

Antenatal maternal corticosteroid administration and markers of oxidative stress and inflammation in umbilical cord blood from very low birth weight preterm newborn infants

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Abstract

Objective: To investigate the association between antenatal maternal corticosteroid administration and blood levels of reactive oxygen intermediates (ROI), reduced glutathione (GR) and interleukin-6 (IL-6) in preterm, very low birth weight infants.

Methods: This was a cohort study in which cord blood samples were used for the following tests: baseline and stimulated granulocyte ROI were measured by flow cytometry; GR was assayed by spectrophotometry; and IL-6 by enzyme-linked immunosorbent assay. Two different comparative analyses of antenatal corticosteroid (betamethasone) were conducted: the first compared administration against no administration and the second compared mothers who received the complete cycle with those given only a partial antenatal corticosteroid cycle. Maternal and neonatal variables were analyzed in order to compare groups. Categorical variables were compared using the chi-square or Fischer tests, and blood marker test results were compared using the Mann-Whitney test.

Results: The different corticoid therapy groups were similar in terms of all of the maternal and neonatal variables with the exception of vaginal delivery, which was significantly associated with not receiving antenatal corticosteroid. The results for ROI, GR and IL-6 did not differ when the comparison was based on simple presence or absence of administration of the steroid. However, when the complete cycle was compared against incomplete administration, median ROI and IL-6 were lower among those given the complete cycle.

Conclusion: Administration of the complete cycle of betamethasone to the mother had a suppressive effect on baseline ROI and IL-6 production in very low birth weight preterm newborn infants.

J Pediatr (Rio J). 2012;88(1):61-6: Newborn, oxidative stress, reactive oxygen species, interleukin-6, glutathione.

Introduction

Antenatal corticosteroid treatment is recommended for expectant mothers at risk of premature labor before 34 weeks' gestation and is effective for accelerating maturation of the fetal lungs.¹ Systematic reviews have demonstrated that it is associated with a reduction in global neonatal mortality and reduced risk of respiratory distress syndrome, of necrotizing enterocolitis and of periventricular and

intraventricular hemorrhage. Furthermore, administration of this drug does not increase maternal risk of chorioamnionitis or puerperal sepsis.^{2,3}

The steroids exert this protective function in the fetus/newborn (NB) through a variety of mechanisms, both anatomic and related to influence over enzymatic systems. One of the drug's effects impacts free radical production.

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Newborns, especially preterm NB, are highly susceptible to tissue and organ damage from free radicals, in particular from oxygen-derived radicals, and superoxide anions are the most important type of intermediate oxygen radicals reactive oxygen intermediates (ROI), produced in response to hypoxic-ischemic or inflammatory stimuli.^{4,5} Furthermore, gestation is itself a physiological state in which metabolic demands and tissue oxygen requirements are increased and, in the event that gestational abnormalities occur, there may be oxidative imbalances with excess free radicals that can damage the fetus, which already has a deficient antioxidant defense system.⁶ With relation to this aspect, it is important to point out that production of the principal intracellular antioxidant component, glutathione, only peaks at the end of gestation.⁷

Several experimental studies have demonstrated the action of corticosteroids on production of reactive oxygen species. Dandona et al.⁸ demonstrated that, in adults, a single injection of hydrocortisone was capable of causing a significant reduction in the production of free radicals derived from O₂, reaching a low point between 2 and 8 hours after administration and returning to normal after 24 hours.

A study using preterm newborn lambs that had been given betamethasone during the antenatal period demonstrated that this steroid provoked a reduction in production of hydrogen peroxide and interleukin-6 (IL-6) and diminished phagocytosis capacity. These effects were time dependent and were no longer observed 7 days after administration.⁹

During the last twenty years it has also been demonstrated that there is a close relationship between mediators of oxidative stress and biochemical markers of inflammation, such as the interleukins, with the observation of a reciprocal effect of induction of synthesis of free radicals and inflammatory mediators.^{5,10}

Nevertheless, the effect of steroids administered to the mother on fetal production of ROI by polymorphonuclear leukocytes has not yet been studied in human beings. Improved understanding of the pathophysiologic mechanisms involved in the effects of antenatal corticosteroid treatment is important for defining potential targets for efforts to reduce or even prevent the harmful consequences of free radical activity.¹¹

Therefore, the objective of this study was to investigate the association between antenatal administration of corticosteroid to the mother and blood ROI, reduced glutathione (GR) and IL-6 concentrations in preterm, very low birth weight NB.

Methodology

This was a cohort study that recruited all preterm NB born live with birth weight of less than 1,500 g at a tertiary

neonatal care center between May of 2009 and October of 2010. There were no exclusion criteria.

This is a secondary study using the results of blood tests originally performed for a study of the association between periventricular and intraventricular hemorrhage and free radical production and intrauterine inflammation. The original sample size was 125 NB, calculated to obtain a statistical power of 80%. Therefore, for the present study test power was recalculated to ensure that there were enough NB to test the chosen outcome.

Cord blood was taken by umbilical puncture at the time of delivery, after the infant had been born and before placental expulsion, providing mixed, arterial and venous samples. The material was put into three tubes, two containing anticoagulant and the third empty. The samples were immediately sent to a specialized laboratory in the institution for prompt processing. Serum for the IL-6 assay was frozen at -80 °C until the test was performed with Enzyme Linked Immunosorbent Assay (ELISA, Duo Set, R & D Systems, United States). Results were expressed in pg/mL. Reduced glutathione concentration in erythrocytes was determined using the Beutler et al. method,¹² as modified by Penna,¹³ and results were expressed as mg% with relation to hematocrit. The granulocyte ROI concentration was determined by flow cytometry of whole blood samples that had been collected into tubes containing ethylenediaminetetraacetic acid EDTA.¹⁴ For each patient, ROI liberation by polymorphonuclear cells was studied in two different ways: spontaneous (or baseline), with leukocytes incubated only in Hanks solution, and stimulated, with leukocytes incubated in phorbol-myristate acetate. The results were expressed as nMol of superoxide/10⁶ cells/incubation time.

Characteristics of the mothers and of their NB and biochemical assay results were all grouped by maternal corticosteroid usage.

Antenatal corticosteroid therapy (ACT) was defined as administration of systemic corticosteroid (betamethasone) to the mother during gestation with the objective of accelerating fetal maturation. The first analysis compared mothers who had been given the drug with those who hadn't, irrespective of time of use or number of doses. This analysis therefore compared two groups: "ACT yes" containing mothers who had had the drug and "ACT no" containing those who hadn't. A complete treatment cycle was defined as when an expectant mother was given two 12 mg doses of betamethasone with a 24-hour interval and the child was born at least 24 hours after the second dose of medication. An incomplete cycle was defined as when the mother was given both doses, but the birth took place less than 24 hours after the second dose of betamethasone. Therefore, mothers who were only given one dose of betamethasone were only assessed in the first analysis.

The maximum time between steroid administration and birth was set at 7 days.

For the purposes of comparing different corticoid therapy groups, the following maternal variables were recorded: age, hypertensive disease (pregnancy-specific and/or chronic), eclampsia, diabetes mellitus, labor, type of delivery, fetal presentation, acute fetal suffering, clinical chorioamnionitis and maternal perinatal infection. Clinical chorioamnionitis was defined as maternal fever prior to delivery with no obvious cause and one of the following signs: uterus sensitive to touch, maternal and/or fetal tachycardia, physometra, leukocytosis.¹⁵ Perinatal maternal infection was defined as the presence of a systemic infection in the mother 48-72 hours before birth (urinary tract infection, pneumonia or sepsis).

The following variables related to the NB were also recorded: birth weight, gestational age, nutritional status,¹⁶ sex, 1st and 5th minute Apgar scores and need for resuscitation in the delivery room.

Distributions across categorical variables are expressed as frequencies. Results for serum ROI, GR and IL-6 are expressed as medians and 25th and 75th percentiles. The chi-square test, and/or Fisher's test when necessary, and relative risk with 95% confidence interval (95%CI) were used to compare categorical variables. The Mann-Whitney test was used to compare median ROI, GR and IL-6 against antenatal corticosteroid administration and to analyze potential confounding variables. The significance level was set at $p < 0.05$. The statistical software employed was the Statistical Package for the Social Sciences (SPSS) 15.0 for Windows.

This study was approved by the institutional Research Ethics Committee, under hearing number 971/2008.

Results

During the study period, 138 children were born with weights below 1,500 g at the maternity unit in the Center for Integral Women's Healthcare (Centro de Atenção Integral à Saúde da Mulher, CAISM) at the Universidade Estadual de Campinas (UNICAMP) in Campinas, state of São Paulo, Brazil. Thirteen NB were not included because it was not possible to take the necessary blood samples. A total of 125 children (89.92%) remained for analysis. There were 111 mothers, since there were 18 multiple births (15 of twins and three of triplets). As the inclusion criteria was birth weight, not all of the children from the multiple births were recruited, since seven of these infants had birth weight over the 1,500 g limit. None of the parents refused permission for the cord blood samples.

In all cases the corticosteroid used was betamethasone. It was observed that 92/111 (82.88%) of the mothers were given the drug. Sixteen of them (17.4%) only received one

dose of betamethasone before their child was born, and 82.60% were given the full cycle.

The antenatal betamethasone group and no antenatal betamethasone group were compared with each other and there were no differences in terms of maternal variables or birth variables, with the exception of vaginal delivery, which was significantly associated with no treatment ($p = 0.04$ and relative risk = 1.549, 95%CI 1.048-2.288).

There were no significant associations between antenatal betamethasone use and the neonatal variables analyzed (Table 1). There were no statistically significant differences for any of the maternal or neonatal variables that could alter the levels of the blood markers studied.

Problems with the cord blood samples meant that not all of the 125 biochemical assays of ROI, GR and IL-6 could be performed. Coagulation prevented ROI and GR assays and hemolysis prevented IL-6 assays. A total of 98 samples were assayed for baseline ROI, 99 for stimulated ROI, 100 for GR and 121 for IL-6.

With regard to the effect of antenatal corticosteroid administration on the blood levels of these markers, there were statistically significant differences in the median test results when divided into groups administered and not administered the drug, irrespective of time or dose. When mothers were given the full betamethasone cycle, however, there were significant reductions in median baseline ROI and in median IL-6 (Tables 2 and 3). A high test power was obtained for analysis of baseline ROI and for IL-6 production when the full ACT cycle was used.

Discussion

In this study 83% of the mothers had been given antenatal betamethasone. This result is superior to rates that have been reported in recent years at the same institution, where the average was 67.5% from 2006 to 2009.¹⁷ Data from the Brazilian Neonatal Research Network (Rede Brasileira de Pesquisas Neonatais) for the eight centers that were participating in 2006 to 2010 also show lower rates of antenatal corticosteroid usage, varying from 51 to 57%.¹⁷ This improvement at the institution studied has been made possible by the efforts of the local obstetrics team to offer the treatment to all expectant mothers who at risk of premature delivery before 34 weeks' gestation, associated or not with tocolytics.¹⁸⁻²⁰

The decision to define a "complete cycle" as two doses of betamethasone with a 24-hour interval between them and a further 24-hour interval prior to birth was arbitrary. There is no consensus in the literature on the optimum time needed for the drug to act, although it is recommended that the mother be given two doses of betamethasone. Since this study was investigating the effect of the medication on biochemical markers and not on clinical variables, we hypothesized that there would be a minimum time needed

Table 1 - Distribution of maternal and neonatal variables by antenatal corticosteroid administration

	ACT		p
	Yes (103)	No (22)	
Median maternal age, (p25-p75) (years)	27 (14-44)	22.50 (13-42)	0.2701*
Hypertensive disease	50/103 (48.5)	8/14 (57.1)	0.352 [†]
Eclampsia	2 (1.9)	0	1.00 [†]
Diabetes mellitus	7 (6.7)	0	0.352 [†]
Labor	36(34.5)	13 (59.1)	0.053
Vaginal delivery	16 (15.5)	10 (45.4)	0.004 [‡]
Acute fetal suffering	37 (35.9)	5 (22.7)	0.322 [†]
Maternal infection	14 (13.6)	4 (18.1)	0.521 [†]
Chorioamnionitis	6 (5.8)	4 (18.1)	0.074 [†]
Median birth weight, (p25-p75) (g)	1,010 (855-1,265)	955 (775-1,295)	0.726*
Median gestational age (p25-p75) (weeks)	29 (28-32)	29(26-33)	0.782*
Male sex	55 (53.4)	12 (54.5)	1.00 [†]
1 minute Apgar ≥ 7	70 (67.9)	11 (50.0)	0.089 [‡]
5 minute Apgar ≥ 7	98 (95.1)	18 (81.8)	0.052 [‡]
Resuscitation	36 (34.9)	13 (59.0)	0.053 [†]
SGA	45 (43.7)	8 (36.3)	0.637 [†]

Categorical variables expressed in frequency, n (%), except where indicated otherwise.

ACT = antenatal corticoid therapy; p25-p75 = 25th to 75th percentile; SGA = small for gestational age.

* Mann-Whitney test.

[†] Fisher's test.

[‡] Chi-square test.

Table 2 - Results for baseline and stimulated ROI, glutathione and IL-6 in cord blood from preterm very low weight newborn infants, broken down by maternal corticosteroid administration

	Corticosteroid administration				p*	Test power
	Yes		No			
	Results	n	Results	n		
Baseline ROI	0.4 (0.4-0.5)	81	0.5 (0.4-0.5)	17	0.2512	0.95
Stimulated ROI	33.7 (17.75-106.97)	82	74.3 (25.4-214.45)	17	0.1086	0.70
Glutathione	69.31 (63.23-80.79)	84	72.45 (61.78-81.26)	16	0.9439	0.55
IL-6	34.20 (14.12-60.55)	101	30.93 (13.26-63.59)	20	0.9750	0.30

Results shown are median (p25-p75).

Glutathione results in mg%.

IL-6 = interleukin-6 (pg/ml); ROI = reactive oxygen intermediates (nMol/10⁶ cells).

* Mann-Whitney test.

for it to take effect. Since there are no references in the literature that state what this "optimum" time might be, we chose 24 hours as the cutoff. Therefore, mothers who were given only one dose of betamethasone were only included in the analysis that differentiated mothers given ACT from mothers not given ACT.

When we evaluated the effect of betamethasone administration on ROI, GR and IL-6 production, we observed that it only had an effect when the cycle was completed. Baseline ROI and IL-6 production was significantly suppressed by betamethasone administration when the mother was given both doses and birth took place at least

24 hours after the second dose. This implies that the effect of the drug is time-dependent.

This suppressive effect can be considered positive to the extent that the drug suppresses part of the oxidative and inflammatory stress to which the fetus is subjected during labor of premature delivery, resulting in a premature neonatal transition that is less unbalanced and therefore less subject to severe hemodynamic disorders.

The inhibitory effect of steroids on ROI production does not imply a negative immunosuppressor effect, but works as an immunomodulator, since intercurrent conditions during gestation and delivery can be associated with

Table 3 - Results for baseline and stimulated ROI, glutathione and IL-6 in cord blood from preterm very low weight newborn infants, by complete vs. partial antenatal corticosteroid cycle

	Complete cycle				p*	Test power
	Yes		No			
	Values	n	Values	n		
Baseline ROI	0.40 (0.4-0.5)	64	0.50 (0.4-0.5)	17	0.0389	0.90
Stimulated ROI	28.40 (17.15-102.75)	65	41.80 (19.90-117.75)	17	0.3749	0.40
Glutathione	69.52 (63.03-81.28)	67	69.10 (63.28-76.05)	17	0.9602	0.07
IL-6	30.16 (13.89-57.36)	82	54.83 (16.12-177.27)	19	0.0243	0.91

Results shown are median (p25-p75).

Glutathione results in mg%.

IL-6 = interleukin-6 (pg/ml); ROI = reactive oxygen intermediates (nMol/10⁶ cells).

* Mann-Whitney test.

excessive, intense and prolonged free radical activation and, as a consequence, cell and tissue damage. As has been demonstrated experimentally, fetal human neutrophils are capable of producing superoxide radicals as effectively as adult cells^{21,22} and, in the presence of excessive stimuli, this could be associated with cell and tissue damage.

Stimulated ROI production did not exhibit a statistically significant difference between the corticoid therapy study groups. Citarella et al. conducted a study of the polymorphonuclear leukocytes of full term NB and also demonstrated that production of superoxide radicals increased significantly after stimulation with N-formyl-methionyl-leucyl-phenylalanine, but that there was no additional increase when interleukin-10 or dexamethasone were added to the culture.²³

It is possible that an increased sample size could be needed to verify whether this finding would be maintained, since the statistical power of the sample was below 80%. On the other hand, one could also argue that since this was *in vitro* stimulation it does not reflect the complex conditions of biological *in vivo* stimuli. Furthermore, the functions of neutrophils change as they move from the circulation into tissues.^{23,24}

We did not observe any suppressive effect of betamethasone on glutathione production. This finding could be considered protective, since the absence of attenuation of this system that neutralized free radicals of oxygen in combination with reduced production of superoxide radicals, should mean there are less chances of oxidative injuries to the fetus/NB. Furthermore, the absence of suppression is beneficial given that glutathione levels drop off rapidly during the first days of life of preterm NB because of the increased oxidative stress associated with birth.^{25,26} Notwithstanding, the low statistical power does not permit extrapolation of these results and a larger sample is required to better assess this effect.

Interleukin-6 is thought to be the principal cytokine involved in fetal inflammatory response syndrome, which is even defined on the basis of serum cytokine levels in combination with chorioamnionitis and funisitis.^{27,28}

The effects of antenatal betamethasone on fetal IL-6 production have been little studied. Kavelaars et al.²⁹ failed to detect suppression of IL-6 synthesis in cells from the umbilical cords of full term and preterm human fetuses. However, Kramer et al.⁹ conducted experimental studies using with neutrophils from full-term lambs and demonstrated that betamethasone administered to the mother suppressed IL-6 production 15 hours later.

Arad et al.³⁰ showed that when the drug was administered to women during premature labor, it was associated with a lower number of NB with elevated IL-6 levels (defined as > 11 pg/mL).

In our study there was a significant association between umbilical serum IL-6 levels and administration of a complete betamethasone cycle to the mother, which could be because this is the *in vivo* effect, in the presence of other active stimulants that are absent in cell culture conditions. This effect could be considered to be beneficial, on the basis that it suppresses the excess inflammatory mediators that are present during premature labor. A meta-analysis of antenatal steroid administration demonstrated a protective effect reducing the incidence of neonatal systemic infection during the first 48 hours of life (relative risk [RR] = 0.56 95%CI 0.38-0.85).²

One limitation of our study is that the analyses were performed on cord blood samples, which reflects the perinatal conditions related to premature birth. This study design was chosen since the objective was to assess the fetal effects of betamethasone administered to the mother. Notwithstanding, a comparison of the same markers assayed in postnatal blood would be a valuable contribution, since the drug still exerts a variable degree of effect at this point

and it would be of use to investigate the effects of birth and of extrauterine conditions on inflammatory mediators and oxidative stress.

Another limitation to our study is that the statistical power was inadequate for some of the analyses since the original sample size calculation was based on the clinical outcome of periventricular and intraventricular hemorrhage.

Therefore, the biochemical effect on ROI and IL-6 production of corticosteroid administered to the mother could be the factor that determines the protection conferred by the drug against neonatal diseases that are associated with oxidative stress and inflammation, such as periventricular and intraventricular hemorrhage and early hemodynamic disorders.

In summary, administration of a complete betamethasone cycle to the mother had a suppressive effect on baseline ROI production and on IL-6 production in preterm very low weight NB. Therefore, this study serves as a basis for evaluation of antenatal corticosteroid use, of the markers studied and of the clinical progress of these NB, especially with relation to diseases caused by the action of free radicals and intrauterine inflammation.

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