A Clinical Randomized Trial on Endocervical Inflammatory Cytokines and Betamethasone in Prime-Gravid Pregnant Women at Risk of Preterm Labor

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ABSTRACT

Background: There are strong evidences suggesting the secretion of different cytokines in cervical fluid during preterm labor. Betamethasone is widely administered for several reasons in preterm conditions. **Objective**: To Investigate the possible effect of betamethasone on endocervical cytokine concentration of women at risk of preterm labor. **Methods**: In a randomized clinical trial of 80 prime-gravid women in preterm labor between 34 and 37 weeks of gestation, cervical fluid was collected. Endocervical concentration of inflammatory cytokines were analyzed before and 48 hours after betamethasone treatment for the evaluation of IL-8, IL-17, IFN-γ and TGF-β. Wilcoxon and Mann-Whitney tests were employed for statistical analysis. $\chi 2$ and Student's *t* tests were used whenever needed. **Results**: All the measured cytokines showed significant changes in the betamethasone treated group. IL-17 ($p=0.001$), IL-8 ($p=0.001$), and IFN-γ ($p<0.05$) decreased significantly, while TGF-β had a significant increase ($p<0.05$). In the patients who delivered before or on the 7th day of admission, IL-17, IL-8, and IFN-γ levels were all significantly higher. However, TGF-β decreased significantly in the same samples in the betamethasone treated group ($p<0.05$). **Conclusion**: Betamethasone significantly decreases the endocervical pro-inflammatory cytokine concentrations in patients with preterm labor.

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Keywords: Cytokine, IL-8, IL-17, IFN-γ, Preterm Delivery, TGF-β

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INTRODUCTION

Preterm deliveries before 37 weeks of gestation are mostly due to spontaneous preterm birth or preterm premature rupture of membranes in almost 80% of the cases (1). The increasing incidence of preterm birth has stayed high despite the improvements in fetal/maternal surveillance. Complications of prematurity caused by preterm labor account for 70-85% of fetal, neonatal and infantile mortalities (2). There is no definite diagnostic indicator or treatment for preterm delivery (3). As a multi-factorial condition, genetic and environmental factors are both involved in preterm birth (4).

Administration of glucocorticoids, dexamethasone or betamethasone (BTM), in women with threatening preterm delivery has significantly decreased the rate of mortality and respiratory distress syndrome (RDS) (5) Betamethasone is suggested as an ideal steroid due to its ability to cross the placenta, little immunosuppressive and mineralocorticoid effects, and long-term activity (6). Prenatal exposure to betamethasone is shown to contribute to lung maturation of preterm neonates preventing the RDS (5). It may also reduce the severity of RDS (7).

Controversial biological interactions during the parturition have made the mechanism poorly definable (8). Many patients with preterm labor encounter the infection of intra or extra uterine tissues (9). It has been reported that 16% of the patients with preterm labor delivery had positive cultures of amniotic fluid (10). The inflammatory reaction due to infection is related to the etiology of preterm birth. Several studies have suggested that bacterial infection may begin the premature labor and preterm premature rupture of the membranes, whereas the host inflammatory response may actually cause it (1). However, inflammatory processes have been reported in the absence of infection in patients at risk of preterm labor (11).

Cytokines are a group of proteins that mediate inflammatory responses to infection and tissue damage (12). There is strong evidence that IL-8 plays an important role in partitution through induction of cervical secretion in preterm labor (13). Besides, there is increasing evidence that pro inflammatory cytokines like IL-17 and IFN-γ are secreted to a notable degree in endocervical fluid of patients at risk of preterm labor (14). We have previously reported (15) dramatic changes in the inflammatory cytokine concentrations after BTM administration in preterm labor. Therefore, we decided to investigate the effect of BTM on some other endocervical cytokines like IL-8, IL-17, IFN-γ and TGF-β in patients at risk of preterm labor.

MATERIALS AND METHODS

Study Design and Participants. In a placebo-controlled, double-blind, parallel-group randomized clinical trial carried out in Iran, a total number of 80 patients were studied. Written informed consent was signed by all the participants who had been fully informed about the study.

From June 2006 to July 2010, patients at risk of preterm labor, with a labor pain between 34 to 37 weeks of gestation as determined by routine ultrasound examination in the first trimester were admitted to the obstetric emergency department of Vali-e-Asr Hospital, Tehran, Iran and were included in this study. The patients were randomly divided into 2 equal groups, the BTM and the saline group. Sequential numbers were assigned for patients and randomization was done by computer-generated sequence with

a block size of 4 patients. A research assistant who was the only one who had access to the randomization list, received the assigned numbers. Each patient was treated with the material provided in the numbered envelopes.

The routine examination for signs and symptoms of preterm labor were performed as follows: palpable uterine contractions every 5-8 minutes and Bishop score of 4 and higher associated with cervical dilatation of more than 1 cm and at least 50% of effacement. Prime-gravid women with preterm labor were included.

Women with systemic diseases, maternal hypertension before or during pregnancy, uterine tenderness, chorioamniotitis signs, symptomatic vaginal infection, rupture of membranes, current use of antibiotics, induced pregnancy, and history of smoking were excluded (14). The follow up visits were done until the time of delivery. All patients had no clinical signs of infection during pregnancy as well as the parturition period.

General and obstetric examinations were conducted on all l 80 patients upon admission. Three milliliters of venous blood was taken from all participants immediately on admission. Routine laboratory tests were requested.

Swab samples were prepared during the primary examination for both groups. In BTM group, intramuscular injection of 12 mg/day of BTM was done immediately after swap sampling, and another dose of 12 mg BTM was injected after 24 hours. Forty eight hours after the second injection, the second swab sampling was performed. The other group received saline and sampling was done in the same manner.

Dacron swabs were used for endocervical secretion samplings (16). The samples were kept at -70°C, and transferred to laboratory at -20°C within 1-2 hours.

The study was approved by the Biomedical Ethics Committee of Tehran University of Medical Sciences according to the Helsinki Declaration.

Determination of Cytokine Levels. Human ELISA Development Kits (R&D Systems, Minneapolis, USA) were used to measure IL-8, IL-17, IFN-γ and TGF beta levels. The wash buffer consisted of 0.05% Tween-20 in PBS and a solution of 1% BSA in PBS was utilized as the block buffer (15).

Determination of WBC and CRP. Determination of WBC in blood was done in the routine laboratory of Vali-e-Asr Hospital (Tehran, Iran). Measurement of C-reactive protein (CRP) in sera was performed by photometric method using quantitative diagnostic kit (high sensitivity CRP; Negar-Darou, Tehran, Iran) as previously described (15).

Statistical Analysis. SPSS version 16 (SPSS Inc. Chicago, IL, USA) was employed for data analysis. Wilcoxon and Mann-Whitney tests were used to analyze the related and independent data respectively, if they did not have normal distribution on Kolmorogov-Smirnov test. In order to analyze the parametric data, Students'*t-test* and $\chi 2$ test were used. $p<0.05$ was considered significant.

RESULTS

A total number of 5 cases, all from the BTM group, were excluded from the study. Two of them had delivery before the second sampling, 1 refused to continue her collaboration, and 2 had increased blood pressure.

Table 1 shows the summary of the demographic data. Age, BMI, blood hemoglobin (Hb), systolic and diastolic blood pressure did not show any significant difference, comparing the saline and BTM group. The same was true about WBC and CRP levels.

The median value of CRP was 2.40 mg/L (0.00 to 9.00) in the BTM treated group while it was 2.46 mg/L (0.73-18) in the saline group (p=0.493). The median value of WBC count was 8350 cells/µL (6100-10400) in the BTM group in comparison with 7600 cells/µL (5000-13000) in the saline group (p=0.618). None of the cytokine measurements showed any significant difference between the two groups at the time of primary sampling (p>0.05, all groups) (Table 1).

Table 1. Background variables comparing the betamethasone and saline treated group, measurements are done before any intervention.

	Betamethasone N=35	Saline N=40	P Value
Age (years)	24 (16-37)	24 (17-35)	1.000
BMI (kg/m^2)	22.48 (16.38-31.59)	22.37 (16.27-30.19)	0.722
Systolic blood pressure (mmHg)	120 (90-140)	110 (90-140)	0.388
Diastolic blood pressure (mmHg)	70 (60-110)	70 (60-100)	0.485
HB (gm/dL)	11.80 (8.90-15.00)	12.35 (9.40-14.30)	0.172
WBC (cells/µL)	8400 (5500-10350)	7600 (5200-11650)	0.269
CRP (mg/L)	2.40 (0.00-9.80)	2.30 (0.00-10.00)	0.730
TGF-β	17.39 (8.93-29.11)	17.13 (9.64-28.60)	0.907
IL-17	6.38 (3.26-9.05)	6.37(3.99-8.57)	0.323
IL-8	1103.00 (13.00-2537.00)	1198.00 (250.00-2232.00)	0.663
IFN-γ	7.18 (5.53-9.98)	7.45 (5.08-9.78)	0.941

Values are Median (Min-Max)

All the measured cytokines had significant changes in the BTM treated group (Table 2a). TGF-β was elevated at the time of second sampling (p=0.013). However, IL-17, IL-8, and IFN-γ decreased after BTM administration (p=0.001, 0.001, and 0.004, respectively). In the control group treated with saline, none of the measured cytokines showed any statistically significant changes (Table 2b).

Table 2a. Cytokine levels (ng/dL) measured on admission compared to measurements done 72 hours later in the betamethasone treated group (N=35).

	Primary samples			Samples 72 hrs after			P Value
	Median	Min	Max	Median	Min	Max	
TGF-β	17.39	8.93	29.11	17.98	10.05	30.84	0.013
IL-17	6.38	3.26	9.05	5.96	3.03	8.45	0.001
IL-8	1103.00	13.00	2537.00	926.00	36.00	2616.00	0.001
IFN-γ	7.18	5.53	9.98	7.01	5.29	9.28	0.004

Table 2b. Cytokine levels (ng/dL) measured on admission compared to the measurements after 72 hours in the saline treated group (N=40).

	Primary samples			Samples 72 hrs after			P Value
	Median	Min	Max	Median	Min	Max	
TGF-β	17.13	9.64	28.60	19.80	9.25	26.07	0.778
IL-17	6.37	3.99	8.57	6.26	3.64	11.64	0.122
IL-8	1198.00	250.00	2232.00	1252.00	119.00	2357.00	0.164
IFN-γ	7.45	5.08	9.78	7.93	4.87	10.45	0.162

While the number of cases delivering over a one week period was higher in both groups, the difference was not significant (p=0.142). A total of 38% of the pregnant women had deliveries under a week after primary measurements (Table 3).

Table 3. Comparison of the effects of betamethasone and saline on the number of patients who delivered before and after 1 week following admission.

	Betamethasone	Saline	P Value
>1week n(%)	25 (71)	22 (55)	0.142
≤1week n(%)	10 (29)	18 (45)	
Total	35	40	

In neither the BTM group nor the control group, TGF-β was not significantly different between the patients who delivered before and after one week period from the primary sampling, both in the primary and the second samples. However, it was lower in patients who delivered under a 1 week from primary sampling. IL-17, IL-8, and INF-γ levels were all significantly higher in patients who delivered in less than 1 week, in any of the groups or in any of the samples (Table 4a, b). However, IFN-γ showed the highest increase in the BTM group (p=0.028) in both the primary sample and the second sample (p=0.015).

DISCUSSION

We previously described (15) the possible effect of glucocorticostroids on the endocervical secretion of inflammatory cytokines. We had reported the important role of betamethasone on the regulation of the production of cytokines. In this report, we investigated the effect of betamethasone on other pro- and anti-inflammatory cytokines. There are very few studies investigating the endocervical concentration of cytokines in pregnant women without any sign of infection (11,15). The prominent effect of inflammatory cytokines in labor process remains challengeable. However, there are studies which analyzed the combination of these cytokines with other risk factors in order to identify women with the highest risk of preterm labor (14).

Cytokines themselves might mediate leukocyte attraction that occurs at the time of parturition. Cytokines might mediate leukocytic infiltration, either directly (in the case of IL-8) or via up-regulation of cell adhesion molecules (IL-1 and TNF-α). It has been demonstrated that there is a massive up-regulation of adhesion molecule expression at the onset of labor (17). Additionally, concentrations of IL-1, IL-6, and TNF-α in amniotic fluid are correlated with the degree of leukocytic infiltration in the placenta and membranes (180). These data raise the possible role of a positive feedback mechanism, whereby pro-inflammatory cytokines attract leukocytes into reproductive tissues at the time of parturition. The leukocytes themselves also generate pro-inflammatory cytokines, attracting more leukocytes.

Table 4a. Cytokine levels (ng/dL) measured on admission day and 72 hours after admission in the betamethasone treated group, with regard to delivery time (before and after 1 week time following admission).

	Primary samples			Samples 72 hrs after		
	>1week N=25	≤1week N=10	P Value	>1week N=25	≤1week N=10	P Value
	Median (min-max)	Median (min-max)		Median (min-max)	Median (min-max)	
TGF-β	19.74 (10.53-29.11)	16.10 (8.93-24.03)	0.122	21.64 (10.51-30.84)	16.29 (10.05-21.95)	0.028
IL-17	5.89 (3.26-8.83)	7.72 (5.82-9.05)	<0.001	5.68 (3.03-8.30)	7.29 (3.94-8.45)	0.003
IL-8	639.00 (13.00-2198.00)	1991.50 (735.00-2537.00)	<0.001	605.00 (36.00-2185.00)	1851.50 (788.00-2616.00)	0.001
IFN-γ	6.97 (6.10-9.51)	8.54 (5.53-9.98)	0.028	6.90 (5.55-9.28)	8.30 (5.29-8.99)	0.015

Table 4b. Cytokine levels (ng/dL) measured on admission day and 72 hours after admission in the saline treated group, with regard to delivery time (before and after 1 week time following admission).

	Primary samples			Samples 72 hrs after		
	>1week N=22	≤1week N=18	P Value	>1week N=22	≤1week N=18	P Value
	Median (min-max)	Median (min-max)		Median (min-max)	Median (min-max)	
TGF-β	19.48 (10.56-28.60)	16.31 (9.64-25.13)	0.396	20.84 (9.25-26.07)	19.45 (12.53-23.43)	0.581
IL-17	5.45 (3.99-6.91)	6.74 (5.11-8.57)	<0.001	5.31 (3.64-6.70)	7.36 (4.10-11.64)	<0.001
IL-8	715.00 (250.00-2232.00)	1650 (739.00-2200.00)	<0.001	745.00 (119.00-2128.00)	1651.00 (817.00-2357.00)	<0.001
IFN-γ	5.98 (5.08-9.78)	8.96 (5.33-9.71)	<0.001	6.90 (4.87-9.12)	8.29 (5.53-10.45)	<0.001

TGF-β is known to have inhibitory effects on T and B cells. Moreover, it expresses its anti inflammatory response by limiting IL-2 and IFN-γ production (19). It also plays a role in the extracellular matrix regulation by decreasing the degradation of matrix proteins. That is done through the reduction in protease synthesis and the increase in the synthesis of protease inhibitors (20). Like many cytokines, TGF-β has both pro- and anti-inflammatory effects. It acts as a biological switch, antagonizing or modifying the action of other cytokines or growth factors. The presence of other cytokines may modulate the cellular response to TGF-β. The effect may differ depending on the activation state of the cell (21).

TGF-β often exhibits disparate effects such as immune-enhancing activity in local tissues and immune-suppressive activity in the systemic circulation (20).

In our study, we found that TGF-β significantly increases after BTM injection. In women who delivered within a week after primary sampling, its concentration significantly decreased compared with the patients who delivered after a week. These data provoke the hypothesis that during the inflammatory state, TGF-β, as a anti inflammatory cytokine, could inhibit the leukocyte proliferation in order to decompensate the pro-inflammatory cytokines release cascade.

TGF-β suppresses the proliferation and differentiation of T and B cells, and also limits the production of IL-2, IFN-γ, and TNF. TGF-β acts as a monocyte/macrophage deactivator in a manner similar to IL-10. However, TGF-β is a less potent inhibitor than IL-10 and has little or no effect on IL-1 production (22). The severe and uncontrolled inflammatory reactions observed in the TGF-β knockout mice confirm the physiologic role of TGF-β as an endogenous anti-inflammatory cytokine (23).

IFN-γ, as a macrophage activating factor, is the most important product in orchestration of leukocyte aggregation and differentiation to many cell types (24) it is believed that corticosteroids could inhibit the in vitro production of cytokines by T lymphocytes. Notably, they resulted in inhibition of IFN-γ via a dose dependent mechanism (25). The production of pro-inflammatory cytokine, like IFN-γ, is especially up-regulated by IL-12 and IL-18 secretion (26) It is down-regulated by production of IL-4, IL- 10, TGF-β, and glucocorticoids (27) We found that betamethasone can significantly decrease the production of IFN-γ in women delivered within or after a week of sampling. It has been previously described that a class III glucocorticoid remarkably decreases the cutaneous expression of IFN-γ in an animal model of atopic dermatitis (28) which is in agreement with our findings. However, the in vivo dose dependent effect of such a treatment is still obscure.

IL-17, as a main product of Th17, a third T-cell lineage, is a pro-inflammatory cytokine which supports mucosal barriers and induces neutrophil expansions (29). The process is differentiated by IFN-γ and blocked by TGF- β. It is clearly shown that BTM effects results in down-regulation of IL-17 in women at risk of preterm labor (Table 2a). Interestingly, BTM could suppress both Th1 mediated cytokines like IFN-γ, and Th17 main product. However, TGF-β concentration, as the other Th17 product, was elevated after BTM injection.

The production of IL-8, a pro-inflammatory cytokine, has been extensively reviewed during pregnancy. It is known as the mediator of cervical ripening during parturition due to migration of neutrophils toward cervix in order to excrete the MMP-8 (30). It is reported that epithelial and stromal cells within myometrium and fetal membranes are responsible for IL-8 secretion (31).

Like other pro-inflammatory cytokines, BTM significantly decreased the endocervical concentration of IL-8. It seems that reduction of IL-8 concentration as a main mediator of cervical ripening would be an important factor to delay the parturition in patients at risk of preterm labor. It is known that infiltration of macrophages and neutrophils targeting myometrium in the time of parturition (32) and the production of IL-8 increases during labor. These findings could bring us to the point to consider that the infiltration process is the main trigger of IL-8 production. BTM, as a glucocorticoid, is known to inhibit the activation of macrophages and neutrophils during inflammatory process (33). Therefore, by this mechanism, it could inhibit the expression of IL-8 in endocervical fluid.

In summary, BTM could diminish the endocervical secretion of pro-inflammatory cytokines like IL-8, IFN-γ, and IL-17. On the other hand, TGF-β increased after BTM administration. We can postulate that BTM, as a glucocorticoid, could significantly change the balance of endocervical cytokines. It could be beneficial for reduction of the inflammatory state. This condition might postpone the parturition to some extent, but we cannot definitely demonstrate this significant delay. We suggested further investigation on different classes of glucocorticoids and variable doses to define the optimum influence with minimum side effects. The measurement of such cytokines in the amniotic fluid could also be of interest for further studies. This study just showed the in vivo effect of BTM on endocervical inflammatory cytokines of patients at risk of preterm labor. However, the exact mechanism still remains obscure.

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