OXTR and ZEB1 expression before and after progesterone dosing in pregnant women with threatened premature labor

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Abstract. – **OBJECTIVE:** To investigate changes in the peripheral blood mRNA levels of oxytocin receptor (OXTR) and Zinc finger E-box-binding homeobox 1 (ZEB1) before and after progesterone dosing in pregnant women with threatened premature labor.

PATIENTS AND METHODS: Blood samples were collected from 30 healthy pregnant women with 28- to 33+6-week gestational age and singleton pregnancy (group A) and from 30 pregnant women with singleton pregnancy and threatened premature labor before and 48 hours after progesterone dosing (groups B and C, respectively) for quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) assay to assess the OXTR and ZEB1 mRNA levels.

RESULTS: The OXTR mRNA level was higher in the group B than in the groups A and C, and the ZEB1 mRNA level was lower in the group B than in the groups A and C. Notably, no significant difference was found in the mRNA level of OXTR or ZEB1 between group A and group C.

CONCLUSIONS: The peripheral blood mRNA level of OXTR was increased, and that of ZEB1 was decreased in pregnant women with threatened premature labor. Progesterone helped to maintain pregnancy by readjusting the mRNA levels of OXTR and ZEB1.

Key Words:
Premature labor, Peripheral blood mRNA, OXTR, ZEB1.

Introduction

Premature labor is defined as labor before 37 weeks of gestational age. With an incidence of 10% to 12%, premature labor is the leading cause of neonatal mortality and morbidity. In recent years, more researchers have been investigating the mechanism of premature labor and have shown that premature labor is the result of interactions among many complex factors. Additionally, the unique nature of normal pregnancy and the delivery mechanism makes it challenging to understand how full-term labor is initiated and how uterine contraction is intensified during premature labor[1-3]; consequently, there remains a need to develop effective measures to prevent premature labor[4,5]. At present, tocolytics are the primary drugs for premature labor, but they have no significant effect in improving newborn outcomes[6]. Studies[3,7-9] have shown that the progesterone level plays a key role in maintaining the relative quiescent state of the uterus during pregnancy; a decrease in the progesterone level coupled with the activation of oxytocin receptor (OXTR) leads to intense inflammation, transforming the uterus from the quiescent state to the activated state and resulting in delivery or premature labor. Mammalian animal studies have shown that the balance between progesterone and its receptor plays a critical role in maintaining normal pregnancy[10]; other studies have shown that progesterone maintains uterus stability by inhibiting OXTR[11].

Renthal et al[12] used a mouse model of premature labor and showed that in the presence of progesterone, its receptor, zinc finger E-box binding homeobox 1 (ZEB1), a transcription inhibitor, binds to the ZEB1 mRNA initiation factor and increases its expression level. Moreover, ZEB1 inhibits the expression level of uterine contraction-related proteins, such as OXTR, and decreases binding of oxytocin to OXTR, thus terminating uterine contractions and preventing premature labor. ZEB1 plays a critical role in this

mechanism[1,13,14]. Given the results of animal models, we suggested that during pregnancy, the imbalance between the peripheral blood mRNA level of ZEB1 and other factors related to premature labor and low progesterone level lead to uterine and cervical inflammation, cervical ripening, and increased expression of uterine contraction-related proteins and, ultimately, premature labor. To date, however, few studies have been conducted on myometrium or the maternal peripheral blood mRNA level of ZEB1 in humans.

In this study, we investigated changes in the peripheral blood mRNA level of ZEB1 and OXTR before and after allylestrenol (progesterone) dosing in pregnant women and the effect of allylestrenol on threatened premature labor, thus providing a reference for clinical treatment to reduce birth morbidity and mortality.

Patients and Methods

Source of Specimens
(1) A total of 30 healthy pregnant women with singleton pregnancy who underwent perinatal care at the Third Affiliated Hospital of Zhengzhou University, China, between August and December 2015 were enrolled in this study; a peripheral blood sample (5 mL) was collected into an EDTA-treated tube. (2) A total of 30 pregnant women with singleton pregnancy hospitalized at the same hospital for threatened premature labor between August and December 2015 were enrolled in this study; a peripheral blood sample (5 mL each) was collected before progesterone dosing and 48 hours after progesterone dosing into an EDTA-treated tube. The normal pregnancy group was 27.50 ± 2.589 years old, with a gestational age of 31.11 ± 1.42 weeks, and the premature labor group was 27.43 ± 2.77 years old, with a gestational age of 31.08 ± 1.75 weeks; no significant between-group difference was observed (Table I). Blood samples from these two groups were used to test the mRNA levels of OXTR and ZEB1 and determine any changes in

the premature labor group. Each pregnant woman signed an informed consent form, and this study complied with the requirements of the Ethics Committee of the Third Affiliated Hospital of Zhengzhou University.

Detection of the Maternal Blood mRNA Levels of OXTR and ZEB1

Processing of Specimens
Once collected, the blood samples were immediately processed or temporarily stored at -80°C.

Peripheral Blood Total RNA Extraction and Reverse Transcription
Peripheral blood total RNA was extracted with TRI reagents (MRC, USA) according to the kit manual; once the RNA concentration and purity were verified with a spectrophotometer, reverse transcription was performed with a SuperRT cDNA first strand synthesis kit (Beijing Kangwei Shiji Biotechnology, Co., Ltd., China) as follows: the total reaction volume was 20 µL, including 4 µL of dNTP mix (2.5 Mm each), 2 µL of primer mix, 8 µL of RNA, and 6 µL of RNase-free water. After 10 minutes at 70°C, the reaction sample was placed in an ice bath for 2 minutes; next, 4 µL of 5 × RT buffer and then 1 µL of SuperRT (200 U/µL) were added and gently mixed with pipetting. After 50 minutes at 42°C and 5 minutes at 85°C, the product was stored at -20°C. All reaction solutions were prepared on ice. Unless otherwise specified, the centrifugal conditions were 12 000 g at 4°C for 10 minutes.

Quantitative Real-Time PCR
We searched the National Center for Biotechnology Information (NCBI) database for OXTR and ZEB1 sequences and used Primer Express 2.0 to design PCR primers to be synthesized by Generay Biotechnology Co., Ltd. (Shanghai, China). The primer sequences are shown in Table II. An UltraSYBR Mixture (Low ROX) kit (Beijing Kangwei Shiji Biotechnology, Co., Ltd., China) was

Table I. Age and gestational age of group A and group B/C.

Group	N	Age (years)	Gestational age (weeks)
1A	30	27.50 ± 2.58	31.11 ± 1.42
B/C	30	27.43 ± 2.77	31.08 ± 1.75
t		0.096	0.81
p		0.924	0.936

Table II. PCR primer sequences.

Primer name	Primer sequence
OXTR	Upstream: 5'-TGCTACGGCCTTATCAGCTT-3'
	Downstream: 5'-CTCCACATCTGCACGAAGAA-3'
ZEB1	Upstream: 5'-AAGTGGCGGTAGATGGTAA-3'
	Downstream: 5'-TTGTTGTATGGGTGAAGCA-3'
β-actin	Upstream: 5'-GACATCCGCAAAGACCTG-3'
	Downstream: 5'-GGAAGGTGGACAGCGAG-3'

used for the quantitative real-time PCR, and β-actin was used as the internal reference. The total reaction volume was 20 μL, including 10 μL of 2× UltraSYBR mixture, 0.4 μL each of the upstream and downstream primers, 2 μL of cDNA, and 7.2 μL of ddH$_2$O. The reaction condition was 95°C for 10 minutes; and 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 40 seconds. The fluorescence signal was detected at 60°C. Each sample was tested in triplicate on a Stratagene Mx3000P (Real-time PCR instrument; Invitrogen Life Science Technologies, Carlsbad, CA, USA). The final results were calculated as $2^{-\Delta Ct}$.

Statistical Analysis

SPSS (version 17.0; SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The data are expressed as the mean ± standard deviation ($\bar{x} \pm s$). Two independent sample t-tests were performed for between-group comparison of basic quantitative data, and the least significant difference test was performed for pairwise comparison of the mRNA level of OXTR and ZEB1. $\alpha = 0.05$ was set as the significance level.

Results

Assessment of Experimental Quality

All PCR products showed single-peak dissolution curves, with no non-specific amplification. The detection rate was 100% for maternal plasma OXTR, ZEB1, and β-actin mRNA.

The Maternal Peripheral Blood OXTR and ZEB1 mRNA Levels in Groups A, B, and C

The peripheral blood mRNA level of OXTR was higher in the group B than in the groups A and C ($p < 0.001$), and the mRNA level of ZEB1 was lower in the group B than in the groups A and C ($p < 0.001$) (Table III).

The Maternal Peripheral Blood OXTR and ZEB1 mRNA Levels in Groups A, B, and C

The relative mRNA level of OXTR was higher in group B than in groups A and C ($p < 0.001$), and the mRNA level of ZEB1 was lower in group B than in groups A and C ($p = 0.040$, $p = 0.002$) (Figure 1).
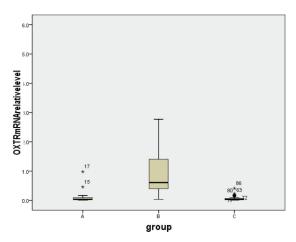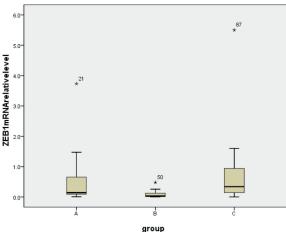
Discussion

Labor and delivery is a complex physiological process that is driven by both maternal and fetal factors[7]. In this study, we collected peripheral blood samples of pregnant women and found that in pregnant women with threatened premature labor, the mRNA level of ZEB1 was lower and the mRNA level of OXTR was higher than those of normal controls. At 48 hours after allylestrenol (progesterone) dosing, the peripheral blood mRNA levels of ZEB1 and OXTR were similar to those in the normal pregnancy group. Cortes-Priéto et al[15] showed that allylestrenol reduced myometrial sensitivity to oxytocin and stimulated underperforming placenta to secrete progesterone, thereby increasing the concentration of circulating progesterone and preventing premature labor.

Table III. The relative peripheral blood mRNA level of ZEB1 and OXTR.

Group	ZEB1 mRNA	OXTR mRNA
A	0.469 ± 0.725*	0.932 ± 0.188#
B	0.079 ± 0.108*	1.509 ± 2.308#
C	0.667 ± 1.021*	0.656 ± 0.859#
F	5.094	11.429
p	0.008	< 0.001

*$p_{A,B} = 0.040$, $p_{A,C} = 0.294$, $p_{B,C} = 0.002$; #$p_{A,B} < 0.001$, $p_{A,C} = 0.937$, $p_{B,C} = < 0.001$.

Figure 1. The maternal peripheral blood OXTR and ZEB1 mRNA levels in 3 groups.

gesterone dosing, the mRNA level of ZEB1 was close to normal. Thus, we concluded that ZEB1 mRNA can be used to predict and assess premature labor and that progesterone helps to prevent premature labor.

OXTR, an important inflammatory factor, plays a key role in the initiation and maintenance of delivery[6,23]. Fuchs et al[24] studied myometrium and decidua from pregnant women who underwent caesarean section or hysterectomy during pregnancy, and the results showed that OXTR level was low in early pregnancy, whereas in pregnant women with at least 37-week gestational age or premature labor, OXTR level and the sensitivity to oxytocin were significantly higher. In pregnant women with prolonged pregnancy (> 42-week gestational age), the OXTR level was significantly lower. These results indicate that OXTR plays an important role in pregnancy and delivery. In this study, we analyzed the maternal peripheral blood mRNA level of OXTR and found that it was significantly higher in pregnant women with premature labor than in those with normal pregnancy. Moreover, at 48 hours after progesterone dosing, the mRNA level of OXTR was similar between the two groups of pregnant women, which was consistent with the results from the animal study[12]. Hence, the maternal peripheral blood mRNA level of OXTR (per RT-PCR) may become a simple, useful indicator for predicting premature labor.

Brabletz et al[16] and Lehmann et al[17] found that ZEB1 played an important role in cell systems with epithelial to mesenchymal transformation (EMT). Abnormal EMT activation caused cancer, whereas ZEB1 inhibited intercellular E-cadherin, thereby reducing intercellular adhesion and resulting in the spread of cancer cells or metastasis[18]. Scholars[14,19-22] have shown that ZEB1 is overexpressed in breast cancer, prostate cancer, lung cancer, liver cancer, and uterine cancer. Renthal et al[12] used a mouse myometrium model of premature labor to show that ZEB1 mRNA was involved in cancer development and progression and deduced that ZEB1 mRNA may become an interventional target for premature labor in humans. In this investigation, we tested peripheral blood samples from pregnant women and showed that the mRNA level of ZEB1 was abnormally decreased in pregnant women with threatened premature labor, whereas at 48 hours after pro-

Conclusions

The mRNA levels of ZEB1 and OXTR are related to premature labor. Further, progesterone and its receptor directly act on ZEB1 to increase its expression and inhibit the mRNA level of OXTR, thus preventing premature labor. Premature labor is a terrible, persistent public health issue and must be further explored with bold innovative approaches.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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