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Correlation between circulating biomarkers of oxidative stress of maternal and umbilical cord blood at birth

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Abstract
The objective of the work was to study the relationship between the oxidative state of the mother and the newborn at the moment of birth. We measured oxidative stress markers (carbonyl groups, lipid peroxides and total antioxidant capacity (TAC)) and found a good correlation between the oxidative state of the normal mother and the neonate, since a high mother oxidative stress corresponds to an even higher oxidative stress of the newborn in umbilical cord blood. We also found that smoking mothers and their newborns had a higher concentration of the carbonyl group, lipid peroxides and less TAC. Newborns from these mothers weighed significantly less than others at birth. These data suggest a need for interest in monitoring the oxidative state of mothers during the pregnancy period, especially taking into account that the oxidative level could be involved in later risks of metabolic diseases for both mother and newborn.

Keywords: *Oxidative stress biomarkers, newborns, carbonyl group, lipid peroxides, total antioxidant capacity, birth*

Introduction

The reduction-oxidation (redox) state constitutes a potential mechanism for the regulation of many metabolic processes through modulation of signaling pathways. Oxidative reactions are an essential part of several biological systems; however, they can also have toxic effects depending on a critical balance between the oxidative stimulus and the antioxidant defense mechanisms available [1]. This could be critical under some conditions, such as during the neonate period. Interestingly, normal pregnancy is a physiological condition where an increase of free radicals is produced, probably because of high energy demands of many bodily functions, with the increase in lipid peroxidation [2–4]. Moreover, in pregnancy complications, such as preeclampsia and diabetes, there is a significant increase in free radicals and, consequently, oxidative damage increases [5–7].

The process of childbirth is accompanied by an increase in oxidative aggression. The fetus goes from an intrauterine hypoxic environment with pO_2 of 20–25 mm Hg into an extrauterine environment with pO_2 of 100 mm Hg [8]. This sudden augmentation in alveolar oxygen concentration and arterial pO_2 after delivery increases the formation of reactive oxygen species (ROS) in lungs and other organs [9]. The input of free radicals depends not only on the ambient oxygen being respired, but also on its conversion to free radicals (e.g. by activated polymorphonuclear cells in inflammatory conditions, during resuscitation after hypoxia by damaged mitochondria or the activated xanthine oxidase enzyme). There are many studies showing that, at birth, the neonate presents

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an increase in markers of oxidative stress and a decrease in antioxidant defenses [10,11]. The oxidative aggression undergone by the neonate is counteracted by the maturation of effective antioxidant mechanisms such as the enzymatic systems (superoxide dismutase, catalase, glutathione peroxidase, etc.). In an experimental study, Frank et al. [12] determined that the increase in antioxidant mechanisms at the pulmonary level is produced at the end of gestation. This process is concurrent with the increase in the substrates derived from the increase in free radicals.

Increased production of free radicals is a feature of most neonatal diseases [13,14]. A large body of evidence has involved ROS formation in hypoxia. ROS generated during hypoxia was demonstrated in fetal guinea pig brain [15] preterm hypoxic babies [16] and in perinatal brain damage [17]. The hypoxia of newborn infants increases the substrate for xanthine oxidase reaction leading to increased ROS generation [18]. These facts point out the importance of protecting the fetus before the birth process.

In order to design intervention strategies to counteract oxidative stress of the fetus, we studied the relationship between the oxidative state of the mother and the fetus at the moment of birth in order to know if the oxidative state of the former influences the oxidative state of the latter. Therefore, we studied the oxidative state of the mother at the beginning of delivery and the oxidative state of the neonate by determining the oxidative parameters of the umbilical cord blood. We found that a high oxidative state of the mother corresponded to a high oxidative state of the newborn in the umbilical cord blood. Taking into account the low level of resistance of a newborn against oxidative stress, it would be interesting to study whether modulation of the mother's oxidative stress could prevent that same level of stress for the child.

Materials and methods

Chemicals

Xylenol orange, butylated hydroxytoluene, sulfuric acid, ammonium ferrous sulfate, sodium dodecyl sulfate, 2,4-dinitrophenylhydrazine (2,4-DNPH), trifluoroacetic acid, trichloroacetic acid, guanidine hydrochloride, 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in the crystallized diammonium salt form, horseradish peroxidase type VI-A, hydrogen peroxide (30%, v/v) were obtained from Sigma (St Louis, MO, USA). All reagents were of analytical grade.

Subjects and blood sample collection

The study protocol was approved by the Ethics Committee for Clinical Research of the Puerta del Mar Hospital. Informed consent, described herein, was obtained from all the women. Subjects were selected from women preparing to deliver at the Obstetric and Gynecology Department of this hospital. The eligibility criteria excluded abnormal pregnancies such as those women with toxaemia, hypertension, thyroid disease, bronchial asthma, active hepatitis and chronic renal failure, as well as heart failure, multiple pregnancies, and those with medical complications including autoimmune disorders, diabetes mellitus, inflammatory conditions and preeclampsia. A questionnaire given to the women included questions about smoking cigarettes before and during the pregnancy, dietary patterns and employment histories. Age, height, weight, date of the last menstrual period, medical history, and reproductive history were obtained from the subject's medical records. Peripheral venous blood samples (10 ml) were taken from fasting subjects from antecubital veins during the antepartum period. Five millilitre of umbilical blood was drawn from a double-clamped segment of umbilical cord before the first breath of the neonate was drawn. The collected blood samples were left at room temperature for 2 h. After clotting, the blood was centrifuged at 2000*g*, and the separated serum was divided into four portions and placed in eppendorf test tubes. The material thus obtained was placed in a nitrogen atmosphere and stored at -80°C. Each assay was done from newly thawed portions of serum.

Determination of lipid hydroperoxide by oxidation of Fe^{2+} in the presence of xylenol orange (FOX reagent)

Lipid hydroperoxides were determined as previously described [19]. FOX reagent was prepared with 100 μmol/l xylenol orange, 4 mM butylated hydroxytoluene, 25 mM sulfuric acid and 250 μM ammonium ferrous sulfate. The samples were mixed with 900 μl of FOX reagent and 55 μl of methanol. After mixing, the samples were incubated at room temperature for 30 min. The vials were centrifuged at 2400*g* for 10 min. Absorbance of the supernatant was measured at 560 nm ($\varepsilon = 4.3 \times 10^4\,M^{-1}\,cm^{-1}$).

Spectrofotometric DNPH assay for carbonyl content determination

Carbonyl groups were determined as described by Levine et al. [20]. Briefly, samples of serum extracts were treated with 24% sodium dodecyl sulfate and boiled for 5 min. A solution of 10 mM 2,4-dinitrophenylhydrazine (2,4-DNPH) in 10% trifluoroacetic acid was added to the samples (1:1 v/v). Proteins were precipitated with cold trichloroacetic acid (15%, final concentration). Protein pellets were washed three times with 1 ml portions of ethanol/ethyl acetate (1:1, v/v) to remove any free 2,4-DNPH. Samples were resuspended in 6 M guanidine hydrochloride in 50% formic acid overnight at room temperature.

Carbonyl content was determined from the absorbance at 366 nm using a molar absorption coefficient of 22,000 $M^{-1}\,cm^{-1}$.

Measurement of total antioxidant capacity (TAC)

The TAC assay was determined using the ABTS method described by Villaño et al. 2005 [21]. Pre-oxidation of ABTS was performed in the presence of H_2O_2 and peroxidase. Once $ABTS^{\cdot+}$ was formed, the reaction was started by adding an aliquot of the sample. Absorbance at 414 nm was measured. Standard Trolox solutions were also evaluated against the radical.

Protein determination

Protein content of the samples was measured by the method of Lowry using BSA as the standard [22].

Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's test were used to compare the results. The index risk was calculated using the SPSS statistical software package (SPSS 12 Inc., Chicago, USA).

Results

Stress biomarkers of mother and newborn

Descriptions of clinical characteristics of the group studied are described in Table I. Table II shows information on maternal and neonatal stress biomarkers, which provide information on the general oxidative stress status. Newborn lipid peroxide serum (LP) was significantly higher compared to maternal levels (0.915 ± 0.096 vs 0.558 ± 0.050 nmol/mg protein, $p = 0.0013$). Also, the carbonyl group serum (CO) was significantly higher in the newborn compared to the mother (3.68 ± 0.349 vs 2.66 ± 0.279 nmol/mg protein, $p = 0.043$). Levels of total antioxidant capacity (TAC) were found to be significantly lower in newborns than in their mothers (0.57 ± 0.021 vs 0.70 ± 0.025 mmol Trolox equivalent/l, $p = 0.0007$).

Table I. Description of the clinical characteristics of study groups.

	Mean ± SEM
Mother	
Maternal age (years)	28.22 ± 0.98
Glucose (g/dl)	80.70 ± 3.35
Urea (mg/dl)	21.72 ± 1.35
Creatinine (mg/dl)	0.620 ± 0.03
Hemoglobin (g/dl)	12.72 ± 0.22
Platelet × 10^3 (μl)	242.5 ± 13.6
Hematocrits (%)	36.45 ± 0.71
Mean corpuscular volume (fl)	85.33 ± 0.34
Mean corpuscular hemoglobin (pg/cell)	30.74 ± 0.46
Leucocytes × 10^3 (μl)	11.42 ± 0.75
Sodium (mmol/l)	135.6 ± 0.55
Potassium (mmol/l)	3.880 ± 0.10
Fibrinogen (mg/dl)	505.7 ± 37.9
Newborn	
Gestational age (wk)	39.00 ± 0.36
Birth weight (g)	3113.1 ± 118

Table II. LP, CO and TAC in serum of mothers and umbilical cord blood of newborn.

	Mothers	Newborn
LP	0.558 ± 0.050	0.915 ± 0.096†
	(0.81 ± 0.084)	(1.20 ± 0.15⋆)
CO	2.660 ± 0.279	3.680 ± 0.349⋆
	(10.7 ± 1.010)	(16.0 ± 2.30⋆)
TAC	0.700 ± 0.025	0.570 ± 0.021‡
	(0.53 ± 0.014)	(0.46 ± 0.02⋆)

LP, CO and TAC were measured as described in materials and methods. Number of samples = 39. Each sample was analysed three times. LP and CO are expressed as nmoles/mg protein and TAC in mmol Trolox equivalent/1. Numbers in parenthesis show LP, CO as uM and TAC as mM. ⋆$p < 0.05$. †$p < 0.005$. ‡$p < 0.0005$ (multifactor ANOVA followed by Tukey's test).

Correlations between stress biomarker

Table III shows the correlation of oxidative stress biomarkers. A significant positive correlation was found between LP and CO in the mother ($r = 0.70, p < 0.0001$) and newborn ($r = 0.46$, $p < 0.05$). Also, LP and TAC were found to correlate negatively in the mother ($r = 0.36$, $p < 0.05$) and newborn ($r = 0.50$, $p < 0.005$), and there was not a statistically significant relationship between CO and TAC in mother and newborn.

The correlation of oxidative stress biomarkers between mother and newborn (Table IV) shows a positive correlation between the levels of mother LP vs newborn LP and CO ($r = 0.675$, $p < 0.0001$ and $r = 0.688, p < 0.0001$, respectively) and mother CO vs newborn LP and CO ($r = 0.366, p < 0.05$ and $r = 0.87$, $p < 0.0001$, respectively), and mother LP with newborn TAC correlated negatively ($r = 0.348$, $p < 0.05$). The other correlations were not statistically significant.

Levels of LP, CO and TAC of smoking and not-smoking mothers

In Table V averages were compared using one-way analysis of variance (ANOVA, followed by Tukey's test).

Table III. Correlation between LP, CO and TAC.

	LP vs CO	LP vs TAC	CO vs TAC
Mothers	0.70 (0.0001)	−0.36 (0.0312)	−0.08 (0.6200)
Newborns	0.46 (0.0460)	−0.50 (0.0021)	−0.08 (0.6290)

The correlations were performed using the data shown in Table I. *P* values are in parentheses.

Table IV. Correlation between LP, CO and TAC of mothers and newborn.

	Mother		
Newborn	LP	CO	TAC
LP	0.675 (0.0001)	0.366 (0.0300)	−0.330 (0.0513)
CO	0.688 (0.0001)	0.870 (0.0001)	−0.090 (0.5700)
TAC	−0.348 (0.0420)	−0.072 (0.6790)	0.2430 (0.1590)

The correlations were performed using the data shown in Table I. *P* values are in parentheses.

Statistical analysis produced a significant difference in the mean between smoking and non-smoking mothers in all oxidative stress biomarkers. A significant difference was also found between newborns of smoker's and non-smoker's (LP: $p < 0.005$, CO: $p < 0.05$ and TAC: $p < 0.005$). In addition, newborn weight was significantly lower in newborns of smoking mother ($p < 0.05$).

Smoking mothers were at a higher risk of increased oxidative stress with possible damage to lipids (14.3-fold), proteins (5.45-fold) and a decrease of antioxidant capacity (6.87-fold), compared with non-smoking mothers. Also, newborns of smoking mothers had higher risk of oxidative stress biomarkers for damage to lipid (57-fold), protein (78-fold) and a decreased antioxidant capacity (2.44-fold). These index risks were determined using SPSS 12 statistical software package.

Levels of oxidative stress biomarkers in birth types, admission motive to enter hospital and the use of oxytocin

Table VI shows that the level of oxidative stress biomarkers in mothers who go through different types of delivery and their newborns was not statistically significant. Only the mothers admitted in hospital with planned labor have higher levels of LP (0.458 ± 0.015, $p < 0.05$) (Table VI). In general, there was not a statistically significant relationship between induced birth and oxytocin and non-oxytocin (Table VII).

Discussion

We studied the oxidative state of the mother immediately before the delivery process and found, as expected, slightly higher levels of the different blood oxidative stress biomarkers, LP and CO along with a decrease of TAC. These are in agreement with the finding that oxidative stress increases during pregnancy [7,23,24]. This oxidative stress can be similar or worse during the 2nd and 3rd trimester of the pregnancy period, as has been pointed out by Little and Gladen, [7]. The increase in oxidative stress is a physiological change probably a result of the increase in free radicals produced by the placenta. During pregnancy, lipid peroxidation is induced in the human placenta [25, 26] being higher there than the concentration found in the blood [27]. Lipid peroxide, originating from both the trophoblasts and the villous core compartment [28], is secreted into the maternal blood as additional peroxidation cascades are initiated. This is corroborated since the rate of lipid peroxidation in the placenta has been reported as being abnormally high in preeclampsia [29,30]. The lipid peroxides returned to baseline values in the postpartum period [23].

These biomarkers of oxidative stress have special significant to fetus development, having been related to intrauterine growth retardation during pregnancy [31–34]. We also studied oxidative stress of the fetus immediately after birth, measuring the stress biomarkers in the umbilical cord blood. We found that the fetus had more oxidative stress than the mother. The umbilical cord blood had more concentration of LP and CO along with lower TAC than that in the mother's blood. Taking into account the correlation studied, the LP could be a more representative parameter to study oxidative stress in fetus blood than CO. We found a good correlation between LP vs CO in the mother, but not in the fetus. Other authors have also described that in the fetus, concentration of CO did not increase similar to other oxidative parameters [35]. The good correlation between mother and fetus in both parameters, LP and CO, suggested a relationship between the oxidative

Table V. LP, CO and TAC in serum of smoking and not-smoking mothers and umbilical cord blood of newborn.

	Mothers		Newborn	
	Smoker	Non-smoker	Smoking mother	Non-smoking mother
LP	0.670 ± 0.064 (2.03 ± 0.64)	0.330 ± 0.035† (0.82 ± 0.12†)	1.160 ± 0.300 (2.70 ± 0.70)	0.530 ± 0.032† (1.02 ± 0.16⋆)
CO	3.160 ± 0.380 (26.5 ± 7.50)	1.660 ± 0.230⋆ (11.0 ± 1.80†)	4.070 ± 0.430 (32.5 ± 8.60)	2.600 ± 0.460⋆ (12.6 ± 2.80†)
TAC	0.650 ± 0.026 (0.51 ± 0.018)	0.800 ± 0.045⋆ (0.57 ± 0.009‡)	0.530 ± 0.024 (0.44 ± 0.14)	0.670 ± 0.034† (0.53 ± 0.02†)
Newborn weight (g)			2835.0 ± 233	3431.1 ± 131⋆

LP, CO and TAC were measured as described in Materials and Methods. Number of samples were 28 and 11, respectively. Results are means ± SEM. LP and CO are expressed as nmoles/mg protein and TAC in mmol Trolox equivalent/I. Numbers in parenthesis show LP, CO as uM and TAC as mM. ⋆$p < 0.05$. †$p < 0.005$. ‡ $p < 0.0005$ (multifactor ANOVA followed by Tukey's test).

Table VI. LP, CO and TAC levels in different birth types* and admission motive to enter hospital†.

	Mothers			Newborn		
	Vaginal delivery	Schedule cesarean delivery	Emergency cesarean delivery	Vaginal delivery	Schedule cesarean delivery	Emergency cesarean delivery
LP	0.417 ± 0.053	0.512 ± 0.118	0.605 ± 0.095	0.738 ± 0.152	0.734 ± 0.114	1.179 ± 0.361
CO	1.650 ± 0.200	2.170 ± 0.750	2.250 ± 0.500	2.650 ± 0.430	3.720 ± 1.100	3.430 ± 0.690
TAC	0.660 ± 0.050	0.760 ± 0.040	0.780 ± 0.060	0.600 ± 0.045	0.530 ± 0.035	0.490 ± 0.062
	Rupture of membranes	Latent phase of labor	Cervical dilatation	Rupture of membranes	Latent phase of labor	Cervical dilatation
LP	0.296 ± 0.058	0.458 ± 0.015*	0.437 ± 0.073	0.505 ± 0.050	0.630 ± 0.057	0.916 ± 0.354
CO	2.060 ± 0.410	1.290 ± 0.280	1.820 ± 0.270	3.550 ± 0.760	1.760 ± 0.400	1.880 ± 0.060
TAC	0.840 ± 0.042	0.550 ± 0.031	0.680 ± 0.086	0.692 ± 0.040	0.580 ± 0.128	0.552 ± 0.060

LP, CO and TAC were measured as described in materials and methods. *Number of samples were between 5–29. †Number of samples were between 5 to 10. Results are means ± SEM. LP and CO are expressed as nmoles/mg protein and TAC in mmol Trolox equivalent/1.

Table VII. Effect of oxytoxin on the levels of LP, CO and TAC.

	Mothers		Newborn	
	non-oxytocin	oxytocin	non-oxytocin	oxytocin
LP	0.351 ± 0.034	0.446 ± 0.077	0.600 ± 0.068	0.682 ± 0.154
CO	1.810 ± 0.240	1.800 ± 0.240	3.170 ± 0.419	2. 100 ± 0.479
TAC	0.750 ± 0.047	0.788 ± 0.065	0.600 ± 0.039	0.530 ± 0.042

LP, CO and TAC were measured as described in materials and methods. Number of samples were 32 and 7, respectively. Results are means ± SEM. LP and CO are expressed as nmoles/mg protein and TAC in mmol Trolox equivalent/1.

stress between them. We did not find any differences between distinct kinds of delivery: eutopic or cesarean. However, other authors have found some differences. Our results show only a slight increase in the some parameter when delivery was produced by an unplanned cesarean. This could happen because in these cases the labor process is longer before cesarean takes place. However, we did not found any effect by delivery time.

These data point to the importance of measuring the oxidative stress status of the mother in order to detect severe oxidative stress in the fetus during pregnancy. The two oxidative stresses are related, that of the fetus being the highest. It is accepted that oxygen free radicals play a role in normal cell growth [35–37], but also in many neonatal complications [38]. Despite using only healthy pregnant women for this study, the questionnaire was aimed at having the women available to study the influence of their habits on the redox state of both mother and child. Smoking had the strongest influence in our study, corroborating other studies. The mothers who smoke, even if they did not smoke during pregnancy, have a higher oxidative stress parameter. Thus, these mothers have a high concentration of LP, CO and a decreased TAC. Smoking mothers have 14.3-fold more possibility of having oxidative stress in their lipids. Moreover, the fetus of a smoking mother also has more oxidized lipids. In these cases the risk of oxidative increase rises to 57- and 78-fold for LP and CO, respectively. This is an important result since increase of the oxidative state in the mother has been shown, in this case produced by smoking, to produce at least a similar increase in the fetus. It is also important to remember that the newborn from these mothers have a significantly lower weight, which is in agreement with other studies [39]. Considering the results, it is possible to speculate that smoking increases the oxidative stress of the mother and this would interfere with normal fetus growth, the molecular mechanism is not known.

In conclusion, these data, corroborate others' work, and point out the importance of the mother's oxidative state during pregnancy, which has a significant influence on fetus development. Moreover, the increase in oxidative state of the mother could be produced by endogenous factors such as hypertension, diabetes, etc. and by exogenous factor such as smoking or toxins. In all these cases, the study of oxidative stress during pregnancy could represent an efficient way of managing risk for the fetus. We must also point out that there is strong evidence that developmental factors, one of which could be the oxidative state of the fetus during pregnancy, contribute to a later risk of metabolic diseases (i.e. heart disease) as well as having a broader impact on osteoporosis and an unfavorable neurological status [40–43].

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