

# RAGE and HMGB1 expressions in fetal membranes of premature rupture of membranes patients

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#### ABSTRACT

**BACKGROUND** Premature rupture of membranes (PROM) often occurs in pregnancy. The fetal membrane weakening is caused by inflammation involving receptor activation for advanced glycation end-products (RAGE) and high mobility group box protein 1 (HMGB1). The associations between RAGE and HMGB1 with PROM are rarely studied. Hence, this study aimed to determine those associations in fetal membranes with PROM occurrence.

**METHODS** This case-control study was conducted at Dr. Ramelan Central Naval Hospital, Surabaya, Indonesia, from August to November 2019. The subjects, determined using a non-probability sampling method (a saturated sample), were divided into PROM and normal pregnancy with intact fetal membranes (control) groups. Fetal membrane specimens were collected during vaginal and cesarean section deliveries. The expressions of RAGE and HMGB1 were determined using the immunohistochemical method and further analyzed using the Mann-Whitney U test.

**RESULTS** The expression of RAGE in fetal membranes with PROM was significantly higher than the control (52.74% versus 14.9% expression/mm<sup>2</sup>,  $p < 0.001$ ), as well as the expression of HMGB1 (45.9% versus 8.5% expression/mm<sup>2</sup>,  $p < 0.001$ ).

**CONCLUSIONS** The higher expressions of RAGE and HMGB1 in fetal membranes were associated with PROM.

**KEYWORDS** fetal membranes, high mobility group box protein 1, immunohistochemistry, premature rupture of membranes, receptor for advanced glycation end products

Premature rupture of membranes (PROM) is defined as a spontaneous rupture of membranes any time after the 28<sup>th</sup> week of pregnancy but before the onset of labor. It occurs in 5–10% of all pregnancies, contributing to 30–40% of preterm labor cases (15 million cases per year),<sup>1</sup> which increases mortality and morbidity for mother, fetus, and neonate.<sup>2,3</sup> PROM arises from complex, multifaceted pathophysiologic pathways where the inflammation axis plays a crucial role.<sup>4</sup>

Recently, an increasing number of studies have shown the implication of sterile inflammation in the fetal membrane weakening of PROM.<sup>5,6</sup> This concept is dependent on specific molecules called alarmins or damage-associated molecular patterns (DAMP), which are released and recognized by pattern recognition receptors (PRRs), leading to a microbial-free inflammatory response or a sterile inflammation.<sup>1</sup> High-mobility group box 1 (HMGB1), which is a nuclear protein, is a DAMP molecule

that plays the most regulatory role throughout gestation.<sup>1,7</sup> It is an evolutionarily conserved protein that stabilizes nucleosome formation and facilitates gene transcription while localized in the nucleus yet acts as an alarmin when released extracellularly. This alarmin activates innate immune cells via PRR, such as receptors for advanced glycation end-products (RAGE), toll-like receptor (TLR)2, and TLR4 to initiate inflammatory responses.<sup>8</sup> *In vitro* studies of monocytes, neutrophils, dendritic cells, and endothelial cells have demonstrated that HMGB1 promotes the activation of pro-inflammatory transcription factor nuclear factor kappa B (NF- $\kappa$ B) that, in turn, induces the production of pro-inflammatory cytokines, including tumor necrosis factor- $\alpha$ , interleukin (IL)-8, IL-6, and IL-1 $\beta$ . However, the mechanisms whereby HMGB1 induces a sterile inflammatory response in the amniotic cavity are poorly understood.<sup>8</sup>

RAGE, a member of the immunoglobulin superfamily of receptors, is a 55 kDa cell surface receptor that interacts with several ligands (including HMGB1) implicated in the pathogenesis of many inflammatory diseases.<sup>9,10</sup> RAGE is known to activate pro-inflammatory pathways, release cytokines, and contribute to the fetal membrane weakening.<sup>11</sup>

The activation of RAGE and HMGB1 plays a major role in causing PROM. The interactions between RAGE and HMGB1 with several other inflammatory diseases have been widely discussed. However, their association with PROM is rarely studied, and studies on fetal membranes are limited.<sup>4,11-13</sup> This study aimed to discover the expressions of RAGE and HMGB1 in fetal membranes of PROM compared with the control.

## METHODS

### Subject participant

A case-control study was performed to compare the expressions of RAGE and HMGB1 in the fetal membranes of pregnant women. A total of 40 pregnant women aged 17–44 years with a gestational age of 20–41 weeks and singleton pregnancies were recruited at Dr. Ramelan Central Naval Hospital, Surabaya, Indonesia, from August to November 2019. The subjects were divided into PROM and control groups. PROM was defined as spontaneous rupture of the fetal membranes at any time before the onset of regular uterine contractions. The control group consisted of pregnant women who had term and

preterm deliveries with intact fetal membranes and no significant complications. The exclusion criteria were subjects with a history of the previous PROM, uterine malformations, polyhydramnios, preeclampsia, immunological disorders, antepartum hemorrhage, or any maternal systemic infection (pneumonia, urinary infection, etc.), and those who refused to participate in the study. All procedures have been approved by the Ethics Committee of the Faculty of Medicine, Universitas Hang Tuah (No. 1/136/UHT.KEPK.03/VIII/2021), and informed consent was obtained from all patients.

### Sample collection

Fetal membrane specimens collected during vaginal and cesarean section deliveries in both groups were obtained from tissues at a clear distance (1–2 cm) from the edge of the cervix. It was then fixed in 10% formalin, embedded in paraffin, sectioned consecutively at 5  $\mu$ m, and stored until further immunohistochemical staining.

### Immunohistochemical staining

All sections were deparaffinized in xylene and rehydrated via a graded series of ethyl alcohol (90%, 70%, and 50%), followed by a buffer solution of phosphate-buffered saline pH 7.2. Next, antigen retrieval was carried out using pH 10 buffer solution, blocking non-specific bonds with 1% hydrogen peroxide for 15 min, and incubated in 5% goat serum (Cat No. PCN5000, USA) for RAGE and 5% donkey serum (Cat No. 0030-01, USA) for HMGB1 for 1 hour. These were then immersed in goat anti-RAGE polyclonal antibody (Cat No. MBS6453394, Singapore) solution for RAGE and rabbit polyclonal anti-HMGB1 (Cat No. 10829-1-AP, Singapore) for HMGB1 overnight at 4°C, followed by signal amplification by avidin-biotin staining and incubated in a 3,3'-diaminobenzidine solution as a chromogen for staining. The immunohistochemical staining results were assessed by light microscope and analyzed using ImageJ software (National Institutes of Health, USA).

### Statistical analysis

All statistical analyses were performed using SPSS software version 23.0 (IBM Corp., USA). The values were reported as mean (standard deviation). Significant differences (*p*) between groups were evaluated using the Mann–Whitney *U* test, with *p* < 0.05 was considered statistically significant.

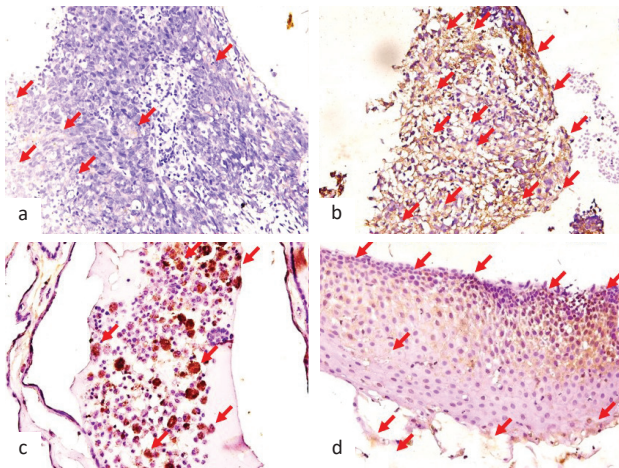
## RESULTS

From both groups, the youngest subjects were 23 years old, while the oldest were 44 years old. Most of the subjects had term gestational age.

**Table 1.** Demographic and clinical characteristics of the subjects

Variables	PROM, n (%) (N = 20)	Control, n (%) (N = 20)
Age (years)		
21–25	5 (25)	3 (15)
26–30	6 (30)	10 (50)
31–35	4 (20)	3 (15)
36–40	2 (10)	3 (15)
>40	3 (15)	1 (5)
Gestational age		
Preterm	6 (30)	2 (10)
Term	14 (70)	18 (90)
Gravida		
Primigravida	6 (30)	7 (35)
Multigravida	14 (70)	13 (65)
Delivery method		
Vaginal delivery	17 (85)	18 (90)
Cesarean section	3 (15)	2 (10)

PROM=premature rupture of membranes

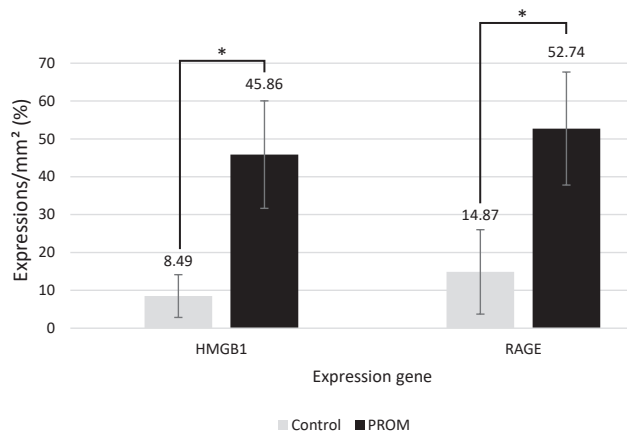


**Figure 1.** Immunohistochemical examination of RAGE and HMGB1 expressions seen under a microscope at 400× magnification. Expression of RAGE in fetal membranes cell in the (a) control and (b) PROM groups; expression of HMGB1 in fetal membranes in the (c) control and (d) PROM groups (positive RAGE and HMGB1 expressions are marked with red arrows). HMGB1=high mobility group box protein 1; PROM=premature rupture of membranes; RAGE=receptors for advanced glycation end-products

Maternal age, gestational age, number of gravidas, and delivery method can be seen in Table 1. The RAGE and HMGB1 expressions were positive in both groups (Figure 1). However, it was higher and more widely distributed in the PROM group than in the control group ( $p < 0.001$ ) (Figure 2).

## DISCUSSION

RAGE and HMGB1 were expressed in both PROM and control cases but were higher in PROM cases. HMGB1-RAGE signaling pathway may be involved in the pathogenesis of preterm PROM. PROM causes accelerated senescence, resulting in a premature aging syndrome of the fetal membranes. Premature aging of the fetal membranes is mediated by DAMP and senescence-associated secretory phenotype (SASP), which are uterotonic and used as a marker of delivery time.<sup>14</sup> HMGB1 protein is normally located in the nucleus; however, the protein will be released into the extracellular space in response to oxidative stress, cytokines, bacterial antigens, damage, and cell death, which could trigger PROM.<sup>15,16</sup> This extracellular HMGB1 protein will then act as an alarmin or DAMP together with SASP to mediate the inflammatory response by diffusion to nearby tissues or entering the systemic pathway. The HMGB1 protein translocation depends on p53, where p53 activation is one of the main pathways for senescence.<sup>16</sup> According to Bredson et al,<sup>17</sup> SASP production requires activation of the transcription factor NF- $\kappa$ B by p38 mitogen-activated protein kinase (MAPK). Meanwhile, HMGB1



**Figure 2.** HMGB1 and RAGE expressions ( $\text{mm}^2$ ) in fetal membranes in the PROM and control groups. HMGB1=high mobility group box protein 1; PROM=premature rupture of membranes; RAGE=receptors for advanced glycation end-products. \*Mann-Whitney U test,  $p < 0.001$

can activate p38 MAPK, which could then form a cycle that worsens the inflammation.<sup>17</sup>

The difference in the mean expression of HMGB1 in this study is in line with Romero et al,<sup>18</sup> who found a higher concentration of HMGB1 in the amniotic fluid of patients with PROM than in patients without PROM. This is also in agreement with the results of Baumbusch et al's<sup>19</sup> study, where cells in fetal membranes with intraamniotic inflammation had a higher HMGB1 expression.

Under normal conditions, RAGE is expressed as a marker of ligands (such as DAMPs and pathogen-associated molecular pattern molecules) in the innate immune response. However, long-term accumulation of ligands will increase the regulation of the RAGE expression.<sup>20,21</sup> This condition indicates that PROM caused by the increased distribution of RAGE expression in fetal membranes does not occur spontaneously but precedes a chronic inflammatory process. In contrast, this receptor reduces the inflammatory process in acute inflammation and can increase cell regeneration in several diseases. However, acute inflammatory pathways other than RAGE-mediated inflammation also appear to induce fetal membrane rupture.<sup>22</sup> PROM caused by significant expression of RAGE on fetal membranes is also in line with a previous study. Choltus et al<sup>23</sup> exposed cigarette smoke condensate (CSC) to fetal membranes and found an increase in toxicity to amniotic epithelial cells, marked by an increased release of lactate dehydrogenase and HMGB1 from the cells. The amniotic epithelial cells in the treatment group were only given CSC, while the second treatment group was given additional RAGE antagonist peptide (RAP). The expression of RAGE and the three intracellular adapter proteins were assessed using reverse transcription polymerase chain reaction, namely TIRAP, MyD88, and diaphanous-1. Without induction through RAGE, the activation of intracellular pathways such as NF- $\kappa$ B, IL-1 $\beta$ , IL-6, and IL-8 was inhibited. In addition, treatment with RAP also inhibited the expression of matrix metalloproteinase (MMP)-2, MMP-9, and gelatinase, which were important in the integrity of the fetal membranes.<sup>23</sup>

This study clearly illustrated the important influence of HMGB1 and RAGE on the continuation of the inflammatory sequence in the fetal membranes that caused PROM.<sup>23</sup> Several intervention methods have been carried out and proven successful in

inhibiting HMGB1-mediated inflammation. For example, recombinant HMGB1 box A protein can inhibit endocytosis of the HMGB1-lipopolysaccharides complexes that mediated by RAGE, peptide P5779 for the HMGB1-TLR4 pathway, resveratrol and dexmedetomidine for TLR4, and anti-HMGB1 monoclonal antibody m2G7 for the HMGB1-RAGE and HMGB1-TLR4 pathways. Meanwhile, thrombomodulin, haptoglobin, metformin, and DNA-conjugated beads have been known to have antagonistic effects on HMGB1.<sup>24</sup> Several inhibitors have also been shown to inhibit RAGE interactions according to their domain location, namely TTP488, 4,6-disubstituted 2-amino pyrimidines, FPS-ZM1, and aptamer-based antagonist in the type V extracellular domain as well as GM-1111 and S100-derived peptide in the V-C1-C2 type extracellular domain. Moreover, a group of 13 compounds inhibited RAGE interaction in the intracellular domain.<sup>14,25</sup> All of these blockages potentially interfere with HMGB1 and RAGE-mediated inflammations. However, further study is needed to find out whether this can be used to prevent PROM.

This study had several limitations. Although this study had shown the difference between RAGE and HMGB1 expressions in the PROM and control subjects, only a limited number of samples were included. Therefore, further research with a more significant number of samples with different methods should be conducted.

In conclusion, the higher expressions of RAGE and HMGB1 in fetal membranes are associated with PROM. These expressions have an important role in the continuation of the inflammatory process in the weakening of fetal membranes. Hence, inhibiting their activities will potentially prevent PROM.

#### Conflict of Interest

The authors affirm no conflict of interest in this study.

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