Serum miR-155, miR-223, miR-17, miR-200a, miR-205, Interleukin 6, and Prostaglandins as Novel Diagnostic Markers for Endometritis in Arabian Mares

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Abstract

Background: So far the intimate link between serum microRNA (miRNA) and uterine inflammation in mares is unknown. We aimed (I) to investigate the expression profile of eca-miR-155, eca-miR-223, eca-miR-17, eca-miR-200a, and eca-miR-205 (II) and to measure the concentrations of interleukin 6 (IL-6), and prostaglandins ($PGF_{2\alpha}$& PGE_2) in serum of Arabian mares with healthy and abnormal uterine status (endometritis).

Methods and Results: This study was conducted on 80 Arabian mares; young (4-7 years), and old (8-14 years). These animals were divided into 48 sub-fertile including 16 young and 32 old mares suspected of endometritis and 32 fertile as control (24 young and 8 old) at stud farms. Serum samples were collected for measuring IL-6, $PGF_{2\alpha}$, and PGE_2 concentrations, as well as serum miRNA isolation and qRT-PCR. Serum concentrations of IL-6, PGE_2, and $PGF_{2\alpha}$ were higher ($P \leq 0.001$) in mares with endometritis (young and old) compared to the control ones. Age of mares had a remarkable effect($0.001 \leq P \leq 0.01$) onIL-6, PGE_2, and $PGF_{2\alpha}$concentrations. The relative abundance of eca-miR-155, eca-miR-223, eca-miR-17, eca-miR-200a, and eca-miR-205 was higher ($P \leq 0.001$) in both young and old mares with endometritis. We noticed that eca-miR-155, eca-miR-223, eca-miR-200a, and eca-miR-205 revealed higher ($0.001 \leq P \leq 0.01$) expression level in old than young mares with endometritis.

Conclusions: To the best of our knowledge, this is the first study revealed that serum miRNA and serum inflammatory mediators (IL-6, PGE_2, and $PGF_{2\alpha}$) could be used as non-invasive gold standard biomarkers, and therefore might be served as an important additional diagnostic tool for endometritis in Arabian mares.

Introduction

For years, equine endometritis considers as the most important cause of infertility in horses [1, 2]. Infectious endometritis is the first cause of equine subfertility and the third most common disease affecting horses[3]. Endometrial infections are directly responsible for decreasing conception rates and indirectly disrupt reproductive outcomes leading to early embryonic loss, abortion and delivery of intrauterine infected foals[4].

An inflammatory response against uterine pathogens is important to control and eliminate the uterine harmful infections, which seems to be the main cause of subfertility and failure of conception[2]. The initial defense mechanisms against endometrial pathogens are directly evoked as an inflammation, which will activate innate and humoral immune system in the mare [5]via series of cytokines and chemokines. Cytokines have an important role in a wide range of reproductive related processes. There are several types of cytokines, where each cytokine is responsible for multiple cellular tropism in an array of different organs and their response showed a different manner according to cell type[6]. Previous studies reported that the RNA gene expression of pro-inflammatory cytokines interleukin 6 (IL-6) and

prostaglandins ($PGF_{2\alpha}$ and PGE_2) in the uterine tissue samples were associated with the development of endometritis in mares[7, 8].

The proper clearance of excess sperm cells, microorganisms, seminal plasma, and other debris from lumen of uterine tissues is an essential step for qualifying uterine milieu for implantation after embryo arrival[2]. This step is a complex and influenced by the host's immune system as well as epigenetics regulation[9]. Nothing is known about the expression pattern of serum miRNA, and post-transcription regulation of inflammatory immune response genes in mares with endometritis. Recently in equine species, many studies revealed that serum miRNA could be used as non-invasive biomarkers either for normal physiological condition (early pregnancy) or disease condition (sarcoid disease)[10, 11]. The miRNAis small non-coding RNA molecules that act as post-transcriptional regulators of gene expression by inhibiting translation or degrading mRNA through partial or complete base pairing with three prime untranslated region(3´-UTR) of the target mRNAs[12]. They are expressed in different cells and tissues, in order to regulate different mechanisms of developmental as well as physiological processes[10, 13]. Furthermore, an extended inflammatory response due to aberrant miRNA expression is likely to impair fundamental cellular processes of the endometrium, affecting uterine receptivity, folliculogenesis, oocyte maturation and ovulation, finally leading to reduced fertility[14, 15].

So far, the expression pattern of free serum miRNA is unknown during endometritis in equine species. We therefore hypothesize that identification and quantification of some candidates serum miRNA from mares with endometritis might serve as useful and implementable clinical biomarkers for early diagnosis of endometiritis. Moreover, any alteration in the endometrial health status might be accompanied with series of pathophysiological changes, which subsequently dysregulate expression pattern of serum miRNA as well as increase the serum levels of inflammatory biomarkers as IL-6, $PGF_{2\alpha}$ and PGE_2.Thus, the current study aimed (I) to investigate the expression profile of eca-miR-155, eca-miR-223, eca-miR-17, eca-miR-200a, and eca-miR-205, and (II) to measure the concentrations of IL-6, $PGF_{2\alpha}$ and PGE_2, in serum of young and old aged Arabian mares with healthy and abnormal uterine status (endometritis).

Material And Methods

Chemicals

All chemicals and reagents were obtained from Qiagen (Hilden, Germany), Thermo Fisher Scientific(Wilmington, USA), unless otherwise stated.

Ethical approval for use of animals

The present study was approved by the Ethical Use and Animal Care Committee of faculty of veterinary medicine, Cairo University.

Animals and management

This study was conducted on 80 Arabian mares; young (4–7 years), and old (8–14 years). These mares were divided into 48 sub-fertile mares {young (n = 16), old (n = 32)} suspected of endometritis (diseased group) and 32 fertile mares {control; young (n = 24), old (n = 8)} not suspected of endometritis that served as control group between November 2019 and April 2020 at a number of stud farms nearby Giza, Egypt. Uterine swabs and blood samples were collected only once from each mare after owner's permission. The selected mares experienced normal physical and vital signs where the normal range of vital signs including rectal body temperatures (37.5–38.5°C), heart rate (36–40 beats/min), respiratory rate (8–15 breaths/min), capillary refill times (1–2 sec) and a moist with a healthy pink color mucus membrane of the buccal cavity. An orthopedic examination was also performed to exclude mares with lameness or active laminitis. None of the mares had dystocia, retained fetal membranes or problems during puerperium. Additionally, none of the mares was in foaling heat. All animals were submitted to transrectal ultrasonographic (US) uterine examination using real-time B-mode machine (Esaote Mylab30-Netherlands) equipped with 5-7.5 MHz linear-array transducer.

Blood and endometrial swabs sampling

Blood samples were collected from the jugular vein into untreated vacutainer tubes and the tubes were maintained for 20 min at room temperature in an inclined position. The samples were subsequently centrifuged at 3000 ×g for 10 min (4°C) until clear serum was separated. Afterwards, serum was divided into two portions; 1st part was kept at – 20°C for measuring IL-6, $PGF_{2\alpha}$ and PGE_2 concentrations, and the 2nd part was kept at – 80°C for RNA isolation.

Endometrial swabs were collected as described before[16] using a sterilized double guarded uterine swab (Minitub GmbH, Germany), Briefly, after removing the feaces, the tail was bandaged, and then vulva and perineum were cleaned with iodopovidine (Betadine, EGIS, Warsaw, Poland), rinsed three times with water, and dried with a paper towel.

The tip of the swab was held and covered in the palm, and using a slight rotatory movement, the hand passed into the vagina till enabling palpation of the external cervical os, the index finger passed gently into the external cervical os followed by the uterine swab.
After passing the cervical canal, the cotton swab pushed forward through the outer then the inner guards to be contacted with the endometrium then gently rotated for 10–15 seconds. Finally, the swab was retracted back inside the inner guard then into the outer guard and the swab directly immersed into commercial transportation media.

Cytological examination

After the collection of uterine smears, the cytological samples were fixed and stained with a special commercial cytological stain; Papanicolaou method (Biodiagnostic, Egypt) according to the instruction's recommendation within two hours at the laboratory. Samples were evaluated in regard to quality of cell morphology, cellularity, number of inflammatory cells per 400× field, as well as any other remarkable features (Zeiss Axioskopmicroscobe, Carl Zeiss, Thornwood, NY)[17].Uterine samples were considered as

marker for inflammation (endometritis) if the amount of PMNs was greater than 2% as described by Aguilar et al.[18], data not shown.

Microbial culturing

Immediately after immersing the uterine smears into the transportation media, samples were transported to the laboratory for further microbial analysis. Both bacterial and fungal culturing protocol were carried out to identify the pathologically infected mares showing endometritis according to the general guidelines[19], data not shown.

Here in the current work, the criteria for mares to be enrolled in the diseased group (endometritis) were that they had been bred three or more times unsuccessfully in the breeding season, or had a history more than one year of reproductive failure. In addition, two or more of the following criteria on a checklist were present: abnormal clinical findings, US scanning showed abnormal fluid in the uterus (echogenic or ≥ 2 cm in diameter), positive endometrial cytology; and bacterial and/or fungal growth, as indicated by Amorim et al. [20].

Serum IL-6, $PGF_{2\alpha}$ and PGE_2 estimation

Serum concentrations of IL-6 were determined by horse IL-6 ELISA kit (SunLong Biotech Co., LTD, Zhejiang, China) and as used according to the manufacturer's instructions.
The assay sensitivity and range were 0.5 pg/ml and 1.6 pg/ml to 100 pg/ml, respectively. For $PGF_{2\alpha}$ measurement, the commercial $PGF_{2\alpha}$ high sensitivity horse prostaglandin F_2 alpha ELISA kit (SunLong Biotech Co., LTD, Zhejiang, China) was used and run according to the manufacturer's instructions. The assay sensitivity and range were 0.5 pg/ml and 3 pg/ml to 210 pg/ml. For PGE_2 measurements, the commercial PGE_2 high sensitivity horse prostaglandin E_2 ELISA kit (SunLong Biotech Co., LTD, Zhejiang, China) was used and run in accordance to the manufacturer's instructions. The assay sensitivity and range were 0.1 pg/ml and 0.8 pg/ml to 50 pg/ml, respectively.

In-silico analysis for the selected candidate miRNA

The miRNA prediction tools such as: DIANA-microT v3.0 (http://diana.cslab.ece.ntua.gr/microT/) and miRecords (http://mirecords.biolead.org/) were used for filterations miRNAs hits according to their potential relevance for targeting inflammatory immune response in mammalian uterine tissue at least in four different search algorithms. We found that of eca-miR-155, eca-miR-223, eca-miR-17, eca-miR-200a, and eca-miR-205could be selected as potential targets. Moreover, these targets were shown different expression pattern in response to inflammation[14, 21–23].

Serum miRNA isolation, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)

For purification of cell-free miRNAs, we used a miRNeasy serum/plasma kit. 200 µl of thawed serum samples on ice were used for purification total RNA according to manufactures protocol. During purification steps, a 3.5 µl of lyophilized *C. elegans* miR-39 miRNA mimic (miRNeasy

Serum/PlasmaSpike-In Control) were added, at concentration 1.6×10^8 copies/µl. In order to elute cell-free RNA, 14µl of RNase-free water was added to the center of the spin column membrane. Afterwards, the purified serum free total RNA was kept at -80°C.The concentration of total RNA was checked by Nano-drop 2000/c (Thermo Fisher Scientific, Wilmington, USA).We selected the samples, which their ratios of absorbance at 260 and 280 nm (A260/280), and 260 and 230 nm (A260/230) were above 1.7. Furthermore, the integrity of RNA was evaluated by denaturing 1.5% agarose gel electrophoresis and ethidium bromide staining.

Around Ten ng of total RNA was reverse transcribed using MultiScribe reverse transcriptase, and RT primers were performed separately for each miRNA according to the supplier's instructions.
Real-time PCR was done in a final volume of 10 µl, using 0.7 µl of RT product, 0.5 µl of specific primers with probes (Table 1), and TaqMan Universal PCR Master Mix II. Amplification was performed with initial denaturation for 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C with Stratagene Mx3000P (Agilent Technologies, USA), PCR reactions were performed in quadruplicates. The data were analyzed by the comparative threshold cycle (ΔCt) method and normalization was performed using geometric means of cel-miR-39-3p, eca-miR-195, and U6.

Table 1
List of miRNA names, miRBase accession numbers and mature sequences.

miR name	Accession number	Mature miRNA sequence
eca-miR-223	MIMAT0013205	UGUCAGUUUGUCAAAUACCCCA
eca-miR-155	MIMAT0013182	UUAAUGCUAAUCGUGAUAGGGGU
eca-miR-200a	MIMAT0012909	UAACACUGUCUGGUAACGAUGU
eca-miR-17	MIMAT0013084	CAAAGUGCUUACAGUGCAGGUAG
eca-miR-205	MIMAT0012962	UCCUUCAUUCCACCGGAGUCUG

Statistical analysis

The NormFinder was used to select the most stable reference gene for normalization miRNA data[24].The raw data of fluorescence values (Rn) were imported into PCR Miner in order to calculate efficiency[25].The normal distribution was checked via Shapiro-Wilk test and Gaussian distribution; and all data was passed normality test (alpha = 0.05), (GraphPad Software, Inc., San Diego, CA, USA). The expression of selected miRNAs and the levels of serum IL-6, $PGF_{2\alpha}$ and PGE_2were analyzed using two-way ANOVA, followed by Sidak's multiple comparisons test. The values shown in graphs are presented as the mean ± standard error of the mean (S.E.M) of at least five independent experiments each done in quadruplicate, *P* values ≤ 0.05 were considered statistically significant. GraphPad Prism 9.0 was used to perform statistical analysis as well as generating bar graphs

Results

Dynamic pattern of serum IL-6, PGE_2, and $PGF_{2\alpha}$in mares with endometritis (diseased) compared to control ones

Serum concentrations of IL-6 were higher ($P < 0.001$) in diseased mares (both young and old) compared to the control ones. For both healthy and diseased mares, there was a remarkable increase ($P < 0.01$) in the serum IL-6 levels in the old compared to the young mares (Fig. 1a). In the same sense, serum concentrations of PGE_2 displayed higher values ($P < 0.001$) in the diseased mares (both young and old) compared to the healthy ones. Old healthy and diseased mares showed increased values ($P < 0.001$) of serum PGE_2 compared to the young ones (Fig. 1b). Serum levels of $PGF_{2\alpha}$ recorded a marked increase ($P < 0.001$) in the diseased mares (both young and old) compared to the healthy ones. In addition, young mares (control and diseased) exhibited a higher value ($P < 0.01$) of the serum concentration of $PGF_{2\alpha}$ compared to the old mares (Fig. 1c).In addition, PGE_2: $PGF_{2\alpha}$ ratio was significantly ($P < 0.05$) higher in the young and old mares with endometritis compared to the control mares (Fig. 2).

Fold regulation of serum miRNA in mares with endometritis compared to normal healthy ones

In figure (3), the relative abundance of eca-miR-155, eca-miR-223, eca-miR-17, eca-miR-200a, and eca-miR-205 was higher ($P \leq 0.001$) in both young and old diseased mares, compared to control healthy mares (young and old). We noticed that eca-miR-155, eca-miR-223, eca-miR-200a, and eca-miR-205 revealed higher ($0.001 \leq P \leq 0.01$) expression in old diseased than young diseased mares, in comparison to control healthy mares.

Taken together, there were significant ($0.001 \leq P \leq 0.05$) interactions among groups; control healthy young, control healthy old, diseased young, and diseased old mares as shown by Sidak's multiple comparisons test.

Discussion

As far as we know, this is the first report measured the serum concentrations of IL-6, $PGF_{2\alpha}$ and PGE_2 in mares with endometritis compared to the healthy Arabian mares. In mares with endometritis the serum concentrations of IL-6, $PGF_{2\alpha}$ and PGE_2 were significantly higher compared with mares that did not suffer from endometritis. In addition to these clear points, the present study recorded that the inflammatory response could be different, with respect to the systemic cytokines, according to the age of the mares. Hurtgen[26]in his review, mentioned that the equine endometritis is classified into more than one category, which are differing according to the causative agent and severity. In addition, he mentioned that the prevention and control of endometritis in mares will be achieved according to the understanding and follow-up the pathophysiology of equine endometritis through its different predisposing factors. Woodward et al. [27]described that the age of the diseased mares with endometritis as well as the seasonal changes were potential predisposing factors to the susceptibility to endometritis in mares.

In the present study, the serum concentrations of IL-6 were markedly increased in mares with endometritis compared with healthy mares. In several species, serum concentrations of IL-6 showed a significant increase in subclinical and clinical endometritis in cows [28] and ewes [29] It is well-known that IL-6 considers as the most important pro-inflammatory cytokine through the inflammation cascade. IL-6 has a supporting and modulatory role during the inflammation of the tissues, where IL-6 stimulates and potentiates the immune response as well as the action of the others cytokines as IL-10[30, 31].

Prostaglandins (PGs) have a great crucial role during inflammation. PGs considered as main modulators for the inflammatory response against pathogens, where it contributes in the control of the pathogens and alleviate its side effects in different species [32]. In the present study, serum concentrations of $PGF_{2\alpha}$ and PGE_2 were markedly increased in mares with endometritis compared to healthy mares. The considerable increase in the concentration of these biomarkers is an indicator of strong response of inflammatory cells against inflammatory status of the endometrial cells in Arabian mares [1]. In addition, Le Blanc and Causey [4] investigated that the uterine concentrations of PGE_2 showed a remarkable increase 30 min post intrauterine bacterial inoculation in mares. Interestingly, PGE_2 is a potent uterine vasodilator, which is responsible for hyperemia of uterine blood vessels and chemotactic pattern for immune cells against uterine pathogens [33].

No doubt that proper uterine function is regulated by multiple arrays of miRNA[34], which responsible for activation or switched off certain target genes, depending on the physiological and pathological conditions of the animal. Thus, uterine infection does not only affect female fertility by perturbing uterine function, but also by prolonging ovarian cycle [35]. Therefore, to tackle the continuing fertility problems associated with uterine inflammation, understanding the molecular regulatory mechanisms associated with the inflammatory immune response is crucial to design appropriate therapeutic drugs. Among the many molecular marks, the free serum miRNA expression patterns could be potential diagnostic as well as prognostic indicators of mares affected by endometrial inflammation. Herein, there were a profound over-expression ineca-miR-155, eca-miR-223, eca-miR-17, eca-miR-200a, and eca-miR-205 in both young and old diseased mares, compared to control healthy mares (young and old). These findings are in agreement with previous studies, which revealed host cells responses to infection via activation the inflammatory immune response mediators, in order to overcome infection[14, 15, 21]. These inflammatory mediators could induce aberration of miRNA expression, which might be associated with an imbalance between pro-inflammatory and anti-inflammatory mediators[21, 36, 37]. Interestingly, there was a clear influence of mare age on the expression pattern of serum microRNA. Whereas, old diseased mares showed higher expression level ofeca-miR-155, eca-miR-223, eca-miR-200a, and eca-miR-205 than young diseased mares, compared to control healthy ones. This might be due to increase fluid retention by mare age, and subsequently associated with clear changes in systemic immune response[27].To the best of our knowledge, this is the first study investigate the expression profile of eca-miR-155, eca-miR-223, eca-miR-17, eca-miR-200a, and eca-miR-205 in mares serum during endometritis.

In conclusion, to the best of our knowledge, this is the first study revealed that serum miRNA and serum inflammatory mediators(IL-6, PGE_2, and $PGF_{2\alpha}$) could be used as non-invasive gold standard biomarkers, and therefore might be served as an important additional diagnostic tool for endometritis in Arabian mares. Moreover, estimation of the serum concentrations of serum miRNA, IL-6, PGE_2, and $PGF_{2\alpha}$ is a promising recommended tool, during the breeding soundness examination in mares.

Declarations

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Conflicts of Interest:

The authors declare that they don't have any conflict of interest.

Author contribution:

Sally Ibrahim: conceptualization, methodology, statistics analysis and writing& editing the manuscript. Mohamed Hedia: methodology and writing & editing the manuscript. Mohamed O. Taqi: data curation, statistics analysis and writing & editing the manuscript. Mohamed K. Derbala: methodology. Karima Gh. M. Mahmoud: conceptualization and review the manuscript. Youssef Ahmed: methodology. Sayed Ismail and Mohamed El-Belely review the manuscript. All authors have read and agreed to the published version of the manuscript.

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Figures

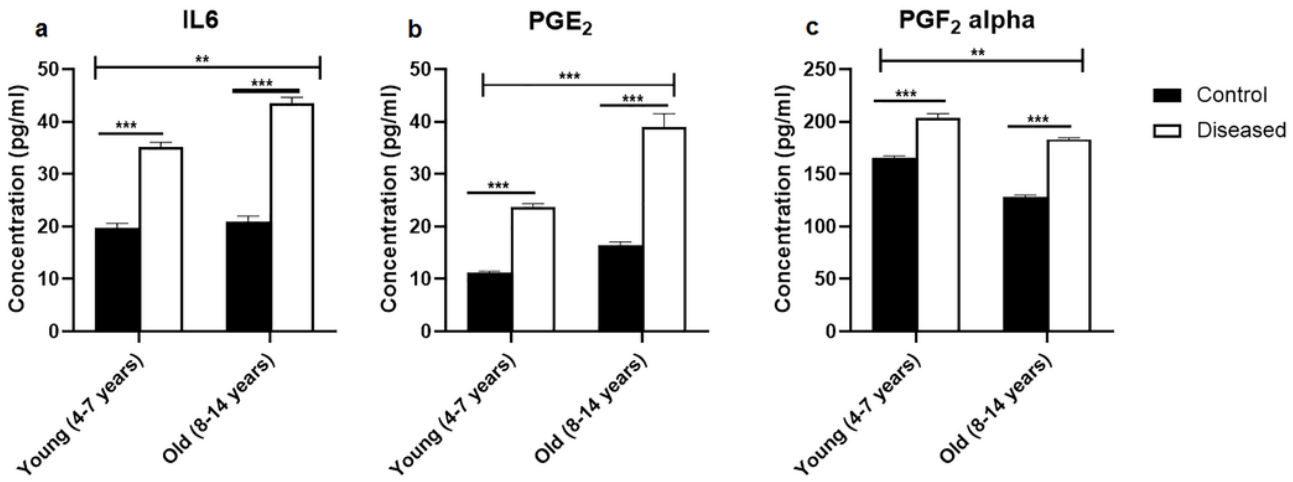

Figure 1

The levels of IL-6 and prostaglandins (mean±SEM) in serum of mares {young (4-7 years) and old (8-14 years)} with endometritis compared to control ones. (A) The serum concentration of IL-6. (B) The level of PGE2in serum. (C) The serum level of PGF2α. Statistical significance was defined as values of P<0.05. Statistical differences among groups are marked with asterisks (**P<0.01, ***P<0.001).

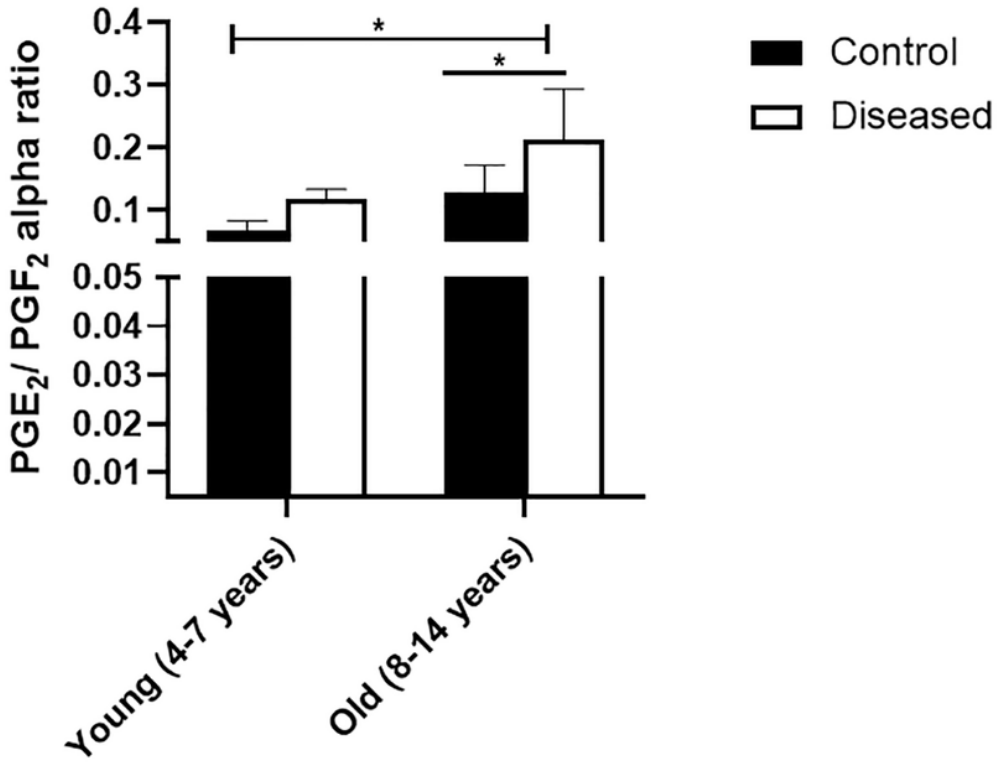

Figure 2

Ratio (mean±SEM) of serum PGE2:PGF2α concentrations in serum of mares {young (4-7 years) and old (8-14 years)} with endometritis compared to control healthy ones. Statistical significance was defined as values of P<0.05. Statistical differences among groups are marked with asterisks (*P<0.05).

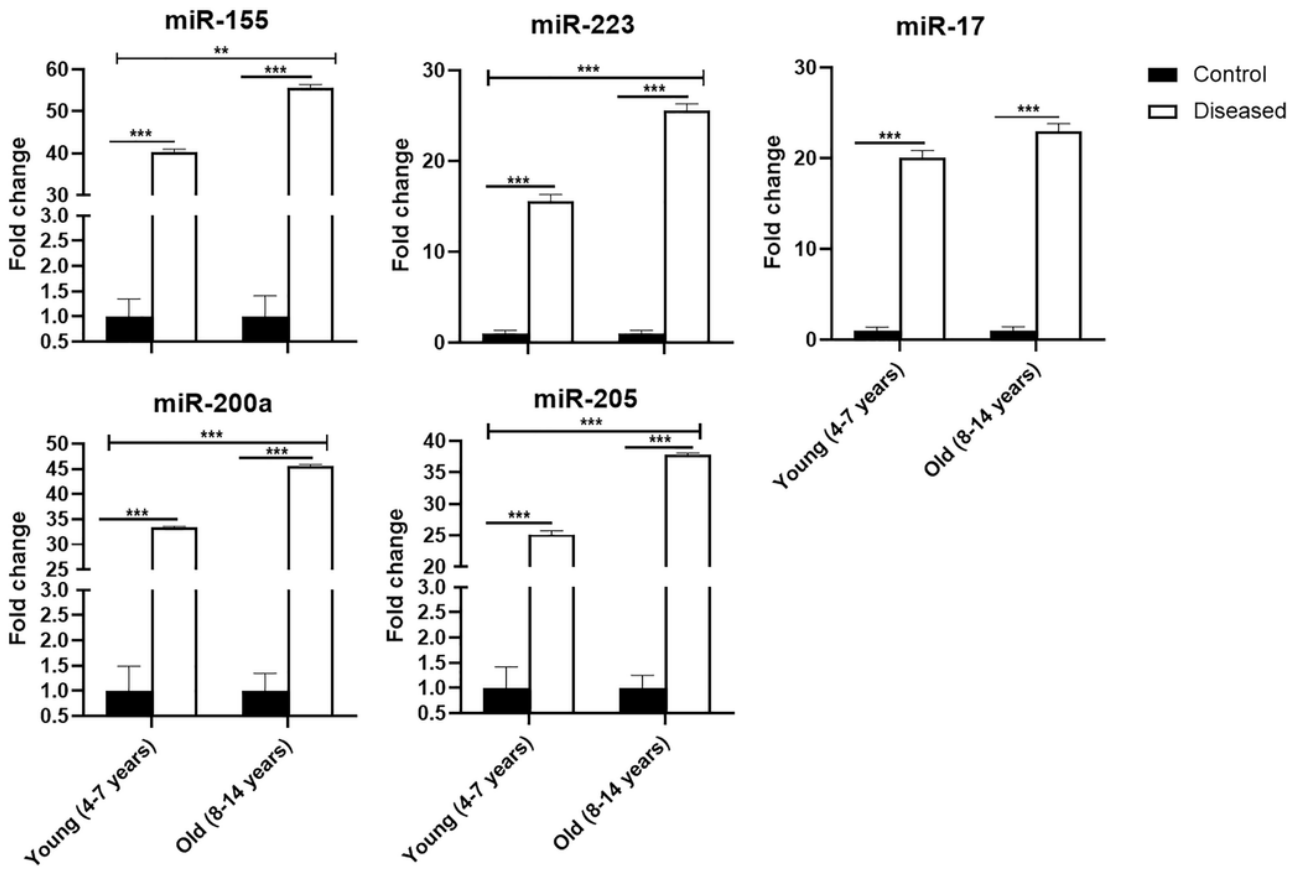

Figure 3

Expression profile of eca-miR-155, eca-miR-223, eca-miR-17, eca-miR-200a, and eca-miR-205in serum of mares {young (4-7 years) and old (8-14 years)} with endometritis compared to control ones. Bars are presented as mean±SEM. Asterisk(s) represent statistical significance;**P<0.01, ***P<0.001.