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Plasma microRNAs as non-invasive biomarkers in bovine endometritis caused by Gram-negative and Gram-positive bacteria

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Abstract

The purpose was to identify differentially expressed plasma microRNAs (miRNAs) in cows with clinical and subclinical endometritis. In this study clinical endometritis (CE; n = 23) based on vaginal discharge score (VDS), subclinical endometritis (SCE; n = 17) based on VDS (0), and endometrial cytology (the presence of 8.00% polymorphonuclear neutrophils (PMN) on days 21-31 and 5.00% on days 41-51 days in milk (DIM) and healthy cows (n = 21) based on vaginal discharge score (0), and endometrial cytology (< 5.00% PMN on days 21 - 31 and < 5.00% on days 41 - 51 DIM) were selected. The results showed that the expression level of miR-146a was significantly higher in the CE (19.17-fold), and SCE (6.22-fold) groups than those of healthy cows. The relative transcript abundance of miR-223 was considerably down-regulated in the CE (0.26-fold) and SCE (0.06-fold) compared to the healthy cows. The expression levels of miR-146a and miR-223 were significantly higher in the CE group which could be caused by Gram-negative bacterial infection. Our results showed that the expression level of plasma miRNAs postpartum could be used as a reliable marker to distinguish between SCE, CE and healthy cows.

Introduction

The postpartum period is prone to uterine contamination and inflammation due to the open cervix and physical damage to the reproductive tract and metabolic changes in high-producing dairy cows.[1] This is unfavorable for uterine health, reproductive performance, ovarian endocrine function and uterine involution.[2,3] Delay in uterine involution leads to uterine inflammation and induces the progression of pathogenic bacteria which causes the loss of embryos an increased incidence of clinical endometritis (CE), and subclinical endometritis (SCE).[4,5] Endometritis is a chronic inflammation of the endometrium that impairs fertility.[6-8] It appears to be caused by one or both of dysregulation of inflammation and persistent infection with *Escherichia coli* in the first week postpartum or *Trueperella pyogenes* in the second week following parturition.[9,10] Lipo-polysaccharides (LPS) from Gram-negative bacteria and lipopeptides from Gram-positive bacterial activate the innate immune system in the endometrium and lead to increase acute phase protein and inflammation.[11,12]

The CE in dairy cows is defined as visually identifiable mucopurulent (approximately 50.00% pus and 50.00% mucus) or purulent (estimated pus content > 50.00%) vaginal discharge 21 days or more after parturition in the absence of systemic signs.[13] In contrast, SCE is defined as the proportion of polymorphonuclear neutrophils (PMN) in the superficial endometrium which is related to a decrease in the reproductive efficiency, however, mucopurulent discharge and systemic signs are absent.[14]

In this regard, early diagnosis and treatment promote early recovery without reducing the reproductive efficiency. There are a variety of commonly used methods for the diagnosis of CE including rectal palpation, ultrasound, cytobrush, biopsy, metricheck device and vaginoscopy[15-17] while endometrial cytology or histopathology are required for the diagnosis of SCE.[18] However, as the interpretation of all diagnostics is dependent on the examiner, there is currently no gold standard diagnostic method to identify inflammation of the endometrium.[15] Therefore, it is necessary to identify specific and sensitive biomarkers for early detection of the endometritis.

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The microRNAs (miRNAs) are a family of small noncoding RNA (20-24 nucleotides) and have been shown to be involved in cell differentiation, regulation of inflammation, immune response, apoptosis and biological development.[19] The regulatory roles of miRNAs have been reported in the pathophysiology of uterus and ovaries such as oogenesis, folliculogenesis, steroidogenesis, implantation, pregnancy, polycystic ovary, uterine infection, embryonic death, and abortion,[20] and their roles in the innate immune response during bovine endometritis has been discussed in recent years.[21] Several researchers demonstrated that enhancement alterations in endometrial transcript and miRNAs profiles of cows with CE and SCE had a significant impacted on uterine homeostasis and receptivity.[22]

The miRNAs are a class of promoters that involved in multilayer enhancement of Toll-like receptors (TLR) signaling pathways including modulation of TLR expression, TLR-related adapter enzymes, signaling molecules and inflammatory cytokines.[23] Ibrahim *et al*. demonstrated that Gram-negative bacterial challenge resulted in differential expression of miRNAs in bovine fallopian tubes cells, which led to an impaired embryo development.[24] Recent miRNAs profile analysis has indicated that miR-146a is strongly induced in LPS–challenged THP-1 cells and negatively regulates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) by inhibiting the expression of interleukin-1 beta (IL1β) interleukin-1 receptor-associated kinase1 (IRAK1) and tumor necrosis factor alpha (TNFα) receptor-associated factor 6 (TRAF6) through lowering the levels of LPS and bacterial-induced production of chemokines and cytokines.[25-27]

Recently, RNA sequencing data has demonstrated large number of miRNAs, especially miR-223, a highly conserved miRNA among species, showing a high expression level in bovine endometritis.[28,29] These results indicated that miR-146a and miR-223 might play protective roles against inflammatory damages and could be considered as a non-invasive biomarkers for early detection of endometritis in cows. Therefore, the expression profiles of miR-146a and miR-223 were investigated in dairy cattle with CE and SCE.

Materials and Methods

Animals and experimental design. This study was conducted in a commercial dairy farm (n = 2,200) near Tabriz, East Azerbaijan province, Iran. The cows in the second to eighth parity, milked three times daily, with an average milk production of 9,700 - 11,500 kg per cow in 305 days were included in the study. These cows calved in open shed stalls with straw bedding. Cows were fed on total mixed rations with a certain amount of concentrates, corn, and alfalfa silage using a feeder. None of the cows was treated with antibiotics or hormone from calving to postpartum checks (locally or systemically). Animal use was approved by the Veterinary Ethics Committee of the University of Tabriz (IR.FVM.REC.1398.138). A total number of 220 Holstein dairy cows were examined for detection of endometritis before inclusion in the experiment. All cows were followed from parturition (Day 0) to 60 days postpartum (DPP). The cows were conducted on 21 - 31 and 41 – 51 days after calving for evaluation of endometritis. A total number of 61 cows were enrolled in the study based on the findings of the reproductive tract examination. Cows were grouped into the three groups as follow: The control or healthy group (n = 21) cows with a clear or translucent vaginal discharge that was not fetid or mucopurulent and PMN level of < 5.00% by cytological examination at both time points were classified as healthy. The CE group (n = 23), Cows with mucopurulent or purulent discharge. Cows exhibiting mucopurulent or worse vaginal discharge without signs of systemic illness were classified as having CE. The SCE group (n = 17), animals without mucopurulent discharge (scores 0) were classified as SCE with a cutoff at ≥ 8.00% PMN on days 21 - 31 and > 5.00% on days 41 - 51 postpartum**.**

Diagnosis of clinical endometritis. Transrectal evaluation of the cervix and uterus involves the estimation of size, symmetry and consistency. Ultrasonography (Agroscan, Angouleme, France) was used for the objective measurement of the diameter of uterine horns and cervix and visualization of mucus and pus within the uterine lumen.[15,30] Manual vaginal examination was used for evaluation vaginal discharge. Vaginal discharge was scored as previously described.[10] Score 0 = no or transparent mucus, score 1 = mucus with flecks of pus, score 2 = mucopurulent discharge less than 50.00% pus, and score 3 = purulent discharge greater than 50.00% pus.[31]

Diagnosis of subclinical endometritis. The cows without mucopurulent discharge (scores 0) on 21 - 31 and 41 - 51 DPP were evaluated base on the endometrial cytology for diagnosis of SCE.

Cytological examination. To perform endometrial cytology, intrauterine endometrial samples were collected using the 'cytobrush technique.[15] The cytobrush (Heinz Herenz Medizinalbedarf GmbH, Hamburg, Germany; 20.00 mm length and 7.00 mm diameter) was screwed on a metal rod. The brush was moved forward carefully and rolled along the uterine wall. Then, the brushes were placed in a tube with phosphate buffered saline solution and shipped to the laboratory. In brief, slides were fixed with methanol for 3 min, stained with 5.00% Giemsa (Merck, Darmstadt, Germany) for 3 min, and finally dried. The smears were examined by counting 300 cells (endometrial epithelial cells, PMNs, and lymphocytes) using a light microscope (CX22; Olympus Corporation, Tokyo, Japan) with 400× magnification to record the

proportion of neutrophils cells. Animals without mucopurulent discharge (scores 0) were classified as SCE with a cutoff at ≥ 8.00% PMN on days 21-31 and > 5.00% on days 41-51 postpartum and cows with a clear or translucent vaginal discharge that was not fetid or mucopurulent and PMN level of < 5.00% by cytological examination at both time points were classified as healthy.[5,18]

Bacteriological culture. After vaginal inspection, 61 intrauterine endometrial samples were collected using the cytobrush technique for bacteriological examination (CE n = 23; SCE n = 17; healthy n = 21).[15] The samples were immediately rolled on sheep blood agar (Oxoid, Hampshire, UK) and MacConkey agar (Merck) and incubated at 37.00 ˚C for 24 hr under aerobic conditions. For anaerobic culture, samples were plated on pre-equilibrated sheep blood agar plates for up to 48 hr and incubated at 37.00 ˚C. Aerobic culture plates were examined for the presence of bacterial growth at 24 hr whereas anaerobic culture plates were inspected at 48 hr. Gram-positive and negative bacteria were identified based on the characteristics of the colony, morphology, Gram stain, hemolysis, biochemical profiles, and oxidase tests according to standard protocols.[32]

Plasma preparation. Peripheral blood samples (n = 61) were collected from the coccygeal vein from CE, SCE, and healthy cows and transported to the laboratory within 2 hr. Briefly, the samples were centrifuged at 1,500 *g* (BH-1200; Behdad, Tehran, Iran) for 10 min to discard the blood cells and the supernatants were moved to new tubes followed by another centrifugation at 1,700 *g* for 5 min. Finally, a third centrifugation was carried out at 2,000 *g* for 5 min. The plasma was collected from the upper layer, moved to RNase/DNase-free tubes and kept at – 80.00 ˚C until analysis.

miRNA extraction. Total miRNA of the of plasma was extracted with a commercial kit a Favorgen miRNA Isolation Kit (Taiwan Biotech, Taoyuan, Taiwan) according to manufacturer's instructions. All extractions were performed in duplicate for each animal. Genomic DNA contamination of all RNA samples was removed with a DNase I (Yekta Tajhiz Azma, Tehran, Iran). The concentration of extracted RNA was measured using a NanoDrop Spectrophotometer (ND-2000; Thermo Scientific, Waltham, USA) and the purity of the extract was evaluated by calculating the ratio of absorbance at 260 and 280 nm. A ratio of 1.80 and 2.00 was considered acceptable.[33]

Quantitative real-time PCR analysis. For cDNA synthesis, 5.00 µL (1.00 µg) of RNA was initially polyadenylated using *E. coli* poly (A) polymerase (New England BioLab, Ipswich, USA) in a total reaction volume of 20.00 µL. The reaction solution was incubated at 37.00 ˚C for 10 min, followed by 20 min at 65.00 ˚C. Then, the polyadenylated RNA was reverse transcribed using a Prime Script RT reagent Kit (TaKaRa Bio Inc., Dalian, China) in a total volume of 10.00 µL. The reaction was incubated at 37.00 ˚C for 60 min, followed by 5 min at 85.00 ˚C, and then stored at – 80.00 ˚C until further experiments. After cDNA synthesis, qRT-PCR was performed in a Roche LightCycler 96 system (Roche, Mannheim, Germany). Sequences of mature miRNAs were obtained from the database (http://www.mirbase.org/). The reaction included 0.50 µL of diluted RT product, 500 µL of SYBR Green Master mix (Ampliqon, Odense, Denmark), 1.00 µL of the primer (Table 1), and 3.70 µL of RNase-free water in each well. The amplification state was used in real-time PCR consisted of one cycle of initial denaturation at 95.00 ˚C for 15 min followed by 40 cycles of the quantification stage, including denaturation at 95.00 ˚C for 10 sec, annealing 60.00 ˚C for 60 sec, and cyclic extraction at 72.00 ˚C for 30 sec, followed by final extraction at 72.00 ˚C for 5 min. Levels of miR-146a and miR-223 were measured and normalized against internal control (SNORD48 or U48) using the $2^{-\Delta\Delta ct}$ method.[34]

Statistical analysis. The results were statistically analyzed using SPSS software (Version 22.0; IBM Corp., Armonk, USA). Data were examined for normal distribution using the Shapiro-Wilk test. Normalized and calibrated values of mRNA levels were analyzed with the Mann-Whitney U test to compare the expression abundance of candidate genes between CE, SCE, and healthy cows. The gene expression levels were compared between CE, SCE and healthy control cows at 21 - 31 and 41 - 51 DPP using the two-way Repeated Measures analysis of variance (ANOVA) followed by Tukey's post hoc test. Bacterial infections were compared between SCE, healthy cows and between CE with different degrees at 21 - 31 and 41 - 51 DPP and analyzed using the Chi-Square test. Data were presented as the mean ± SEM and values of $p < 0.05$ were considered statistically significant.

Table 1. Sequences of the oligonucleotides for use as primers to analyze the miRNAs expression via RT-qPCR.

Name	Sequences (5' - 3')	Temperature (˚C)	Oligonucleotide ID
miR-223	F: CGCAGTGTCAGTTTGTCA	54.00	1905296Boo4F1249/59
miR-223	R: CCAGTTTTTTTTTTTTTTTGCGGTA	58.00	190529B004G1250/59
miR-146a	F: GCAGTGAGAACTGAATTCCATAG	61.00	190529B004H1251/59
miR-146a	R: CCTCCAGTTTTTTTTTTTTTTTACAAC	59.00	190529B023C0852/59
U48	F: CCUGGAUGAUGAUAGCAAAUGCUGACUGA	59.10	GH06J031813
U48	R: ACAUGAAGGUCUUAAUUAGCUCUAACUGACU	59.10	GH06J031813

Results

Microbiological analysis. As shown in Table 2, the characteristics of bacteria isolated from the uterus in healthy cows and those of CE and SCE on days 21 - 31 and 41-51 are presented. According to the bacteriological examinations at the 3rd and 7th weeks, some positive samples were identified on bacteria culture, 23 of which were identified for CE, 17 for SCE and 21 Healthy cows the remaining 11 samples showed no bacterial growth at the both intervals in three groups.

A study of the frequency of bacterial infections in healthy cows 21 - 31 days postpartum revealed that *E. coli* (11.76%) was the most common Gram-negative bacteria isolated from the uterus. In uterus healthy cows, the most common Gram-positive bacteria were *Corynebacterium spp* (11.76%) *a*nd *Bacillus spp* (11.76%). *Fusobacterium necrophorum* (25.00%) *and E. coli* (25.00%) were the major Gram-negative bacteria isolated from the uterus of cows with CE on days 21-31, while *T. pyogenes* (16.67%) was the major Gram-positive bacteria isolated from the uterus of cows with CE on days 21 - 31. The majority of Gram-negative bacteria isolated from the uterus of cows with SCE on days 21 - 31 were *E. coli* (22.22%). At this time, the most common Gram-positive bacterium isolated from SCE was *Streptococcus spp.* (22.22%).

Pseudomonas spp. (25.00%) was the most prevalent Gram-negative bacterium isolated from the uterus of healthy cows on days 41 - 51 postpartum. At the same time, *Carnobacterium spp.* (25.00%) was the most common Gram-positive bacterium isolated from the uterus of healthy cows. *Fusobacterium necrophorum* (36.36%) was the most common Gram-negative bacteria isolated from cows with CE on days 41 - 51. At the same time, most of the Gram-positive bacteria isolated from the uterus of CE cows were *T. pyogenes* (9.09%). Between 41 - 51 days postpartum, *E. coli* (25.00%) dominated the Gram-negative bacteria isolated from the uterus of SCE cows. At the same time, *Lactobacillus spp*. (25.00%) and *Bacillus spp*. (25.00%) were the two most common Gram-positive bacteria isolated from the uterus of SCE cows.

Based on the results, the effect of animal health status (healthy cows, clinical endometritis and subclinical endometritis) on uterine bacteria was not significant ($p > 0.05$). However, the effect of postpartum uterine sampling times (postpartum days of 21 - 31 and 41 - 51) on uterine bacteria was significant ($p < 0.05$). On the other hand, the interaction effect of animal health status by time on uterine bacteria was not significant ($p > 0.05$).

Cytology evaluation. Based on the type of bacteria isolated from the uterus on days 21 - 31 and 41 - 51 postpartum, uterine cytology in cows with SCE was evaluated (Table 3). Statistical analysis showed that there was not significant correlation between type of isolated bacteria and the presence of PMN in SCE cows. On days 21-31 in cows with SCE caused by Gram-negative bacteria and PMN > 8.00%, the cytological results were 100%. At this time, the prevalence of SCE with Gram-positive bacteria with PMN > 8.00% was 83.30% and 16.70% with PMN < 8.00%.

Table 3. Percentage of polymorphonuclear neutrophils (PMN) in cows with subclinical endometritis (SCE; n = 17) according to the type of bacteria isolated from the uterus on days 21 - 31 and 41 - 51 postpartum. Data are presented as absolute values (%).

Variables	Days 21 - 31			Days 41 - 51		
	No.	< 8.00%	> 8.00%	No.	< 5.00%	> 5.00%
Gram-negative	3	0 (0.00)	3 (100)	3	0 (0.00)	3 (100)
Gram-positive	6	1 (16.70)	5 (83.30)	5	1 (20.00)	4 (80.00)
***p*-value**		0.546			0.740	

Pearson Chi-Square showed no significant correlation between type of bacteria and the presence of PMN.

Table 2. Categorization of bacteria isolated from healthy cows, clinical endometritis (CE) and subclinical endometritis (SCE) on days 21-31 and 41-51 postpartum. Data are presented as frequency (%).

Bacteria	Recognized uterine pathogens on days 21 - 31			Recognized uterine pathogens on days 41 - 51		
	Healthy (n = 17)	CE (n = 12)	SCE (n = 9)	Healthy (n = 4)	CE (n = 11)	SCE (n = 8)
Gram negative						
Fusobacteriumnecrophorum	0	3 (25.00%)	1 (11.11%)	0	4 (36.36%)	1 (12.50%)
E. coli	2 (11.76%)	3 (25.00%)	2 (22.22%)	0	3 (27.27%)	2 (25.00%)
Pseudomonas spp.	0	1 (8.33%)	1 (11.11%)	1 (25.00%)	1 (9.09%)	0
Bacteroides spp.	0	2 (16.67%)	0	0	2 (18.18%)	0
Total	2 (11.76%)	9 (75.00%)	4 (44.44%)	1 (25.00%)	10 (90.90%)	3 (37.50%)
Gram positive						
Bacillus spp.	2 (11.76%)	0	0	2 (50.00%)	0	2 (25.00%)
Trueperella spp.	0	2 (16.67%)	1 (11.11%)	0	1 (9.09%)	0
Streptococcus spp.	0	1 (8.33%)	2 (22.22%)	0	0	0
Carnobacterium spp.	2 (11.76%)	0	0	1 (25.00%)	0	0
Lactobacillus spp.	0	0	2 (22.22%)	0	0	2 (25.00%)
Lactococcuslactis	0	0	0	0	0	1 (12.50%)
Total	4 (23.53%)	3 (25.00%)	5 (55.56%)	3 (75.00)	1 (9.09%)	5 (62.50%)
No growth	11 (64.71%)	0	0	0	0	0

Vaginal discharge evaluation. In cows with CE, vaginal discharge score (VDS) was evaluated according to the type of bacteria isolated from the uterus on days 21 - 31 and 41 - 51 postpartum (Table 4). Data analysis showed that there is not significant correlation between type of isolated bacteria and the VDS in CE cows. According to results, in CE cows with Gram-negative bacterial, about 11.10% of cows had grade 0 to 1 vaginal discharge, while 88.90% had 2 to 3 vaginal discharges on days 21 - 31 postpartum. Furthermore, the results of VDS showed that about 100% of cows with CE with Gram-positive displayed grade 2 to 3 vaginal discharge within 21 - 31 postpartum. On days 41 - 51 postpartum in CE cows with Gram-negative bacteria, results showed that about 10.00% of cows had grade 0 to 1 vaginal discharge and 90.00% had grade 2 to 3 vaginal discharge. In addition, results showed that about 100% of CE cows with Gram-positive bacteria showed grade 2 to 3 vaginal discharge at 41 - 51 postpartum.

Table 4. Vaginal discharge score in cows with clinical endometritis (CE; n = 23) according to the type of bacteria isolated from the uterus on days 21 - 31 and 41 - 51 postpartum. Data are presented as absolute values (%).

Gram	Days 21 - 31			Days 41 - 51		
	No.	0 - 1	2 - 3	No.	0 - 1	2 - 3
Negative	9	1 (11.10%)	8 (88.90%)	10	1 (10.00%)	9 (90.00%)
Positive	3	0 (0.00%)	3 (100%)	1	0 (0.00%)	1 (100%)
***p*-value**		0.453			0.408	

Pearson Chi-Square shows no significant correlation between type of bacteria and vaginal discharge score.

mRNA expression of miR-146a and miR-223. The data obtained from the molecular assays revealed that miR-146a and miR-223 could be detected in the plasma of all the animals. As illustrated in Figure 1, the mRNA levels of miR-146a were significantly higher in the cows affected by CE (19.17-fold; 95.00% CI; $p < 0.0001$) and SCE (6.22-fold; 95.00% CI; $p < 0.05$) than the healthy cows. Additionally, the results showed that the relative transcript abundance of miR-223 was considerably down-regulated in the CE (0.26-fold, 95.00% CI; $p < 0.001$) and SCE (0.06-fold; 95.00% CI; $p < 0.001$) groups compared to the healthy animals (Fig. 1).

As shown in Figure 2, miR-146a and miR-223 expression patterns were significantly different in the plasma of cows with CE and SCE at the 3th week of postpartum than the 7th week after calving. The results indicated that the mRNA levels of miR-146a were significantly up-regulated ($p < 0.001$) in the plasma of the CE group infected by Gram-negative bacteria compared to the healthy cows. However, no significant difference in the relative transcript abundance of miR-146a was observed in the plasma of CE group infected by Gram-positive bacteria compared to the healthy animals (Fig. 3). Furthermore, the results indicated that the mRNA level of miR-223 was significantly up-regulated ($p < 0.01$) in the plasma of CE cows infected with Gram-negative bacteria compared to that of the healthy cows; however, the CE of animals infected with Gram-positive bacteria did not have significant difference with the healthy animals (Fig. 3).

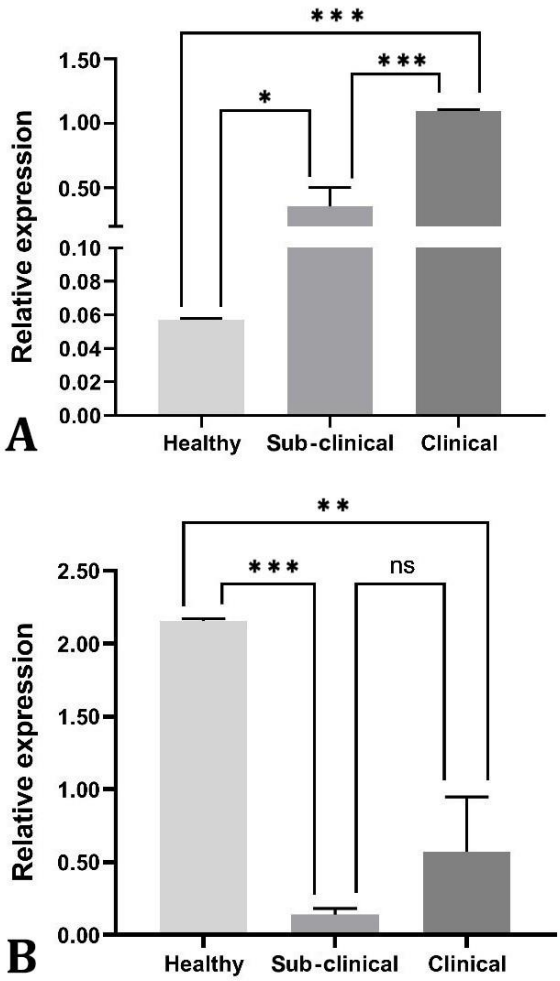

Fig. 1. Changes in **A)** miR-146a and **B)** miR-223 expression levels in plasma samples of cows with clinical endometritis (n = 23) subclinical endometritis (n = 17) and healthy cows (n = 21). *, **, *** indicate significant differences as $p < 0.05$, $p < 0.01$, and $p < 0.001$. ns = not significant $p > 0.05$.

As a shown in Figure 3 our results indicated that the highest expression level of miR-146a was observed in SCE cows infected with Gram-negative bacteria compared to the control group ($p < 0.05$). There was no statistically significant difference between the expression levels of miR-146a in SCE cows infected with Gram-positive and Gram-negative bacteria. The highest expression level of miR-223 was observed in SCE cows infected with Gram-positive bacteria compared to the control group ($p < 0.05$).

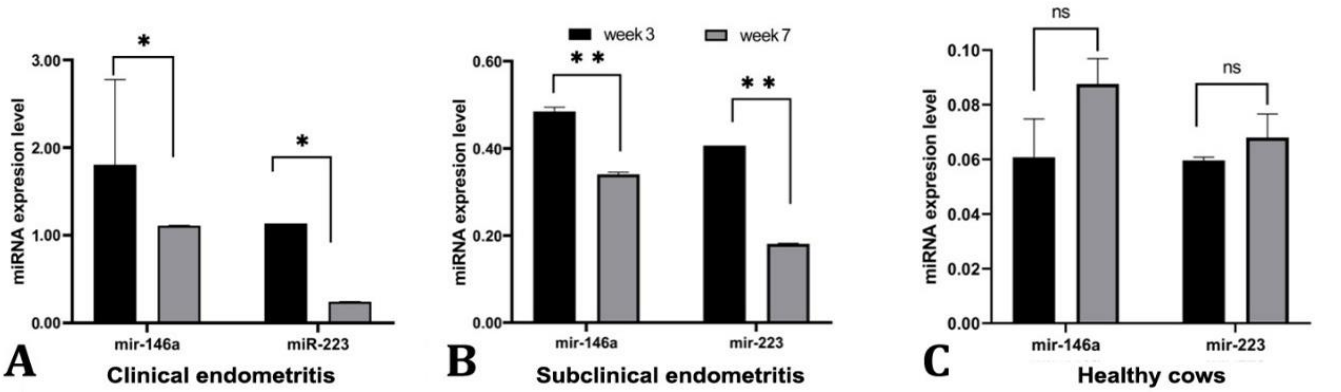

Fig. 2. Expression of miR-146a and miR-223 in the plasma of cows with **A)** clinical, **B)** subclinical endometritis and **C)** healthy cows in 21 - 31 days (week 3) and 41 - 51 (week 7) days postpartum.
*, ** indicate significant differences as $p < 0.05$, and $p < 0.001$. ns = not significant $p > 0.05$.

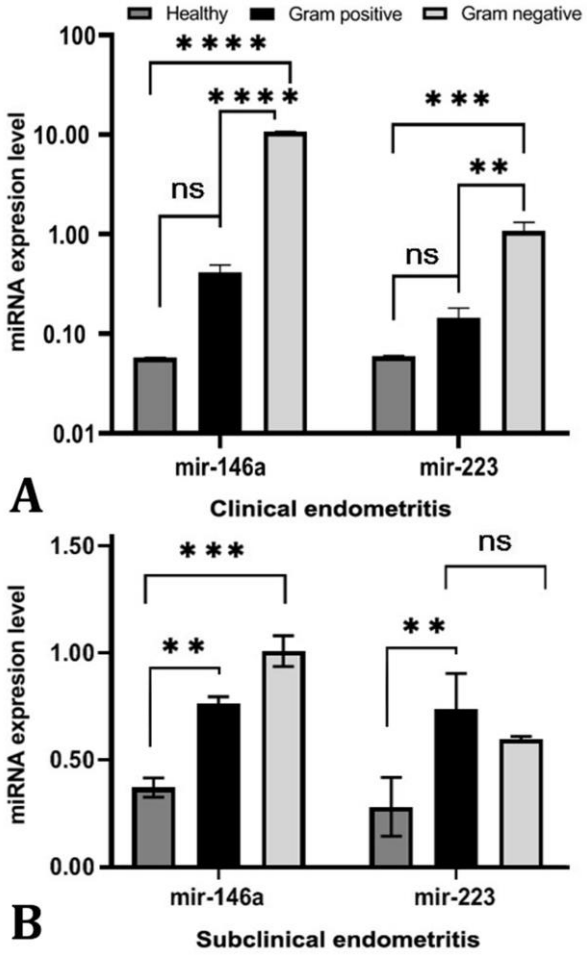

Fig. 3. Changes in the expression level of miR-146a and miR-223 in plasma of cows with **A)** clinical endometritis and **B)** subclinical endometritis infected by Gram-positive bacterial and Gram-negative bacteria. **, ***, **** Values marked by different superscript within Gram-positive and Gram-negative bacteria each microRNA compared to healthy group were significant (**$p < 0.01$; ***$p < 0.001$; **** $p < 0.0001$). ns = not significant $p > 0.05$.

Diagnostic accuracy of circulating miR-146a and miR-223 in discriminating clinical and subclinical endometritis. The overall analysis of CE samples demonstrated that the down expression of miR-223 in plasma could achieve an acceptable diagnostic accuracy to distinguish SCE and CE from the healthy cows with a sensitivity of 98.00% and specificity of 100%. Our findings revealed that mir-146a had a relatively good diagnosis performance in CE with a sensitivity of 75.00% and a specificity of 100%.

Discussion

Endometritis is one of the most important diseases that cause infertility in mammals.[35] The inflammatory response involves a variety of cells and active factors that form a complex network of interactions.[36] Recently, it has clearly been indicated that endometrial transcriptome and miRNAs profiles are altered in bovine CE and SCE, with a significant effect on uterine hemostasis and receptivity.[22,24] However, the expression level of these miRNAs and its relationship with various micro-organisms of uterine infections is unknown. In the present study, alterations in expression profiles of miR-146a and miR-223 associated with inflammation were investigated in bovine endometritis.

It was interesting that the mRNA levels of miR-146a were up-regulated in SCE and CE cows. In line with our results it has also been reported that miR-146a rs2910164 polymorphism genotypes (GG, GC, and CC) was significantly associated with endometriosis severity in patients.[37] miR-146a was first recognized as an immune system controller in a systematic attempt to find miRNAs involved in the responses of mammals to microbial infections.[38] Our data revealed that the expression levels of miR-146a were significantly higher in the plasma of cows affected by Gram-negative bacteria in both SCE and CE groups. Similarly, other studies showed that miRNAs related to inflammation were highly up-regulated in the

blood of cows suffering from Gram-negative bacterial metritis.[39] Similarly, previous findings showed that some miRNAs, such as miR-148b, miR-486-5p, miR-23b, miR-99b, and members of the let-7 families were changed in animals affected by SCE.[22] Overexpression of miR-146a can target TLR4 and regulate inflammatory response that results in the production and release of pro-inflammatory cytokines, type I interferon (IFNs), antibacterial proteins and chemokines to clear the infection.[40] These studies suggested that miR-146a might inhibit NF-κB activation and reduce the undesired effects of pro-inflammatory cytokines including Interleukin 6 (IL6), IL1β and TNFα.[41,42] Overall, the results suggested that mir-146a, as an important regulator immune response gene, leads to the attenuation of the inflammatory condition of endometritis.

Our findings showed that the expression level of miR-223 was increased in animals with SCE associated with gram-positive bacteria. These findings were in accordance with that of a previous study in which the expression levels of miR-223 was increased in the endometrium of horses suffering from endometritis.[43] Furthermore, our results revealed that the expression levels of these miRNAs had no significant differences between the cows with CE caused by gram-positive bacteria compared to the healthy cows. It might be due to the reduced prevalence of gram-positive bacteria in the pathogenesis of endometritis and the distinct patterns of inflammatory responses to these bacteria. Earlier findings revealed that different Toll-like receptors (TLRs) were engaged in cell activity by gram-negative and gram-positive bacteria,= and they might not stimulate similar intracellular signaling pathways.[44] The anti-inflammatory mechanisms of these miRNAs (miR-223) begin with the activation of NF-κB Band by the inhibition of NOD-like receptor (NLRP3) and IL1β to attenuate LPS-induced endometritis.[29] In general, the available evidence demonstrates that miR-223 probably suppresses the production of cytokines and mediates protective effects on LPS-induced endometritis.

In this respect, plasma expression levels of some miRNAs such as miR-146a and miR-223 may involve not only in regulating inflammatory and immune responses but might also associated with the control of intrauterine bacterial infection. In addition, our data demonstrated that the overexpression of miR-146a in plasma could achieve an acceptable diagnostic accuracy to distinguish CE from the healthy cows. These results showed that plasma miR-146a functions as a promising non-invasive screening tool for the detection of CE. However, the findings revealed that mir-146a alone did not achieve sufficient diagnostic reliability to differentiate between the CE and healthy cows with a sensitivity of 75.00% and a specificity of 100%. Further large-scale studies are necessary to validate these results. There were a number of limitations in this study, including wide-scale biomarker feature testing on a large number of samples, as well as the use of a large number of biomarkers and bioinformatics information to communicate between these biomarkers and different forms of endometritis.

In conclusion, the results obtained showed that the miRNAs expression alterations were associated with CE and SCE in blood plasma of dairy cattle. During postpartum, the expression of plasma miRNAs, particularly miR-146a could be used as a reliable marker to distinguish between CE and healthy cows. Increased plasma expression levels of miR-146a and miR-223 were observed when exposed to Gram-negative and Gram-positive bacteria, respectively. Therefore, the functional role of miRNAs involved in the regulation of inflammatory reactions caused by Gram-positive bacteria will be the main objective for future research.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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