ORIGINAL ARTICLE

CONTENTS

Molecular-genetic characteristics of HMGB1 mRNA expression in blood of women with endometriosis associated with infertility

Oksana V. Bakun, Natalya Ya. Muzyka, Svitlana B. Semenenko, Tetiana P. Savchuk, Alla I. Peryzhniak
BUKOVINIAN STATE MEDICAL UNIVERSITY, CHERNIVTSI, UKRAINE

ABSTRACT

Aim: To examine HMGB1 expression in the blood of women with endometriosis-associated infertility and to establish its role in disease progression.

Materials and Methods: We analyzed HMGB1 gene expression levels in two groups: 20 women with endometriosis-associated infertility (main group) and 10 healthy women (control group). The study was conducted at Bukovinian State Medical University and the Centre of Reproductive Medicine. Primary infertility was significantly higher in the main group. We used real-time reverse transcription polymerase chain reaction (RT-PCR) to determine HMGB1 mRNA levels in mononuclear cells isolated from whole blood. Statistical significance was determined using the Student's t-test, with $p < 0.05$ considered significant.

Results: Results indicated a significant increase in HMGB1 mRNA expression in the main group compared to the control group ($p < 0.001$). The relative normalized expression ranged from 1.8924 to 33.426 (median = 8.01). Expression levels were categorized into three groups: borderline with the control, moderate increase, and significant increase. Only one sample (5%) had values equal to the control. Fifteen percent of samples had values slightly above control (1.89–3.45), 30% had moderate increases (3.45–8.01), and 50% had significant increases (>8.01). In 95% of the main group, HMGB1 mRNA expression was elevated, predominantly at high values ($p < 0.001$).

Conclusions: Women with endometriosis-associated infertility show significantly increased HMGB1 mRNA expression, particularly in moderate and severe cases compared to mild cases, indicating HMGB1's role in disease progression ($p < 0.001$).

KEY WORDS: endometriosis, infertility, assisted reproductive technologies, HMGB1

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INTRODUCTION

HMGB1 is a nuclear protein that is present in almost all cell types. It can also be released extracellularly, where it mediates the activation of innate immune responses, including chemotaxis and cytokine release. HMGB1 was originally described as a protein that binds nuclear DNA [1,2]. It is evolutionarily highly conserved and functions as a nuclear cofactor in transcriptional regulation. HMGB1, like many other cofactors, was later found to play another role as an intercellular molecule that is released from various cells into the extracellular environment to act on specific cell surface receptors. In this latter role, HMGB1 is a proinflammatory cytokine that can contribute to many inflammatory diseases, including sepsis [3-5]. Through interaction with immune cell cell surface receptors, HMGB1 activates intracellular cascades that regulate immune cell functions, including chemotaxis and immune modulation. HMGB1 is a critical mediator of lethality in sterile and infectious inflammation. Similar inflammatory responses are initiated by strokes caused by sterile trauma or infection. During infection, innate immunity is activated by foreign molecular products called PAMPs, which include, for example, lipopolysaccharides [6]. During sterile injury or ischemia, the cells themselves are activated under the influence of endogenous DAMPs, which include molecules such as heat shock proteins, uric acid, annexin, and IL1β. HMGB1, released by activated immune cells and damaged or necrotic cells, plays an important role in host responses to both types of threats; thus, it is a critical mediator in the final common pathway to morbidity and mortality during infection and sterile trauma [7-10].

In a recent study (Expression of high-mobility group box-1 in eutopic/ectopic endometrium and correlations with inflammation-related factors in adenomyosis Xiu-Ni Liu and Zhong-Ping Cheng) it was described.

Possible pathogenesis of adenomyosis: the strong HMGB1/TLR4 system may be involved in the inflammatory pathological process of adenomyosis development. It generates a local inflammatory reaction and activates the body's specific immune system, builds

and maintains a stable inflammatory inflammatory microenvironment in the local lesion, and forms a local inflammatory pathology of adenomyopathy, which may involve the pathogenesis of angiomyopathy.

Therefore, HMGB1 may also play an important role in the pathophysiology of endometriosis, a gynecological disease associated with a chronic immunoinflammatory process.

AIM

The aim of our study was to examine the expression HMGB1 in the blood of women with endometriosis-associated and to establish the role of HMGB1 in the progression of endometriosis by analyzing the level of gene expression in the blood of women with infertility-associated infertility, depending on the severity of endometriosis.

MATERIALS AND METHODS

We determined the level of HMGB1 mRNA gene expression in two groups. The first group (main) consisted of 20 women with endometriosis associated with infertility. The control group consists of 10 practically healthy women. The absence of signs of acute inflammatory processes in the reproductive sphere and the negative results of microbiological and virological studies were evidence of a long-overdue inflammatory process that caused tubal obstruction.

This study was conducted at the Bukovyna State Medical University and the clinic "Yuzko medical center".

To analyze the expression of the HMGB1 gene and to determine the relative normalized expression of HMGB1 mRNA, the polymerase chain reaction with reverse transcription in real time (RT-PCR) was used. The object for molecular genetic studies by the RT-PCR method was the fraction of mononuclear cells isolated from the whole blood of patients with endometriosis.

Specific pairs of primers for the analysis of the studied and reference genes were selected using the Primer-BLAST software (www.ncβi.nlm.nih.gov/tools/primer-βlast) and manufactured by Thermo Scientific (USA). The actin, βeta (Actβ) gene was used as a reference gene to determine the relative value of the change in the expression level of the studied genes. The comparative Ct method (ΔΔCt method) was used to express the relative level of gene expression. Calculations were made according to the formulas: ΔCt (target gene) = Ct (target gene) – Ct (calibrator gene / ACT1); ΔΔCt = ΔCt (target gene) – ΔCt (base gene); The relative expression level was expressed as 2–ΔΔCt. Statistical analysis of PCR data was performed using CFX Manager™ software (Bio-Rad, USA). Optimal RT-PCR conditions were selected to achieve a linear relationship between the number of cycles and the number of PCR products. Negative controls were included in the experiment: without addition of cDNA matrix in the PCR reaction, without addition of mRNA matrix in cDNA synthesis, without addition of enzyme in cDNA synthesis. All amplification reactions were performed on individual samples in triplicate. Quantitative variables were evaluated using the Shapiro-Wilk test (if the number of subjects was less than 50) or the Kolmogorov-Smirnov test (when the number of subjects was more than 50).

Comparison of two groups for a quantitative variable according to a normal distribution, under the condition of equality of variances, was performed using the Student's t-test.

Statistical processing of the results of this section was carried out using non-parametric methods, namely the Wilcoxon-Mann-Whitney test. Differences between groups were considered probable at the significance level of $p<0.05$.

RESULTS

In the analysis, depending on the degree of severity of endometriosis, stage I and II were combined into so-called «small» forms of endometriosis, or mild stage, stage III corresponded to the average severity of endometriosis, and stage IV corresponded to the severe stage of the disease.

The results of the study of the relative normalized expression of HMGB1 mRNA in the whole blood of women with endometriosis associated with infertility are shown in Fig. 1.

Based on our research, it was established that the expression of HMGB1 mRNA in the blood of women with endometriosis associated with infertility is significantly increased compared to the indicators of the control group ($p< 0.001$).

The range of all obtained values of the relative normalized amount of mRNA of the HMGB1 gene was 1,8924 – 33,426 (median – 8,01). For a broader characterization of the features of the level of HMGB1 mRNA expression in the blood of women with endometriosis, associated with infertility, and not only the limitation of the data of the average value, conditionally, the ranges of fluctuations of the indicator were separated into the following groups: borderline with the control group, lower than the median (moderate increase HMGB1 expression level) and higher than the median (significant increase in HMGB1 expression level). As can be seen, the lower range of values of the HMGB1 gene is equal to the

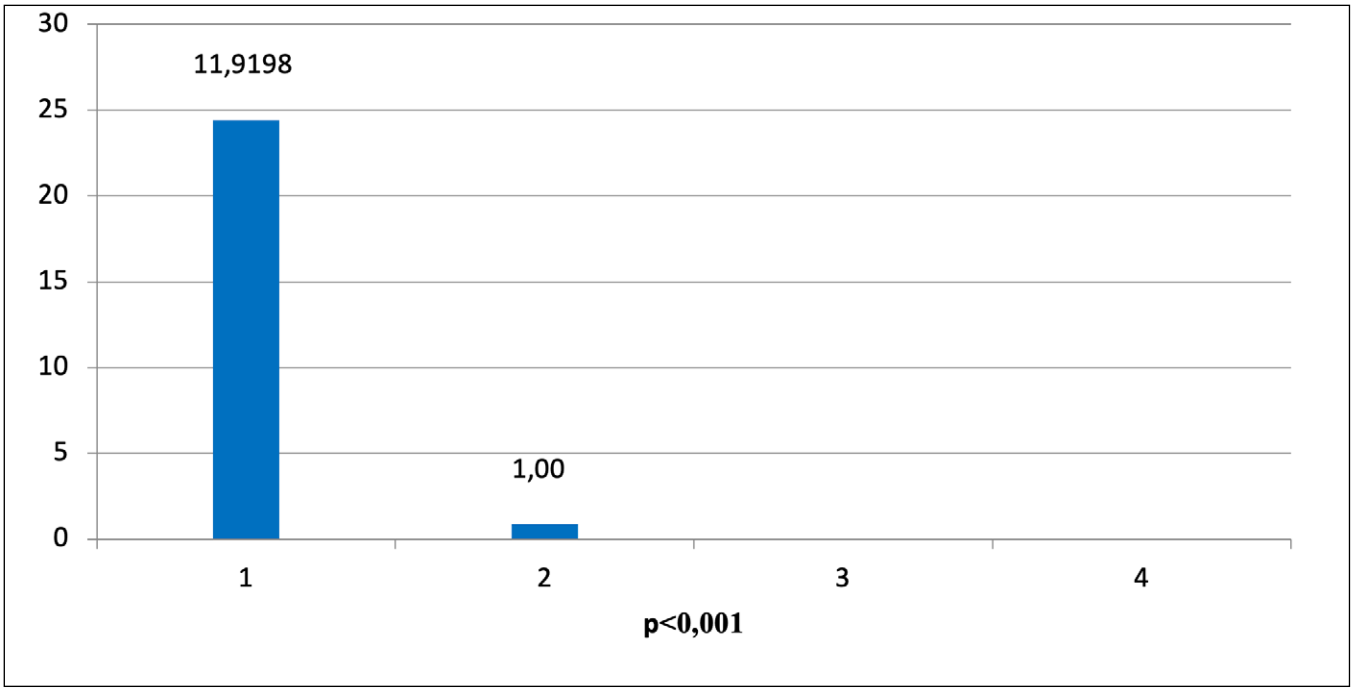

Fig. 1. Relative normalized HMGB1 mRNA gene expression
Normalization by the ΔΔCt method with a reference gene Actin, βeta

control, however, this indicator was measured only in 1 out of 20 studied blood samples, which was 5% of cases (p<0,001). There were only three results that slightly differed from the control (>1,89-<3,45), which was 15% (p<0,001). The number of blood samples in which low values (>3,45-<8,01) of relative normalized expression were found was 6 cases (30%). Relative normalized expression above the median (>8,01) was found in 10 blood samples, which accounted for half of the cases - 50%. Thus, in the blood samples of 95% of the women in the study group, increased expression of the HMGB1 mRNA gene was detected relative to the control with a preference for high values (p<0,001) (Table 1).

Thus, the obtained results indicate an increase in the relative normalized amount of mRNA of the HMGB1 gene in the blood of women with endometriosis associated with infertility. The results of the work indicated in the previous subsection demonstrate a pronounced probable increase in the expression of the HMGB1 gene in the blood of women with endometriosis associated with infertility, which coincides with the trends of scientific works of recent years. Free HMGB1IL participates in all phases of inflammation, from damage to repair. It is able to activate endothelial cells and their precursors, which determines a certain contribution of HMGB1 to angiogenesis in pathological processes.

To implement the tasks of this work unit, patients are divided into three groups depending on the severity of endometriosis. Stages of the disease were determined according to the recommendations of AFS-85, however, in order to simplify the analysis, stages I and II were combined into the so-called "small" forms of endometriosis, or mild stage, stage III corresponded to the average severity of endometriosis, and stage IV corresponded to the severe stage of the disease. The first group included 10 women with mild endometriosis, the second group included 3 patients with moderate endometriosis, and the third group included 7 women with severe endometriosis.

Table 2 shows the results of the HMGB1 mRNA gene expression study in the blood of women depending on the severity of endometriosis.

As can be seen from the table 2, the levels of relative normalized expression of the HMGB1 gene increased in direct proportion to the increasing stage of endometriosis. This was reflected in the statistically significant difference found between the indicators of the first and third, and second and third studied groups, i.e., compared between the groups of women with mild and severe endometriosis. It is worth noting that the range of relative normalized values of HMGB1 gene mRNA in mild cases was 1,89 – 7,32 (median – 4,76). No specific features of the distribution, concentration of low values or predominance of lower values of HMGB1 expression in the group of patients with "small" forms of endometriosis were found - all values of the relative normalized mRNA expression of the studied genes are "scattered" among the entire range of the sample as a whole. In the group of patients with an average degree of endometriosis, the range of values of the relative

Table 1. Comparative characteristics of relative normalized expression HMGB1 gene mRNA in the blood of women with endometriosis is associated with infertility

Groups	HMGB1 mRNA	p
Researched Group	11,9198 ±0,01	p<0.05
Control Group	1,00±0,01	*

Note: Relative normalized amount of HMGB1 gene mRNA. Normalization by the ΔΔCt method with the reference gene Actin βeta p<0,05 reflects a probable difference.* - normalization by relative amount.

Table 2. Expression of the HMGB1 mRNA gene level in the blood of women, of patients with endometriosis associated with infertility, depending on the severity of endometriosis (M±m)

No /p	Groups of patients	Indexes
		HMGB1
1	1 group (mild endometriosis ("small" forms) (n=10))	4,76±0,22
2	2nd group (moderate endometriosis (n=3))	9,94±0,73
3	3 group (severe endometriosis (n=7))	22,97 ± 1,23
	p	p1 > 0,05 p2 <0,001 p3 < 0,001

Notes:p reflects the statistical probability of the difference between the indicators of the studied and control groups; p < 0.05, p < 0.01 - probable difference, p > 0.05 - no probable difference. p1 - the probability of the difference between the indicators of the 1st and 2nd groups, p2 - the probability of the difference between the indicators of the 2nd and 3rd groups, p3 - the probability of the difference between the indicators of the 1st and 3rd groups.

normalized expression of the HMGB1 gene ranged from 8,28 to 12,87 (median – 9,94), without certain trends.

In the group of women with severe endometriosis, the range of relative normalized expression of the HMGB1 gene was 13,94 – 33,42 (median – 22,97). It should also be noted that (100,00%) of the observations in this group belonged to significant deviations from the control and the median (p<0,001). Accordingly, the expressed values of HMGB1 gene expression were concentrated even in groups of women suffering from mild endometriosis (I stage).

Thus, statistical differences in the relative normalized expression of HMGB1 were found between groups of women with mild, moderate, and severe endometriosis, trends indicate an increase in this indicator directly proportional to the increase in the degree of endometrioid lesions. This is consistent with the assumption of some authors that endometrioid growth is supported by the activation of a number of innate immune molecules with subsequent production of factors such as cytokines and vascular growth factors [1, 16, 20, 21].

DISCUSSION

The resulting increase in IL1β expression can be explained from the standpoint that HMGB1 is part of a system that binds to immunogenic nucleotides to activate the innate immune response during tissue damage or microbial infection. The biological activity of extracellular HMGB1 is largely related to the interaction with target cells involved in inflammatory and immune responses, which emphasizes the importance of HMGB1 blood levels as a valuable biomarker of inflammation or inflammatory diseases [11-15]. HMGB1 provokes cell proliferation, invasion and cellular inflammation in the ectopic endometrial environment through Toll-like receptors [16-20]. By itself, HMGB1 has little or no pro-inflammatory activity, but forms complexes with pro-inflammatory factors such as IL 1β or lipopolysaccharide and thus enhances their biological activity [21]. Thus, HMGB1 complexes stimulate the synthesis of pro-inflammatory cytokines. During infection, innate immunity is activated by foreign molecular products called PAMPs (pathogen-associated molecular patterns), which include, lipopolysaccharides. During damage or ischemia, the cells themselves are activated under the influence of endogenous DAMPs (damage associated molecular patterns), which include such molecules as heat shock proteins, annexin, IL1β. HMGB1, released by activated immune cells and damaged or necrotic cells, plays an important role in host responses to both

types of threats; thus, it is a critical mediator in the final common pathway to morbidity during infection and trauma. A possible pathogenesis of adenomyosis a strong HMGB1/TLR4 system may be involved in the inflammatory pathological process of adenomyosis development. It generates a local inflammatory reaction and activates the body's specific immune system, builds and maintains a persistant inflammatory microenvironment in the local lesion, and forms a local inflammatory pathology of adenomyopathy, which may involve the pathogenesis of angiomyopathy, include IL1β. Etracellular matrix acts an endogenous ligand for molecules such as TLR-4 (tumor like receptor) to stimulate the activation of TLR-4-mediated cellular inflammatory signaling and participate in the inflammatory response [17]. It has been proven that HMGB1, TLR-4-mediated inflammatory signaling system is involved in the formation of multiple tumor inflammatory pathological microenvironments and participates in inflammatory tumor proliferation, invasion and metastasis and other pathological mechanisms [18].

CONCLUSIONS

In the blood of women with endometriosis associated with infertility, there is a significant increase in the level of mRNA expression of the HMGB1 gene. In the blood of women with endometriosis associated with infertility, a probable increase in the expression of HMGB1 ($p<0,001$) in women with moderate and severe endometriosis compared to the mild course of the disease and HMGB1 ($p<0.05$) in the groups of women with mild and severe endometriosis, which indicates the role of these factors in the progression of the disease.

REFERENCES

1. Broi MG Da, Ferriani RA, Navarro PA. Ethiopathogenic mechanisms of endometriosis-related infertility. JBRA Assist Reprod. 2019;23:273-80. doi: 10.5935/1518-0557.20190029. DOI
2. Chen WC, Cheng CM, Liao WT, Chang TC. Urinary Biomarkers for Detection of Clinical Endometriosis or Adenomyosis. Biomedicines. 2022;10(4):1-13. doi: 10.3390/biomedicines10040833. DOI
3. Lightbourne A, Foley S, Dempsey M, Cronin M. Living With Endometriosis: A Reflexive Thematic Analysis Examining Women's Experiences With the Irish Healthcare Services. Qual Health Res. 2024;34(4):311-22. doi: 10.1177/10497323231214114. DOI
4. He J, Xu Y, Yi M et al. Involvement of natural killer cells in the pathogenesis of endometriosis in patients with pelvic pain. J Int Med Res. 2020[cited 2024;48(7):300060519871407. doi: 10.1177/0300060519871407. DOI
5. Horne AW, Missmer SA. Pathophysiology, diagnosis, and management of endometriosis. BMJ. 2022;379:e070750. doi: 10.1136/bmj-2022-070750. DOI
6. Allaire C, Bedaiwy MA, Yong PJ. Diagnosis and management of endometriosis. CMAJ. 2023;195(10):363-71. doi: 10.1503/cmaj.220637. DOI
7. Begum MIA, Chuan L, Hong ST, Chae HS. The Pathological Role of miRNAs in Endometriosis. Biomedicines. 2023;11(11):1-27. doi: 10.3390/biomedicines11113087. DOI
8. Taylor HS, Kotlyar AM, Flores VA. Endometriosis is a chronic systemic disease: clinical challenges and novel innovations. Lancet. 2021;397(10276):839-52. doi: 10.1016/S0140-6736(21)00389-5. DOI
9. Dolińska W, Draper H, Othman L et al. Accuracy and utility of blood and urine biomarkers for the noninvasive diagnosis of endometriosis: a systematic literature review and meta-analysi. F&S Reviews. 2023;4(2):116-30. doi: 10.1016/j.xfnr.2022.12.001. DOI
10. Tian Z, Chang XH, Zhao Y, Zhu HL. Current biomarkers for the detection of endometriosis. Chin Med J (Engl). 2020;133(19):2346-52. doi: 10.1097/CM9.0000000000001063. DOI
11. Janša V, Pušić Novak M, Ban Frangež H, Rižner TL. TGFBI as a candidate biomarker for non-invasive diagnosis of early-stage endometriosis. Hum Reprod. 2023;38(7):1284-96. doi: 10.1093/humrep/dead091. DOI
12. Laganà AS, Garzon S, Götte M et al. The Pathogenesis of Endometriosis: Molecular and Cell Biology Insights. Int J Mol Sci. 2019;20(22):5615. doi: 10.3390/ijms20225615. DOI
13. Moga MA, Bălan A, Dimienescu OG et al. Circulating miRNAs as Biomarkers for Endometriosis and Endometriosis-Related Ovarian Cancer-An Overview. J Clin Med. 2019;8(5):1-19. doi: 10.3390/jcm8050735. DOI
14. Cao Y, Liu X, Guo SW. Plasma High Mobility Group Box 1 (HMGB1), Osteopontin (OPN), and Hyaluronic Acid (HA) as Admissible Biomarkers for Endometriosis. Sci Rep. 2019;9(1):1-17. doi: 10.1038/s41598-019-45785-w. DOI
15. Liu XN, Cheng ZP. Expression of high-mobility group box-1 in eutopic/ectopic endometrium and correlations with inflammation-related factors in adenomyosis. Gynecol Endocrinol. 2023;39(1):2269265. doi: 10.1080/09513590.2023.2269265. DOI
16. Yun BH, Chon SJ, Choi YS et al. Pathophysiology of endometriosis: role of high mobility group box-1 and toll-like receptor 4 developing inflammation in endometrium. PLoS One. 2016;11:e0148165. doi: 10.1371/journal.pone.0148165. DOI
17. Yun BH, Chon SJ, Choi YS et al. Correction: Pathophysiology of Endometriosis: Role of High Mobility Group Box-1 and Toll-Like Receptor 4 Developing Inflammation in Endometrium. PLoS One. 2018;13:e0203741. doi: 10.1371/journal.pone.0203741.

18. Ouyang F, Huang H, Zhang M et al. HMGB1 induces apoptosis and EMT in association with increased autophagy following H/R injury in cardiomyocytes. Int J Mol Med. 2016;37:679–89. doi: 10.3892/ijmm.2016.2474. DOI
19. Shimizu K, Kamada Y, Sakamoto A et al. High expression of high-mobility group box 1 in menstrual blood: implications for endometriosis. Reprod Sci. 2017;24:1532–7. doi: 10.1177/1933719117692042. DOI
20. Tang D, Kang R, Livesey KM et al.. Endogenous HMGB1 regulates autophagy. J Cell Biol. 2010;190:881–92. doi: 10.1083/jcb.200911078.
21. Li Q, Yu B, Yang P. Hypoxia-induced HMGB1 in would tissues promotes the osteoblast cell proliferation via activating ERK/JNK signaling. Int J Clin Exp Med. 2015;8:15087.

CONFLICT OF INTEREST

The Authors declare no conflict of interest

CORRESPONDING AUTHOR

Oksana V. Bakun
Bukovinian State Medical University
129 Golovna St, 58000 Chernivtsi, Ukraine
e-mail: kupchanko06@gmail.com

ORCID AND CONTRIBUTIONSHIP

Oksana V. Bakun: 0000-0002-4742-2265 A B C D
Natalya Ya. Muzyka: 0000-0002-3381-1163 E
Svitlana B. Semenenko : 0000-0002-6124-1938 F
Tetiana P. Savchuk: 0000-0002-1782-1812 D
Alla I. Peryzhniak: 0000-0003-0139-0058 E

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