health and sustainable well-being".*

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