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# Expression of High Mobility Group Box-1 (HMGB1) and Nuclear Factor Kappa-B (NF-κB) in Fetal Membranes of Premature Rupture of Membranes

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## Abstract

**Background:** Inflammation, either sterile or infection-related, may lead to premature rupture of membranes (PROM). The non-histone nuclear proteins, high mobility group box-1 (HMGB1), and nuclear factor kappa-B (NF-κB) transcription factors have been extensively investigated in many disorders involving inflammatory reactions. This study aimed to determine the expression of HMGB1 and NF-κB in fetal membranes of PROM compared with non-PROM.

**Methods:** This study was an analytical observational study with a case-control design, performed from November 2021 to January 2022, including 40 fetal membrane samples (20 PROM and 20 non-PROM), which were obtained from pregnant women treated in the emergency unit of a hospital in Surabaya from August to November 2019 using the non-probability sampling method. The HMGB1 and NF-κB expressions were examined using the immunohistochemical method and further viewed under a light microscope (400x magnification), then assessed by Image-J software. The values between PROM and non-PROM were then compared and analyzed using the Mann-Whitney U test.

**Results:** There was a significant difference ($p<0.001$) in the expression of HMGB1 and NF-κB in PROM and non-PROM for HMGB1 45.86±14.21% vs. 8.50± 5.66% expression/mm$^2$; and NF-κB 33.47±5.45%vs.7.29±4.90% expression/mm$^2$, respectively.

**Conclusion:** PROM groups have significantly higher expression of HMGB1 and NF-κB s, indicating higher activity and contribution to PROM.

**Keywords:** Fetal membranes, HMGB1, NF-κB, premature rupture of membranes

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## Introduction

Premature rupture of membranes (PROM) contributes to 30% of preterm births in developed nations, which is one of the leading causes of poor outcomes, including maternal and neonatal death.[1,2] PROM is defined as the rupture of the fetal membranes before labor begins. It is called term PROM if it occurs ≥37 weeks of gestation and preterm PROM if it occurs before 37 weeks of gestation. The prevalence of PROM worldwide ranges from 5–10% of all births.[3]

The fetal membranes are a bilayer tissue composed of an inner dense collagen layer (amnion) and an outer cellular layer (chorion), which protects the fetus until delivery.[4] The presence of biochemical changes and increased physical stress on the fetal membranes causes the fetal membrane integrity to deteriorate, induces apoptosis, decreases collagen components and its activity, and even triggers

an inflammatory response that results in focal damage.[5]

Pregnant women are susceptible to a variety of risk factors that trigger inflammatory responses, including sterile inflammation and infection-related inflammation. The presence of bacterial antigens, oxidative stress, cytokines, tissue damage, and necrosis will cause cells to secrete high mobility group box-1 (HMGB1). HMGB1 is a nuclear protein that binds to chromatin and is present in all cell types. It is normally localized in the nucleus or scattered in the cytoplasm and plays an important role in regulating gene transcription, nuclear homeostasis, and genome stability. However, HMGB1 will be released into the extracellular space in response to various stimuli. HMGB1 can be released passively upon cell death or actively secreted due to inflammatory signals from activated immune cells.[6,7] These will act as damage signals called damage-associated molecular patterns (DAMPs) and interact with various receptors, either HMGB1 itself or forming complexes with other pro-inflammatory molecules.[6,8] HMGB1 mediates the inflammatory process by binding to various receptors, such as the receptor for advanced glycation end-products (RAGE) and toll-like receptors (TLRs). These receptors generate various signaling pathways that will activate nuclear factor kappa-B (NF-κB). NF-κB is a transcription factor that has a major role in the inflammatory process; therefore, its activation results in an increased inflammatory state, that decreases the integrity of the fetal membranes.[6,8,9]

HMGB1 and NF-κB have been shown to play a role in various physiological processes of labor.[10,11] Research on the inflammatory process involving HMGB1 and NF-κB has also been done in various inflammatory diseases, including PROM. However, research on HMGB1 and NF-κB in fetal membranes is still limited. Therefore, this study aimed to find out whether there were differences in the expression of HMGB1 and NF-κB in PROM and non-PROM fetal membranes.

## Methods

This research was an analytical observational study with a case-control design performed from November 2021 to January 2022 in Dr. Ramelan Central Naval Hospital Surabaya, which was a continuation of previous research conducted in 2019 regarding the histopathological examination of foetal membranes from PROM and non-PROM. The sample selection process, written informed consent to research subjects, sampling, and sample paraffinization process were carried out in previous study.

Pregnant women, both preterm (gestational age <37 weeks) and term (gestational age ≥37 weeks) with PROM and non-PROM in the Emergency Unit of Dr. Ramelan Central Naval Hospital Surabaya from August 2019 to November 2019 was recruited using the following criteria. The PROM patients selected should be pregnant women aged 17–44 years, singleton pregnancy with gestational age $20^{0/7}$–$41^{6/7}$ weeks. Pregnant women with preeclampsia, gestational diabetes, foetuses with congenital abnormalities, and subjects who refused to participate were excluded. Meanwhile, non-PROM patients selected should be pregnant women aged 17–44 years, singleton pregnancy with a gestational age of $20^{0/7}$–$41^{6/7}$ weeks, without complications. Pregnant women with a history of childbirth due to previous premature rupture of membranes, infections, foetuses with congenital abnormalities, and study subjects who refused to participate were excluded.

The sample selection method used was non-probability sampling with a saturated sample. All subjects who met the research criteria were sampled consecutively over a set duration, yielding a sample of PROM (n=20) and non-PROM (n=20).

Immunohistochemistry (IHC) staining was performed to detect the expression of HMGB1 and NF-κB in PROM and non-PROM foetal membranes. IHC staining of HMGB1 was done by the following procedure. Five µm foetal membranes of each participant were deparaffinized using xylene. Then rehydration was performed with graded ethanol, followed by potassium phosphate buffer solution (pH 7.2). After antigen retrieval with buffer solution (pH 10) , the sections were soaked for 15 minutes in 1% $H_2O_2$. Then, sections were incubated in 5% donkey serum for one hour, followed by incubation at 4 °C for one night in rabbit polyclonal anti-HMGB1 (1:1,000 dilution). Biotinylated donkey anti-rabbit IgG was used for detection, followed by a signal amplification process using avidin-biotin labeling. Samples were incubated in the chromogen 3,3'-diaminobenzidine (DAB) solution.

IHC staining of NF-κB was done with the following procedure. Fetal membranes with a size of five µm were deparaffinized using xylene, rehydrated with graded ethanol, and washed with running water for five minutes.

Then, the sections were incubated with Link Dako Epitope Retrieval for approximately one hour and soaked in tris buffered saline (TBS) with pH 7.4 for five minutes, followed by blocking with $H_2O_2$ for 5–10 minutes. The slides were rinsed with TBS, blocked using 3% normal horse serum (NHS) for 15 minutes, and rinsed again with TBS. Then, slides were incubated with NF-κB antibody (1:40 dilution) for one hour and rinsed with TBS.The slides were re-incubated in Dako Real Envision Rabbit for 30 minutes, then rinsed with TBS. The sections were immersed in DAB and substrate chromogen solution (20 µL:1,000 µL) for five minutes. Samples were rinsed with running water and counterstained with hematoxylin and rinsed again. After being immersed in lithium carbonate (5% in aqua), the sections were dehydrated with graded ethanol and cleared using xylene. Sections were mounted and attached by cover glass.

Five fields of view were randomly selected from each slide after the IHC staining process was completed and viewed under a light microscope at 400x magnification. The expressions of HMGB1 and NF-κB were then analyzed using Image-J software. Values range from 0% expression/mm$^2$ (no expression per mm$^2$) to 100% expression/mm$^2$ (full expression per mm$^2$). The average expression value was obtained from five fields of view of each slide, then analyzed.

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) v. 23 for windows. The result of the Shapiro-Wilk normality test showed that the data was not normally distributed. The analysis was then continued with the Mann-Whitney U test. The data was statistically significant if $p<0.05$. This study was approved by the Health Research Ethics Committee, Faculty of Medicine, Hang Tuah University, Surabaya (I/140/UHT.KEPK.03/VIII/2021).

## Results

The youngest subject from both groups was 23 years old, the eldest subject from the PROM group was 44 years old, and the eldest subject from the non-PROM group was 41 years old. The eldest gestational age in both groups was 41 weeks, whereas the youngest gestational age in the PROM groups was 29 weeks, and in the non-PROM group was 35 weeks. All subjects of maternal age, gestational age, parity, and delivery method were depicted in Table 1.

HMGB1 expression was positive in both groups. The mean expression in the PROM group (45.86±14.21% expression/mm$^2$) was higher than the non-PROM group (8.50± 5.66% expression/mm$^2$). Figure 1a showed that HMGB1 was more prominently expressed in the cytoplasm of fetal membrane cells and the extracellular space, whereas Figure 1b showed HMGB1 expression in the nucleus and cytoplasm. Mann-Whitney U analysis showed that there was a significant difference in HMGB1 expression in both groups ($p<0.001$). Minimum, maximum, mean, and standard deviation (SD) value were provided in Table 2.

NF-κB expression was positive in both

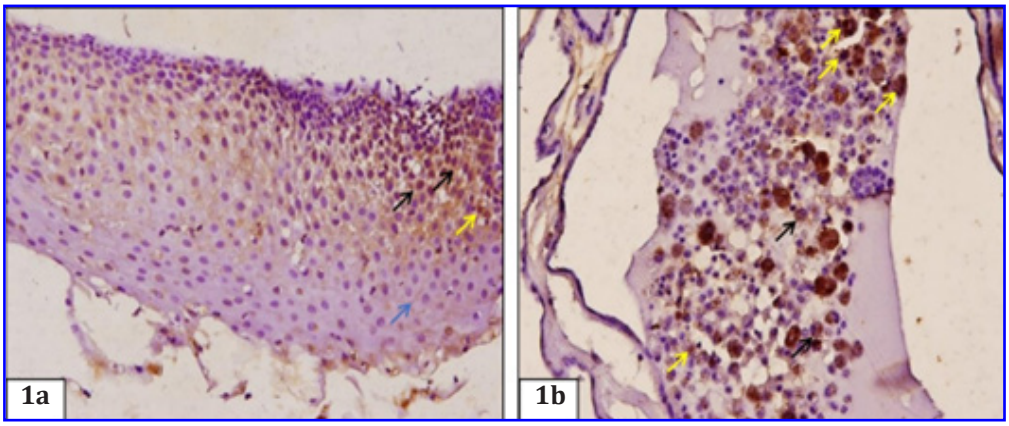


**Figure 1 Immunohistochemical Staining of HMGB1 Expression in Fetal Membranes from PROM (a) and Non-PROM (b) Groups.**

Note: Positive HMGB1 expression showed in brown color, black arrows= stained cytoplasm, yellow arrows= stained nucleus, blue arrows= stained extracellular space)

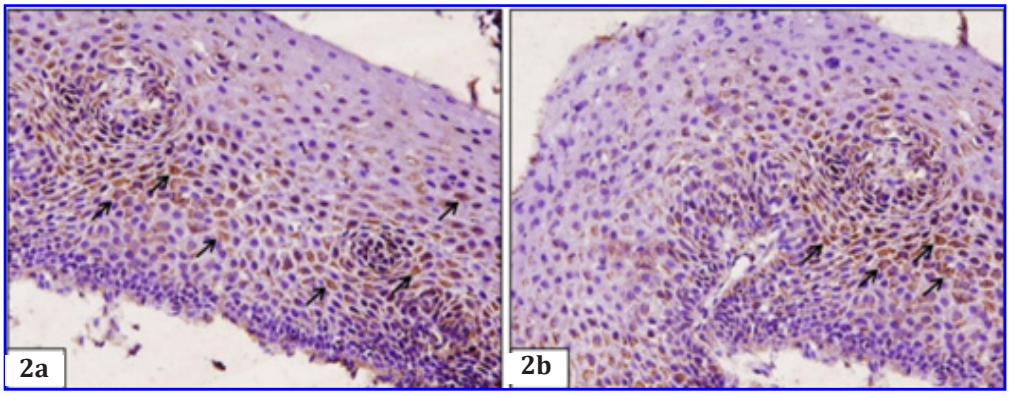


**Figure 2 Immunohistochemical Staining of NF-κB Expression in Fetal Membranes from PROM (a) and non-PROM (b) groups.**

Note: Positive NF-κB expression showed in brown color, black arrows= stained cytoplasm

groups, with the mean expression in the PROM group (33.47±5.45% expression/mm$^2$) being higher than the non-PROM group (7.29±4.90% expression/mm2). Both groups showed NF-κB expression in the cytoplasm of fetal membrane cells (Figure 2a and 2b). There was a significant difference in NF-κB expressions in both groups ($p<0.001$) (Table 2).

## Discussion

This study showed that HMGB1 expression was significantly increased in PROM compared to non-PROM. This finding is in line with other research on placental HMGB1 mRNA expression, serum HMGB1 levels, and concentration of HMGB1 in the amniotic fluid.[9,12]

The expression of HMGB1 is more prominent in the cytoplasm and extracellular space than in the nucleus, meanwhile staining of the nucleus seemed strong (Figure 1). The presence of more HMGB1 in the cytoplasm than in the nucleus in the PROM group indicates that nuclear HMGB1 has translocated from the nucleus to the cytoplasm or extracellular space. In contrast, there is still prominent nuclear staining in non-PROM, indicating that the inflammation is not as severe as PROM and that there is still a lot of nuclear HMGB1 that has not been translocated. Translocated HMGB1 will be packaged in exosomes or through secretory lysosomes to be released from the cell. Before being released into extracellular space, HMGB1 will be acetylated to prevent the HMGB1 from re-entering the

**Table 1 Characteristics of the Subjects from PROM and non-PROM Groups**

| Characteristics | PROM n=20 | Non-PROM n=20 | p-value |
|---|---|---|---|
| Maternal age (years) | 30.75±6.62* | 29.95±4.98* | |
| <20 and >35 (n, %) | 5 (25) | 4 (20) | 0.946 |
| 20–35 (n, %) | 15 (75) | 16 (80) | |
| Gestational age (weeks) | 37.15±3.15* | 39.05±1.67* | |
| Preterm (n, %) | 6 (30) | 2 (10) | 0.028** |
| Term (n, %) | 14 (70) | 18 (90) | |
| Parity | | | |
| Primigravid (n, %) | 6 (30) | 7 (35) | 0.736 |
| Multigravid (n, %) | 14 (70) | 13 (65) | |
| Method of delivery | | | |
| Vaginal birth (n, %) | 17 (85) | 18 (90) | 1.000 |
| Caesarean section (n, %) | 3 (15) | 2 (10) | |

Note: *= mean±SD, **= $p<0.05$ considered as significant

**Table 2 Immunohistochemistry Examination Results of HMGB1 and NF-κB Expressions in Fetal Membranes**

| | PROM | | | Non-PROM | | | p-value |
|---|---|---|---|---|---|---|---|
| | Min | Max | Mean± SD | Min | Max | Mean± SD | |
| HMGB1 (% expression/mm$^2$) | 22.78 | 68.35 | 45.86±14.21 | 0.83 | 17.31 | 8.50±5.66 | <0.001** |
| NF-κB (% expression/mm$^2$) | 26.27 | 47.08 | 33.47±5.45 | 2.58 | 18.52 | 7.29±4.90 | <0.001** |

Note: **= p<0.05 considered as significant

nucleus.[8,13]

Various risk factors associated with preterm birth and premature rupture of membranes (infection, obesity, antioxidant deficiency, malnutrition, smoking, alcohol, and certain drug consumption) can increase the formation of oxidative stress and are often associated with senescence in amniotic membrane cells.[8,14] Another study has proven that exposure to cigarette smoke extract and lipopolysaccharides (LPS) on the placental membrane can cause an increase in oxidative stress, resulting in a sterile inflammatory response and triggering senescence. Senescent cells will secrete DAMPs, in this case represented by HMGB1, which is indicated by the finding of an increase in cytosolic HMGB1; therefore the loss of nuclear HMGB1 can be used as a marker of senescence, which is in accordance with the results found in this study.[15]

Extracellular HMGB1 will produce inflammatory effects on fetal membranes depending on HMGB1 levels, and activation of senescence in fetal membranes occurs mainly through binding HMGB1 to TLR-2 and TLR-4 receptors. Moreover, in the state of infection, bacterial antigens such as polysaccharide peptidoglycan and LPS can also increase the expression of TLR-2, TLR-4, and RAGE receptors, so that the possibility of HMGB1 to bind to these receptors increases. HMGB1 has also been reported to form complexes with LPS derived from gram-negative bacteria, binding TLR-4 and RAGE. The binding of HMGB1 to the receptor causes NF-κB to be activated.[6,8,16]

NF-κB consists of various subunits (p50/p105, p52/p100, p65, c-Rel, and RelB) that have DNA-binding dimers and function as transcription factors. In the inactive state, NF-κB dimers are resting in the cytoplasm. Their activity can then be triggered when certain receptors on the cell trigger a signaling process that results in the translocation of NF-κB into the nucleus, where it then binds to DNA and activates gene transcription to produce various pro-inflammatory cytokines, adhesion molecules, and chemokines.[17,18] This study found that the mean expression of NF-κB was higher in PROM patients, indicating increased NF-κB activity in fetal membrane cells compared to non-PROM patients. These findings are in line with studies on the expression of NF-κBp65 mRNA in the placenta and its concentration in maternal blood.[9,18] Its expression can be seen in Figure 2a and 2b where the expression is localized mainly in the cytoplasm.

Local and hormonal stimuli awaken NF-κB from its dormant state in the cytoplasm, where it binds to the inhibitory protein kappa B protein a (IκBa), thus preventing translocation. When cytokines, prostaglandins, free radicals, and steroid hormones are out of balance before delivery, the activity of NF-κB in the fetal membranes increases, predisposing to preterm labor and PROM.[19] NF-κB induced pro-inflammatory activity has been shown to increase IL-1β, TNF-α, IL-6, IL-8, and IFN-γ which act as pro-inflammatory cytokines and chemokines that recruit many leukocytes, as well as matrix metalloproteinases (MMPs), particularly MMP-1, MMP-2, MMP-8, and MMP-9, which play an important role in degrading the extracellular matrix.[11,14,20] Active NF-κB also promotes RAGE expression, creating an endless pro-inflammatory loop, allowing further damage in fetal membranes.[21]

The results of this study are supported by other studies findings that HMGB1 has a direct impacts on expression and activity of NF-κB.[22] Both play an important role in inducing an inflammatory process that weakens the fetal membranes, thereby allowing rupture. These ruptures are considered irreversible because most patients will give birth within a short time. Fetal membranes with minor rupture, frequently as a result of a medical procedure such as amniocentesis, has been reported to heal on its own. However, large ruptures,

particularly in the zone of altered morphology lying above the cervix, will be difficult or even irreparable.[5,23]

We speculate that inhibiting HMGB1 and NF-κB activity could help prevent fetal membranes rupture in high risk patients. Various interventions that may inhibit pathways mediated by HMGB1 and NF-κB have been investigated in many inflammatory diseases, including preeclampsia, bacterial keratitis, and spinal cord injury.[7,21,24–26] Unfortunately, there is no research on PROM has been conducted. Therefore, further investigation into the possibility of preventing PROM by inhibiting the activity of HMGB1 and NF-κB is required.

Although this study showed a significant difference between HMGB1 and NF-κB expressions in PROM and non-PROM patients, the number of samples provided was limited. Future study with a larger sample size from a larger sampling area is required to represent the total population.

To conclude, the higher HMGB1 and NF-κB expressions in PROM group compared to the non-PROM group indicates their important roles and contributions to PROM, hence it is possible to prevent PROM by inhibiting their activities.

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