IL-10 and IL-12 in The Cord Blood of Preterm Newborns with Respiratory Distress Syndrome

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Abstract:

The development of respiratory distress syndrome (RDS) is closely related to fetal immaturity, although the participation of inflammatory mechanisms also seems to be likely. In this study, we investigated the possible role of IL-10 and IL-12 in preterm newborns with RDS, a disease that is also related to gestational age. The cord blood levels of IL-10 and IL-12 were assessed in 60 preterm infants, 30 of them developed RDS and they were taken as cases, and the other 30 were not distressed and used as a control group.

The results showed that IL-10 was higher and IL-12 was lower in preterm infants with RDS than those without RDS. IL-10 manifested negative correlation with gestational age and birth weight, while IL-12 manifested no correlation with gestational age or birth weight. There was no correlation between IL-10 and IL-12 either in cases or controls. Five cases died from RDS, these cases had significant higher IL-10 levels and lower IL-12 levels than survivors. Non presenting twins of the cases had significant higher IL-10 levels and lower IL-12 levels than presenting twins.

Conclusion: *We concluded from these findings that IL-12 and IL-10 can be used as indicators for the risk of developing RDS in the born preterm. IL-10 and IL-12 levels in cord blood most probably indicate functional immaturity of the preterm.*

Introduction:

Respiratory distress syndrome (RDS) is a severe pulmonary disease with high mortality and morbidity in premature infants. It is due, at least in part, to insufficiency of pulmonary surfactant and occurs primarily in preterm infants.[1] Cytokines 'messenger proteins' are, soluble mediators that allow communication between cells. Together with hormones and neurotransmitters, they contribute to a chemical signaling language that controls development, tissue repair, inflammation and the immune response in multicellular animals.[2]

IL-10 is a cytokine that functions as an important regulator of the immune system. It is mainly produced by the Th2 subset of $CD4^+$ helper cells following their stimulation by lectins. However, it is also produced by some B cells, some Th1 cells, macrophages and some non hematopoietic sources.[3] The act of giving birth is associated with an increase in the body's production of proinflammatory mediators by gestational tissues. Research has shown that IL-10 may play a role in modulating or promoting the termination of inflammation during labor at term and in intrauterine infection-associated preterm labor.[4] IL-10 has been shown also to be a physiologic antagonist of IL-12.

IL-12 is an important regulatory cytokine that has a function central to the initiation and regulation of cellular immune responses. It has the capacity to regulate the differentiation of naive T cells into TH1 cells.[5] IL-12 is produced by macrophages, monocytes, dendritic cells, and B cells in response to bacterial products and intracellular parasites.[6]

Subjects and Methods:

This study was carried out on 60 preterm infants attending to El-Minya University Hospital, during the period from December 2004 to May 2005. Thirty preterm infants developed signs of respiratory distress syndrome and were taken as cases, while the other 30 without distress were taken as controls. All cases with RDS were selected according to clinical and radiological findings with respiratory distress occurring within the first 4 hours of age. The severity of RDS was assessed by both the duration of respiratory distress in days and the scoring system "Clinical scoring for infants with RDS".[7]

All neonates were subjected to the following:

- Resuscitative measures.
- History taking: including: mode of delivery and maternal problems e.g. diabetes mellitus, hypertension, and prolonged rupture of membranes (PROM).
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- Clinical examination:
  -Estimation of gestational age by Dubowitz/Ballard score.
  -Sex
  -Birth weight
  -Assessment of intrauterine growth by growth charts for preterm infants.
  -Chest examination for assessment of the degree of respiratory distress with the aid of the "Clinical scoring for infants with RDS".
- Chest X-ray was done for every case with suspected RDS.
- For all cases and controls in this study, serum IL-10 and IL-12 by enzyme linked immunosorbent assay (ELISA) technique were measured.

Sample Collection:

In this prospective study cord blood samples were collected immediately after delivery from all 60 preterm infants, promptly centrifuged within one hour of collection. Supernatant serum was stored at –60ºC till time of analysis. 30 preterm infants who developed respiratory distress were taken as cases, while the other 30 without distress were taken as controls.

Determination of Serum IL-10:

Human IL-10 ELISA Kit, for the quantitative determination of human interleukin 10 (IL-10) concentrations in serum. [Catalogue Number: EL10027, 96 tests, ANOGEN:2355 Derry Road East, Unit 23, Mississauga, Ontario, Canada L5S 1V6].

Principle of The Assay:

This IL-10 enzyme linked immunosorbent assay (ELISA) applies a technique called a quantitative sandwich immunoassay. The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific to IL-10. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated polyclonal antibody preparation specific for IL-10 and incubated. IL-10 if present, will bind and become immobilized by the antibody pre-coated on the wells and then be "sandwiched" by biotin conjugate. The microtiter plate wells are thoroughly washed to remove unbound IL-10 and other components of the sample. In order to quantitatively determine the amount of IL-10 present in the sample, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Avidin is a tetramer containing four identical subunits that each has a high affinity-binding site for biotin. The wells are thoroughly washed to remove all unbound HRP-conjugated Avidin and a TMB (3, 3'5, 5' tetramethylbenzidine) substrate solution is added to each well. The enzyme (HRP) and substrate are allowed to react over a short incubation period. Only those wells that contain IL-10, biotin conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. In order to measure the concentration of IL-10 in the samples this kit includes two calibration diluents (Calibrator Diluent I for serum/plasma testing and Calibrator Diluent II for cell culture supernatant testing). According to the testing system, the provided

standard is diluted 2-folds with the appropriate Calibrator Diluent and assayed at the same time as the samples. This allows the operator to produce a standard curve of Optical Density (O.D) versus IL-10 concentration (pg/mL). The concentration of IL-10 in the samples is then determined by comparing the O.D of the samples to the standard curve.[8]

Determination of Serum IL-12:

ACCUCYTE® Human IL-12 kits is a competitive enzyme immunoassay (EIA), which measures the natural and recombinant forms of Interleukin-12, p70 variant, (IL-12)[(U.S. Patent No. 5,587,294, European Patent No. EP 0 598 758 B1).

Principle of the Assay:

With the ACCUCYTE® assay system, goat anti-rabbit antibodies are used to capture a specific IL-12 complex in each sample consisting of IL-12 antibody, biotinylated IL-12, and sample/standard. The biotinylated IL-12 conjugate (competitive ligand) and sample or standard compete for IL-12 specific antibody binding sites. Therefore, as the concentration of IL-12 in the sample increases, the amount of biotinylated IL-12 captured by the antibody decreases. The assay is visualized using a streptavidin alkaline phosphatase conjugate and an ensuing chromagenic substrate reaction. The amount of IL-12 detected in each sample is compared to a IL-12 standard curve which demonstrates an inverse relationship between Optical Density (O.D) and cytokine concentration: i.e. the higher the O.D the lower the cytokine concentration in the sample.[9]

Statistical Analysis:

Data were analyzed by the statistical " SPSS for windows version 8.0". Two-tailed tests were used throughout and statistical significance was set at the conventional 95% level.

I- Descriptive statistics:

The range, means and standard deviation were calculated for interval and ordinary variables .

II- Group comparisons:

Comparisons were done by several procedures, depending on type of variable:

* Student's t-test:
Unpaired t-test was used to test the statistical significance of the difference in means of a variable measured on an interval scale.
* Correlation: Correlations between variables measured on the interval or dichotomous scale were carried out by Pearson Product Moment Correlation.

Results:

There was highly significant increase in serum IL-10 ($p<0.0001$), and highly significant decrease in serum IL-12 ($p<0.0001$) in preterm neonates with RDS when compared with their corresponding normal controls (table I).
We found significant correlations between serum IL-10 and gestational age (figure 1), days of respiratory distress (figure 2) and birth weight and non-significant correlations with RDS score and antenatal steroids. There were significant correlations between IL-12 and days of distress ($p=0.04$) (figure 3), but there were non-significant correlations with gestational age, birth weight, RDS score and antenatal steroids (table II).
The outcome of cases was: 25 cases improved representing 83.3%, and 5 cases died (16.7%). There was a significant decrease in gestational age ($p=0.049$), significant increase in IL-10 ($p=0.001$) and a significant decrease in IL-12 ($p=0.001$) in non-survivors compared to survivors, while birth weight was not different (table III)
There was a significant increase in IL-10 ($p=0.002$),a significant decrease in IL-12 ($p=0.02$), and a highly significant increase in days of distress ($p<0.0001$) in first order twin than second order twin.

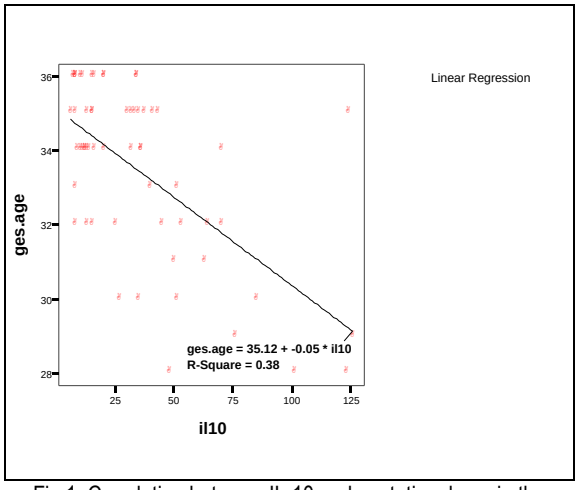

Fig 1. Correlation between IL-10 and gestational age in the preterm cases

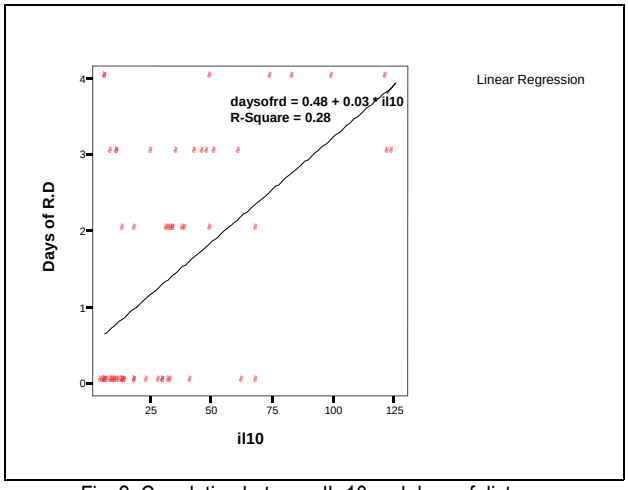

Fig. 2. Correlation between IL-10 and days of distress
R.D.=Respiratory distress

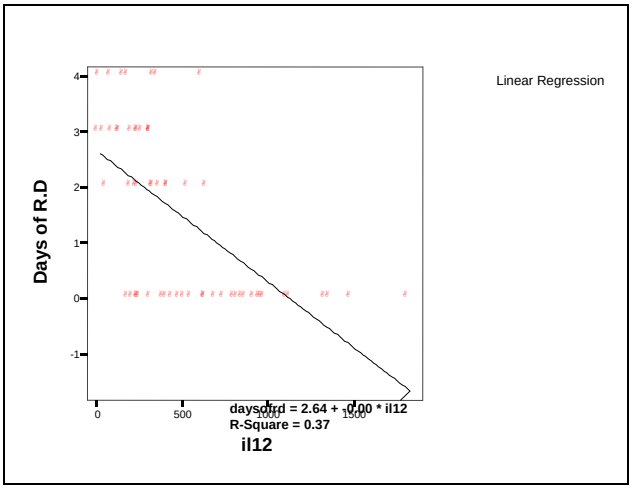

Fig 3. Correlation between IL-12 and days of distress
R.D.=Respiratory distress

Table I: Statistical analysis of IL-10 and IL-12 levels in cases and controls

Item	Cases (Group I) (n=30)		Controls (Group II) (n=30)		P value
	Range	Mean ±SD	Range	Mean ±SD	
Il-10 (pg/ml)	36.3-61.8	49.1±34.1	14.7-26.6	20.6±16	0.0001***
IL-12 (pg/ml)	210.4-332.1	271.3±162.9	496.1-658	577.1±216.8	0.0001***

**** highly significant*

Table II: Correlations between clinical characteristics and IL-10 and IL-12 in cases with RDS

Item	IL-10		IL-12	
	Correlation Coefficient (r)	P value	Correlation Coefficient (r)	P value
Gestational age	-0.59	0.0001***	-0.078	0.68
Weight	-0.39	0.03*	0.13	0.47
RDS score	0.22	0.25	-0.44	0.13
Days of RD	0.31	0.048*	-0.56	0.04*
Antenatal steroids	0.015	0.9	0.29	0.9

**significant ***highly significant RD= respiratory distress*

Table III: Outcome of cases with RDS

Item	Survivors		Non-survivors		P value
	Range	Mean ±SD	Range	Mean ±SD	
Gestational age (in weeks)	31.7-33.6	32.7±2.4	26.2-36.6	31.4±4.2	0.001**
Birth weight (in grams)	1594.3-1901.7	1748±372.4	674.7-2125.3	1400±584.2	0.09
IL-10 (pg/ml)	33.3-57.2	45.3±29	14.1-134.5	67.8±53.7	0.001**
IL-12 (pg/ml)	251.2-371.2	311.2±145.5	20.4-163.2	71.4±73.9	0.001**

**significant ** highly significant*

Discussion:

Respiratory distress syndrome, is the most common respiratory disorder in preterm infants, and is considered as a main cause of morbidity and mortality in the neonatal period, as well as a major contributor of respiratory failure in the pediatric age group.[10]

The development of RDS is closely related to functional immaturity which is the most important factor predisposing to RDS, although participation of inflammatory mechanisms also seems to be likely.[11]

The concept of involvement of an inflammatory process was supported by the findings of increase of complement, increase of the number of alveolar macrophages and increase of released products from polymorphnuclear cells in tracheal aspirates.[12] This local inflammatory reaction leads to increased vascular permeability and protein leakage into alveolar spaces, which interferes with formation of surfactant monolayer.[13] This deficiency or dysfunction leads to alveolar collapse, hypoxia and acute lung injury, with severe epithelial and endothelial lesions in RDS of premature neonate,[14] numerous inflammatory stimuli and cytokine induce and control this inflammatory process.

In this study, IL-10 (known as an anti-inflammatory cytokine) and IL-12 (known as a pro-inflammatory cytokine) were evaluated in the serum of the cord blood in preterm infants suffering from RDS and preterm infants without RDS as a control group.

In the present study, 56.7% (although not significant) of preterm neonates with RDS were males, and this is in agreement with Stoll and Kliegman,[15] in 2000, who stated that the incidence of RDS is highest among preterm male infants.

Also, Elsman et al.,[16] in 2004, reported that there are early gender-related differences in need for ventilatory and circulatory support that may contribute to the worse long-term outcome in prematurely born male infants. This can be explained by an apparent acceleration in lung epithelium maturation as proved to occur sooner in female than in male rats during fetal life.

The relation between maternal risk factors causing preterm delivery and development of RDS in this study were as the following; PROM 43.3% of cases, and in 53.3% of the control group (not significant). This is in agreement with Sims and coworkers,[17] in 2002, who stated that PROM and associated infections may expose the fetus to stress and increase the concentration of different hormones, cytokines, and growth factors which in turn induce surfactant synthesis, accelerates lung maturation, and protect from RDS.

In this study, the incidence of pre-eclampsia in both cases and controls were 16.7% and there are contradictory reports about pre-eclampsia as a risk

factor for RDS. Wolf and coworkers,[18] in 1993, reported higher incidence of RDS in preterm infants of hypertensive than non-hypertensive mothers. However, this can be explained by absence of labor before caesarean section, in other study, preterm infants of mothers with pre-eclampsia had less RDS compared to infants of non-hypertensive mothers.[19]

Multiple pregnancy represented 23% of cases but only 16.7% of the controls, this is in agreement with Donovan and his colleagues,[20] in 1998, who reported higher incidence of morbidity from RDS in preterm twins compared to preterm singletons and corrected for gestational age.

Antepartum hemorrhage represented maternal risk factor in 30% of cases and in 6.7% of controls (p=0.02). This can be explained by asphyxia which is induced by antepartum hemorrhage, with its injurious effects on fetal lung parenchyma .

One preterm with RDS was delivered to a mother with rheumatic heart disease, this could be attributed to the fact that cardiac disease limits the ability to increase cardiac output, so the increase in blood flow to the uterus, expected with advancement in pregnancy is limited resulting in increased incidence of prematurity.[21]

In the present study, IL-10 cord blood levels had negative correlation with gestational age (r=-0.59). This is in agreement with Blanco et al.,[22] in 2000, who suggested that this negative correlation was a result of fetal immaturity and immunosuppression occurring during pregnancy. There was also negative correlation with birth weight (r=-0.39).

IL-10 levels also were found to be significantly higher in preterms with RDS than preterms without RDS (p=0.0001). This is in accordance with Blanco et al.,[11] in 2004, who stated high cord blood IL-10 in preterm neonates with RDS than preterms infants without RDS, they explained increased IL-10 level in infants with RDS by biological immaturity of these infants. There was a positive correlation with RDS severity as presented by duration of distress (r=0.61), while there was no correlation with scoring system for RDS.

We suggested that the increased IL-10 levels in cases with RDS, especially with prolonged respiratory distress, is a protective anti-inflammatory effect against the inflammatory process occurring in RDS.

An alternative explanation, as occurs with other cytokines, is that the effect of IL-10 might not be exclusively anti-inflammatory and different and even contradictory effects of IL-10 in particular situations are known. Mitchell and coworkers,[23] in 2004, had found that IL-10 does exert anti-inflammatory properties in choriodecidua (maternal face of placenta) but that in the adjacent amnion (fetal face of placenta) it has remarkable pro-inflammatory actions.

Even in adult healthy volunteers, it has been reported that after the injection of endotoxin, a high dose of IL-10 increases the effect of proinflammatory cytokines.[24]

Others have shown that the tracheal instillation of IL-10 in animals highly increases bronchial hyperresponsiveness.[25]

Also, at present, there is little evidence supporting a prominent anti-inflammatory role of IL-10 in the prevention of chronic lung disease.[26] After lipopolysaccharide injection, IL-10 knock-out mice undergo a chronic disease similar to human ulcerative colitis, with cachexia, anemia and shock,[27] but it is striking that they did not develop lung disease, as it occurs in TGF- β knock-out mice.[28]

IL-10 seems to be not related to airway inflammation, on the contrary, it has been found associated to recurrent wheezing and bronchial hyper-responsiveness in different experiment models.[29]

In an early study using bronchoalveolar lavage samples from preterm infants with RDS, no soluble IL-10, or IL-10 mRNA cellular expression were detected, whereas most samples from full term infants had positive results.[30] However, later studies showed detectable IL-10 levels in 50 % of bronchoalveolar samples from 17 premature infants.[26]

These contradictory results are not relevant to our findings because bronchoalveolar lavage samples were obtained several days after birth, and, Blanco et al.,[11] in 2004, found that IL-10 level is decreasing later in postnatal life and becoming undetectable in a high percentage of samples.

Cord blood IL-12 serum levels were also assessed in this study. They were significantly lower in cases than controls (p=0.0001) with a negative correlation between IL-12 levels and severity of RDS as presented by duration of distress (r=-0.56), but no correlation was found between IL-12 levels and the scoring system for RDS, gestational age or birth weight. These data are in agreement with Blanco et al.,[11] in 2004, who found lower IL-12 in preterms with RDS than preterms without RDS.

This can be explained by functional immaturity of preterms developing RDS than controls and is not related to gestational age.

IL-12 expression and production was previously reported to be related to maturity of the immune system, as Lee and his colleagues,[31] in 1996, found reduced production of IL-12 from activated cord blood than adult peripheral blood mononuclear cells and this in part contribute to decreased graft-versus-host

rejection following cord blood stem cell transplantation.

Also, Upham and coworkers,[32] in 2002, reported a decrease in the production of IL-12 after birth and in neonatal period and that IL-12 producing capacity develops throughout childhood, they also found that reduced IL-12 level was not related to excessive IL-10 production, and this is in agreement with the present study, as there was no correlation between IL-10 and IL-12 levels in either cases or controls.

In the present study, 5 preterm infants died from RDS representing 16.6% of the total cases, there was a significant difference between survivors and non-survivors preterm neonates with RDS as regards gestational age (p=0.001), but there was no significant difference in birth weight (p=0.09). This is in agreement with Chard and coworkers,[33] in 1997, who concluded that the risk of RDS and neonatal death does not appear to be related to the birth weight of preterm neonates, but related to gestational age.

The present study revealed that IL-10 was significantly higher (p=0.001) and IL-12 was significantly lower (p=0.001) in non-survivors than survivors.

So both IL-10 and IL-12 have better prognostic values than gestational age for detecting the outcome of preterms with RDS.

IL-10 and IL-12 were not correlated with antenatal steroid intake. Bessler and coworkers,[34] in 2001, on the other hand stated that dexamethasone induce a dose-dependent inhibition of IL-10 production by peripheral blood monocytes in adults whereas newborns were mostly unaffected by the drug, but they also reported that dexamethasone caused a dose-dependent inhibition of IL-12 in all age groups.

Also, Borish,[35] in 1998, found that steroids have an inhibitory effect on IL-10. The results in this study can be explained by the fact that some mothers received dexamethasone injection less than 48 hours before the onset of the preterm labor, and this gave no enough time for dexamethasone to exert its effect.

This study showed that IL-10 was significantly higher (p=0.007) and IL-12 was significantly lower (p=0.02) in non-presenting than presenting twins.

This was in agreem0ent with Ziadah and Badria,[36] in 2000, as their study had shown that the second, or the non-presenting twin had a higher risk of RDS compared to presenting twin. This was explained by Arnold and coworkers,[37] in 1987, who reported that the non-presenting twin, in vaginal delivery mainly, does not benefit from salutary effects of labor to the same extent as the presenting twin.

The present study revealed that IL-12 is more sensitive (93.3%) than IL-10 (80%) for RDS, but IL-10 is more specific (63.3%) than IL-12 (53.3%). While Blanco and coworkers,[11] in 2004, found IL-10 sensitivity (95%) and specificity (77%) while IL-12 sensitivity (70%) and specificity (73%).

Conclusion:

We conclude from these findings that IL-10 and IL-12 can be used as indicators for the risk of developing RDS in the preterm infants.

Both IL-10 and IL-12 have good prognostic values for diagnosis and assessing severity of respiratory distress syndrome as assessed by duration of distress and also they have better prognostic values than gestational age and birth weight for detecting the outcome of cases.

IL-10 and IL-12 levels in cord blood most probably indicate functional immaturity of the preterm.

Recommendations:

1. Assessment of IL-10 and IL-12 in cord blood of preterm infants for the prediction of the development of RDS and its severity, and for prediction of the outcome.
2. Further studies are needed to clarify the pathogenesis of the increase in IL-10 and the decrease of IL-12 in preterms with RDS.

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