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Original Article

HMGB1-RAGE signaling pathway in pPROM

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ABSTRACT

Objective: Increased inflammation of the placenta is considered as a risk factor and a promoter of preterm premature rupture of the membranes (pPROM). High-mobility group box 1 (HMGB1) is a recently identified inflammatory cytokine, and HMGB1-RAGE signaling pathway has been associated with many pathophysiological processes. This study aims to reveal the mechanisms of HMGB1-RAGE signaling pathway in pPROM.

Materials and methods: The mRNA levels of relative gene of HMGB1 pathway, HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2, were analyzed by real-time PCR in placentas collected from 60 normal term women, 60 women with PROM and 60 women with pPROM. Additionally, levels of HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 protein were detected in frozen placental specimens by western blot, and the locations of HMGB1, RAGE and NF-κBp65 were evaluated in the well-characterized tissue microarray (TMA) by immunohistochemistry. ELISA was further used to detect HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 level in maternal and cord serum.

Results: Compared with normal term and PROM women, we found that (1) The mRNA expressions of HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 in HMGB1-RAGE pathway of pPROM placentas were higher. (2) The protein levels of HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 in pPROM placentas were higher. (3) HMGB1 and RAGE immunoreactivity in pPROM placenta TMA were increased in the cytoplasm of syncytiotrophoblast (STB), extravillous trophoblast (EVT) and mesenchymal cells, while NF-κBp65 was enhanced in the nucleus of STB and EVT. (4) Maternal serum concentrations of HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 in pPROM group were greater. (5) Cord serum concentrations of HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 among the 3 groups had no significant differences.

Conclusion: HMGB1 nuclear-cytoplasmic translocation in pPROM placenta may lead to the binding of HMGB1 to its receptor RAGE, resulting in provoking NF-κBp65 activity, and then inducing the release of MMP-9 and MMP-2, which all above activities contributed to the process of pPROM. Consequently, HMGB1-RAGE signaling pathway may be involved in the pathogenesis of pPROM.

Introduction

Preterm premature rupture of the membranes (pPROM) is a major pregnancy complication, accounting for more than one-third of pregnancies ending before 37 weeks gestation as well as 1 out of 10 pregnancies of premature rupture of the membranes (PROM) [1,2]. Over the last decade, a substantial understanding of the molecular and pathophysiologic pathways involved in pPROM has been achieved, and several biomarkers have also been identified in women with pPROM [3–5]; however, none of them has lead to a reduction in the delivery of the fetus with pPROM. Inflammations of the placenta, maternal blood, cord blood and amniotic fluid are considered as risk factors and promoters of pPROM, which can reflect the infection status of intrauterine. Inflammatory cytokines

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in amniotic fluid have been investigated in many studies of pPROM [6–8]. However, amniosynthesis is an invasive procedure, which can result in some uncertain damages for mothers and their babies. Although maternal and cord blood can be obtained easier than amniotic fluid for measurement, cytokine levels have shown variable results in many studies in maternal and cord blood of pPROM [6,7,9,10]. This is the reason why we selected placenta, maternal blood and cord blood in our research.

High-mobility group box 1 (HMGB1) is a recently identified inflammatory cytokine, and the over-expression of HMGB1 and receptor for advanced glycation end-products (RAGE), one of specific receptors identified by extracellular HMGB1, have been reported involved in many pathophysiological processes, such as rheumatoid arthritis, liver injury, preeclampsia and tumors [11–14]. The binding of HMGB1 to RAGE is found to be associated with induction of nuclear factor-κB (NF-κB) activation and release of proinflammatory cytokines and chemokines such as tumor necrosis factor, Interleukin-6 and Matrix metalloproteinases (MMPs) [15]. NF-κB family is an upstream regulator of multiple labor-associated processes. During normal pregnancy, extracellular matrix (ECM) plays a pivotal role in the maintenance and structural integrity of membranes. MMPs, including MMP-9 and MMP-2, are known to be main mediators of ECM [16].

However, few studies can be available on the expression of HMGB1-RAGE signaling pathway in pPROM. To explore more precise mechanisms of pPROM, the mRNA and protein expressions of HMGB1-RAGE pathway, HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 in the placenta were investigated. Besides, the locations of HMGB1, RAGE and NF-κBp65 in the placental tissue microarray (TMA) were detected. Furthermore, the concentrations of HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 in maternal and cord serums were evaluated to investigate whether maternal and cord serum levels of them could be useful biomarkers for pPROM.

Materials and methods

Case selection

A total of 180 patients from the Third Affiliated Hospital of Zhengzhou University, from January 2014 to October 2016, were recruited in the study. They were divided into the following 3 groups: a) normal term group: Women at term with intact membranes undergoing cesarean delivery (n = 60); b) pPROM group: women with preterm premature rupture of the membranes (<37 weeks gestation) (n = 60); c) PROM group: women with rupture of the membranes and term birth (n = 60). Patients with dysfunctional of cervix uteri, abnormal fetal position, history of abortion or premature delivery, fetal malformations and multiple gestations were excluded from this study. The local Ethics Committee approved the study (No. 2014-26), and informed consent and written approval were obtained from all of the parents.

Sample collection

Five small separate biopsies were taken from the maternal aspects of placenta within 15 min after delivery, excluding areas of calcification, infarction and necrosis. Blood in the placental samples was removed with sterile filter papers. One sample was washed with 0.9% NaCl and then fixed in 10% formalin for TMA, the others were immediately stored at −80°C for the following experiments. Maternal venous blood samples were collected within 2 h after delivery, cord blood venous samples were obtained immediately after delivery by trained nurses. Blood samples were collected in EDTA tubes and centrifuged at 1500 g for 5 min, then serums were aliquoted and frozen at −80 °C until analysis.

Quantitative RT-PCR

To quantify the mRNA expressions of HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 in the 3 groups, quantitative RT-PCR (qRT-PCR) experiments were performed. RNA was extracted using trizol Reagent (Invitrogen, Carlsbad, California), and reverse transcribed into cDNA using the Reverse Transcriptase M-MLV kit (CWBIO, Beijing, China). The utilized primers were designed by Sangon (Inc, Shanghai, China) and are shown in Table 1.

The qRT-PCR was manipulated on an Applied Biosystems 7500 (ABI, Foster City, California) with the Ultra SYBR Mixture With ROX (CWBIO, Beijing, China). Cycling conditions were as follows: denaturation at 95 °C for 60 s and subsequent cycling (40 times) at 95 °C for 15 s, 60 °C for 15 s, 72 °C for 45 s, then data were collected and melt curve analysis was conducted. All the results were standardized against the expressions of GAPDH and run in triplicate. The $2^{-\Delta\Delta Ct}$ method was used to analysis all the target genes of mRNA expression level.

Western blot analysis

The total protein was extracted from placenta tissues with RIPA buffer containing 1 mmol/L phenylmethylsulfonyl fluoride, a protease inhibitor cocktail (Solarbio, Beijing, China). Crude protein in each sample was measured with a BCA assay (Sangon, Shanghai, China). Following quantification, the samples containing 80 mg of protein were separated by 10% sodium dodecyl sulfate (SDS) sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. The separated proteins were electrophoretically transferred to the nitrocellulose membranes. The membranes were blocked for 2 h at room temperature with blocking buffer (5% skim milk) and incubated overnight at 4 °C with anti-HMGB1 (1:1000 dilution, ab79823, Abcam), anti-RAGE (1:1000 dilution, ab37647, Abcam), anti-NF-κBp65 (1:500 dilution, ab32536, Abcam), anti-MMP-9 (1 μg/ml dilution, ab73734, Abcam) and anti-MMP-2 (0.5 μg/ml dilution, ab37150, Abcam) respectively. β-actin (1:1000 dilution, ab8227, Abcam) was used as an internal control for each sample.Then the membranes were incubated with the secondary fluorescent antibodies (1:5000 dilution, Odyssey CIX) for 1 h at room temperature. The fluorescence intensity was detected by Infrared Laser Scanning Imaging System (Odyssey CIX, LINCOLN, USA).

Immunohistochemistry in placenta

To determine the location and expression of HMGB1, RAGE and NF-κBp65 in the placenta, tissues were harvested, immediately frozen, and tissue microarray (TMA) technology was used, then prepared for immunohistochemistry. TMA technology can provide an opportunity to detect tissues on a large scale in a consistent manner. The process was as follows: TMA slides were deparaffinized

Table 1
Primers used for qRT-PCR quantifications.

Gene name	Product size	Direction	Primer sequence
HMGB1	248	Forward	TGAGCTCCATAGAGACAGCG
		Reverse	GCAGACATGGTCTTCCACCT
RAGE	225	Forward	GCTGTCAGCATCAGCATCAT
		Reverse	ATTCAGTTCTGCACGCTCCT
NF-κBp65	100	Forward	TCAATGGCTACTCAGGACCA
		Reverse	CGGCAGTCCTTTCCTACAAG
MMP-9	139	Forward	GGGACGCAGACATCGTCATC
		Reverse	TCGTCATCGTCGAAATGGGC
MMP-2	323	Forward	ACAGTGCATCTCAGCCCACATAGT
		Reverse	AAGCTCTGACCTTTCCAGCAGACA
GAPDH	220	Forward	GAGAAGGCTGGGGCTCATTT
		Reverse	GGACTGTGGTCATGAGTCCT

Table 2
Patients characteristics for in normal term (N), pPROM and PROM group.

Variables	N (n = 60)	pPROM (n = 60)	PROM (n = 60)	p
Maternal age (years)	27.40 ± 3.93	28.33 ± 4.91	27.80 ± 4.27	0.507
Gestational age (days)	274.68 ± 6.34	234.65 ± 19.86	270.43 ± 7.42	<0.001
Maternal BMI (kg/m^2)	29.84 ± 3.64	29.30 ± 3.45	30.55 ± 2.91	0.125
Neonatal weight (g)	3423.41 ± 459.26	2848.26 ± 513.58	3406.25 ± 663.53	<0.001
Apgar score (1 min)	9.82 ± 0.39	8.87 ± 0.54	9.65 ± 0.55	<0.001
Apgar score (5 min)	9.92 ± 0.33	9.28 ± 1.97	9.90 ± 0.35	0.004
Number of pregnancies	2.23 ± 0.95	2.38 ± 0.98	2.43 ± 1.06	0.521
Number of children	1.23 ± 0.50	1.27 ± 0.61	1.43 ± 0.67	0.148

twice with xylene for 5 min each, then sequentially transferred through 100%, 95%, 85%, 75% alcohol. Then, antigen recapture was performed on tissue sections using microwave treatment in 10 mmol/L sodium citrate buffer (pH 6.0) for 10 min. Immunohistochemistry was performed using the Cell and Tissue Staining Kit (R&D, California, USA). The sections were incubated overnight at 4 °C with anti-HMGB1 (1:150 dilution, ab79823, Abcam, USA), anti-RAGE (1:200 dilution, ab37647, Abcam, USA), and anti-NF-κBp65 (1:200 dilution, ab32536, Abcam, USA). TMA slides stained with PBS served as the controls. Hematoxylin was employed for counter staining the nuclei. The slides were examined using an inversion fluorescence microscope (OLYMPUS IX-71, Tokyo, Japan). Immunostaining of HMGB1, RAGE and NF-κBp65 in the placenta were graded on a semi-quantitative scale: 0, absent staining/no color; 1, weak staining/pale brown color; 2, distinct staining/dark brown color; 3, strong staining/brownish-black color. The intensity of immunostaining of each slide was separately evaluated by 2 pathologists.

ELISA Immunoassay

To investigate maternal and cord serum levels of HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 in pPROM, sandwich enzyme-linked immunosorbent assay (ELISA) was further employed in our study. The assays were strictly performed according to the manufacturers' protocol (USCN-Life, Science Inc, Wuhan, China). All of the specimens were tested in duplicate.

Statistical analysis

All data were reported as mean ± SD. Data comparisons between 2 groups were performed using *t*-test or a nonparametric Mann–Whitney, and one way analysis of variance (ANOVA) was used when comparing among 3 groups. For IHC in tissue microarray, the intensity of staining in placental tissues between the 3 groups was compared using the chi-square test. $p < 0.05$ was considered statistically significant. All of the statistical analyses were performed by SPSS 20.0.

Results

Clinical characteristics of the study population

Table 2 presents the clinical characteristics of patients with pPROM, PROM and normal term. Among the 3 groups, no significant differences were observed with regard to the following variables: maternal age, BMI at delivery, number of pregnancies and number of children. However, gestational age at delivery, the neonatal weight and the Apgar score of newborns in patients with pPROM were significantly lower compared with PROM and normal term patients.

HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 mRNA expression in the placenta

Results from our qRT-PCR experiments demonstrated mRNA expressions of HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 in the placentas were higher in pPROM group compared with PROM and normal term group ($p < 0.05$, Fig. 1). However, there was no significant difference between PROM and normal term group ($p > 0.05$).

HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 protein expression in the placenta

To determine the protein expression of HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 protein, western blot analysis was performed. Western blot confirmed the high expressions of HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 in pPROM when compared with the PROM and normal term groups ($p < 0.05$, Fig. 2).

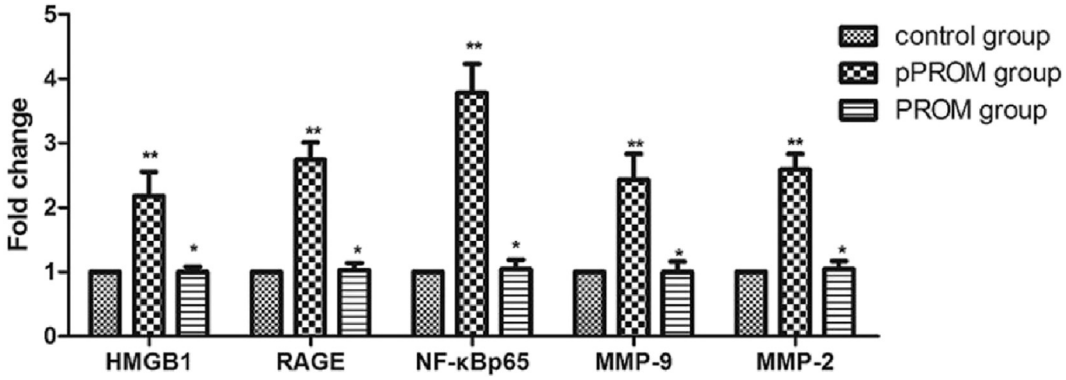

Fig. 1. Quantitative real-time PCR of HMGB1, RAGE, NF-κBp65, MMP-9, MMP-2 mRNA expression in placentas of the three groups. Compared with normal term group and PROM group, mRNA of HMGB1, RAGE, NF-κBp65, MMP-9, MMP-2 were upregulated in pPRPM group. For comparison of the three groups, the values of the normal term were arbitrarily set at 1. Bars indicated the means ± SD of the cases performed in triplicate. GAPDH was used as an internal control. ** versus normal term and PROM, $p < 0.001$; * versus normal term, $p > 0.05$.

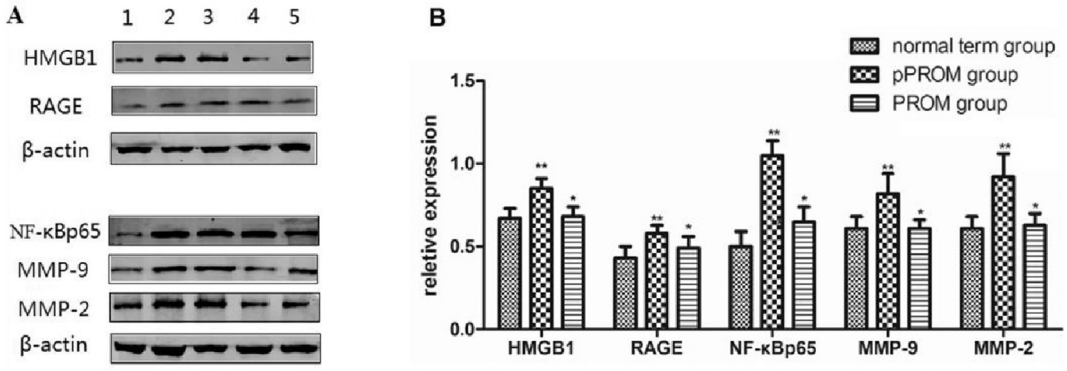

Fig. 2. The protein levels of HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 in the placentas by Western blot. A, Representative examples of HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 in placentas from normal term group, pPROM and PROM. 1, normal term; 2, 3, pPROM; 4, 5, PROM. **B,** Quantification of HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 Western blot data obtained from the 3 groups. Bars represented the mean values; error bars indicated SD. ** versus normal term and PROM, $p < 0.001$, * versus normal term, $p > 0.05$.

Similarly, protein relative expressions of HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 between PROM and normal term group had no statistical difference ($p > 0.05$).

Localizations of HMGB1, RAGE and NF-κBp65 in the placenta

Compared with normal term and PROM group, the immunoreactivity of HMGB1, RAGE and NF-κBp65 were significantly increased in pPROM patients ($c^2 = 22.29, 15.46, 24.09$, $p < 0.001$). In normal term and PROM group, HMGB1 mainly existed in nucleus of syncytiotrophoblast (STB) and extravillous trophoblast (EVT), and a weak immunolabeling of HMGB1 was present in nucleus of mesenchyme cells, while cytoplasmic HMGB1 expression was increased in patients with pPROM ($p < 0.05$). RAGE was mainly localized in the cytoplasm and at the membrane of the STB and EVT, while in pPROM group, RAGE was observed to be stronger in

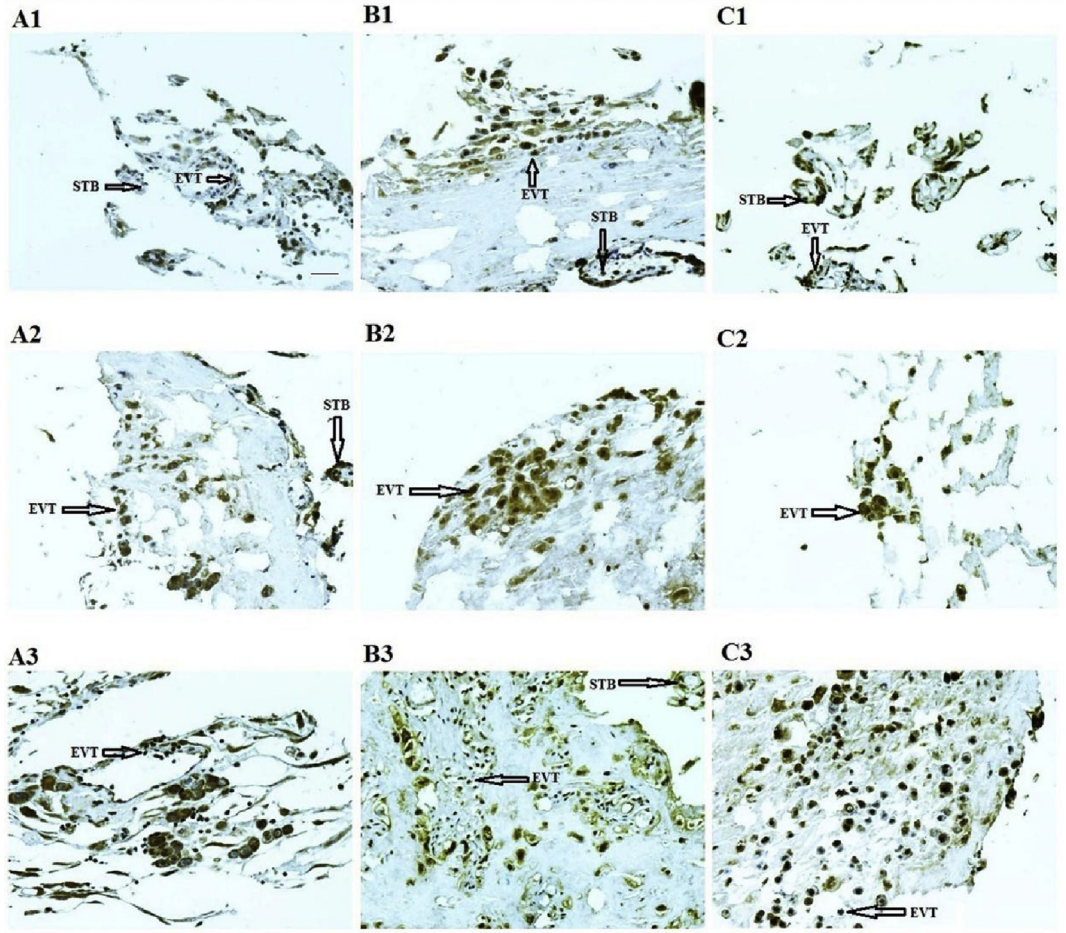

Fig. 3. HMGB1, RAGE and NF-κBp65 staining of placental tissue sections. A1, A2 and A3, HMGB1 staining of placental tissue sections in normal term pPROM and PROM group. B1, B2 and B3, RAGE staining of placental tissue sections in normal term pPROM and PROM group. C1, C2 and C3 NF-κBp65 staining of placental tissue sections in normal term pPROM and PROM group.

Table 3
Immunostaining of cytoplasmic HMGB1, RAGE and NF-κBp65 in normal term (N), pPROM and PROM group.

Immunostaining		Absent staining	Weak staining	Distinct staining	Strong staining	χ^2	p
HMGB1	N (n = 60)	12	28	14	6	22.29	<0.001
	pPROM (n = 60)	2	15	28	15		
	PROM (n = 60)	11	27	15	7		
RAGE	N (n = 60)	10	26	16	8	15.46	<0.001
	pPROM (n = 60)	3	14	27	16		
	PROM (n = 60)	12	24	15	9		
NF-κBp65	N (n = 60)	10	29	14	7	24.09	<0.001
	pPROM (n = 60)	1	16	24	19		
	PROM (n = 60)	11	28	13	8		

cytoplasm of above cells. NF-κBp65 was labeled in the nucleus of STB and EVT in normal term and PROM group, in addition, nuclear NF-κBp65 was increased in patients with pPROM ($p < 0.05$). What's more, there were no statistical differences of HMGB1, RAGE and NF-κBp65 expressions between women with PROM and normal term group respectively ($p < 0.05$, Fig. 3, Table 3).

In normal term and PROM, HMGB1 mainly existed in nucleus, while cytoplasmic HMGB1 expression was increased in patients with pPROM ($p < 0.05$). Compared with normal term and PROM, RAGE expression was significantly increased in pPROM patients ($p < 0.01$). Besides, nuclear NF-κBp65 was greatly increased in patients with pPROM ($p < 0.01$). STB indicated syncytiotrophoblast; EVT indicated extravillous trophoblast. Bars 20 mm.

HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 concentrations in serum

To assess whether HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 could be useful biomarkers for pPROM, concentrations of them in maternal and cord serum were measured. Interestingly, HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 levels in maternal serum were higher in pPROM patients compared to PROM and the normal term group ($p < 0.05$, Fig. 4). However, it is disappointing that, there were no obvious differences of HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 concentrations in cord serum among pPROM, PROM and the normal term groups ($p > 0.05$, Fig. 4).

Discussion

HMGB1 is a kind of non-histone protein, which primarily presents in eukaryotic cells [17,18]. As a major component of the nuclear protein group, the functions of HMGB1 are pleiotropic, such as genes transcription, DNA repair, cell differentiation and extracellular signal transduction, which depend on tissue type, location, post translational modifications and the receptor type utilized for signal transduction [19,20]. Recently, HMGB1 is identified as a transducer of innate immune system and causes inflammation-associated pathologies in many diseases [21–23]. Our data showed that the mRNA and protein levels of HMGB1 in placentas were significantly enhanced in patients with pPROM compared with PROM or normal term. Besides, increasing cytoplasmic HMGB1 of STB and EVT were detected in pPROM placenta, while in PROM and normal term group, HMGB1 mainly exists in nucleus of above cells, the result suggested that HMGB1 nuclear-cytoplasmic translocation may participate in the pathophysiological process of pPROM, which is in line with a recently report by Romero [24]. Furthermore, the maternal serum concentration of HMGB1 of pPROM is greatly increased compared to PROM and normal term pregnancy. This phenomenon probably indicates that HMGB1 may be a forecast molecule in pPROM.

Roberto discovered that pPROM patients with intrauterine infection had a higher median amniotic fluid concentrations of sRAGE and esRAGE than those without intrauterine infection [25]. Our data exhibited that RAGE was greatly enhanced in maternal serum of pPROM women. Similarly, both mRNA and protein levels of RAGE were increased in pPROM placenta. Besides, RAGE expression was significantly increased in the STB and EVT in patients with pPROM. The findings justify RAGE's role in the formation of placental disorders during inflammation, which was in line with previous studies [26,27].

Activation of RAGE includes a sequence of NF-κB binding motifs, thus making NF-κB an important regulator of RAGE [28]. Hofmann revealed that when RAGE signaling was inhibited, NF-κB activation in mononuclear phagocytes was suppressed [29]. In our study, high levels of mRNA and protein NF-κBp65 in pPROM placenta as well as enhanced maternal blood level in pPROM were noted when compared with normal term and PROM group. In addition, NF-κBp65 in the nucleus of STB and EVT was increased in patients with pPROM. This phenomenon probably indicates that NF-κBp65 was activated during pPROM. However, we didn't find significant changes of HMGB1, RAGE and NF-κBp65 between PROM and normal term groups, which could reveal that the pattern of placental dysfunction and increased inflammation of maternal serum in the preterm gestation can be significantly different from that in the term gestation regardless of rupture of membrane.

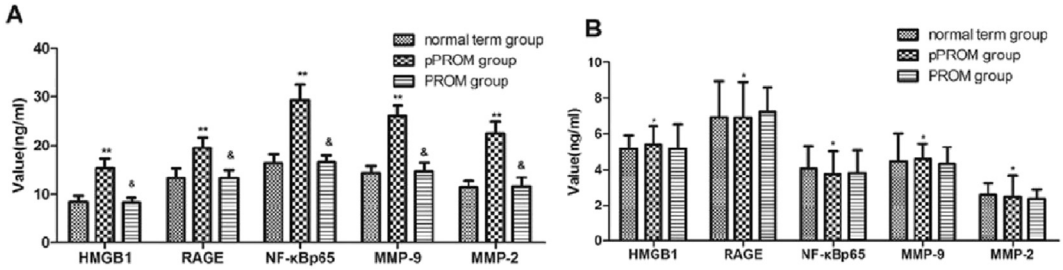

Fig. 4. HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 concentrations in maternal and cord serum of the three groups. A, HMGB1, RAGE, NF-κBp65, MMP-9, MMP-2 concentrations in maternal serum; **B,** HMGB1, RAGE, NF-κBp65, MMP-9, MMP-2 concentrations in cord serum. ** versus normal term and PROM $p < 0.001$; & versus normal term $p > 0.05$; * versus normal term and PROM $p > 0.05$.

MMP-9 and MMP-2 can regulate the catabolism of fetal membrane extracellular matrix proteins in amniotic membranes. Maymon indicated that PROM in the absence of infection is associated with a significant increase in the amniotic fluid concentration of MMP-9 [30]. Fortunato reported that MMP-2 was elevated in pPROM [31]. In our study, increased levels of MMP-9 and MMP-2 were noted in maternal serum [32]. Besides, our data exhibited that the mRNA expressions of MMP-9 and MMP-2 in placentas of pPROM patients were higher than those of PROM and normal pregnancies. Total protein expressions of MMP-9 and MMP-2 in placentas were also ascended in pPROM patients compared with other two groups. These results suggested that MMP-2 and MMP-9 played vital roles in the maintenance and structural integrity of membranes.

Cord blood can be extracted from discarded placentas immediately after delivery. Cord blood markers can reflect the intrauterine fetal environment and is associated with subsequent infections of mothers and newborns. In this study, we are trying to find potential cord blood markers of pPROM cytokine. It is disappointing that no obvious differences were found in the cord blood levels of HMGB1, RAGE, NF-κBp65, MMP-9 and MMP-2 among the 3 groups, suggesting that cord levels of these cytokines in HMGB1-RAGE signaling pathway demonstrated low predictive values to be used clinically.

Thus taken together, this data supported that in HMGB1-RAGE signaling pathway, HMGB1 nuclear-cytoplasmic translocation in pPROM placenta may lead to the binding of HMGB1 to its receptor RAGE, resulting in provoking NF-κBp65 activity, and then inducing the release of MMP-9 and MMP-2, which all above activities contributed to the process of pPROM. Consequently, HMGB1-RAGE signaling pathway may be involved in the pathogenesis of pPROM.

Conflict of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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