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Exosomal *miR-433* regulates the secretion of inflammatory factors in endometrium epithelial cells of dairy cows with endometritis

Naihan Yuan, Longfei Xiao, Kaijun Guo and Xiangguo Wang*

Animal Science and Technology College, Beijing University of Agriculture, Beijing, China

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ABSTRACT

Endometritis is a common postpartum reproductive system disease, which causes reproductive disorders and even infertility in dairy cows. High-throughput sequencing revealed that exosomal *miR-433* is differentially expressed in the peripheral blood of dairy cows with endometritis. However, the specific roles of *miR-433* in the occurrence and development of endometritis in dairy cows are still unclear. Therefore, a *miR-433*-labeled probe was used initially and found that *miR-433* is mainly located in uterine cavity epithelial cells and stromal cells. The fluorescence expression of *miR-433* in the uterine cavity epithelium and stromal tissues of dairy cows with endometritis is significantly weaker than that in the healthy tissues. The qRT-PCR results showed that the expressions of *miR-433* in the uterine tissues of dairy cows with endometritis, *LPS* stimulated endometrial epithelial cells (*EECs*), and their derived exosomes were significantly lower than those in the uterine tissues of healthy dairy cows, *EECs* and their derived exosomes. Exosomal *miR-433* derived from *EECs* transports into neighboring *LPS*-stimulated *EECs* by exosome vesicles fusion, regulating the secretion of inflammatory factors within the endometrial epithelium of dairy cows with endometritis, further influencing the development of endometritis. In conclusion, the intensity of *miR-433* expression decreased in the epithelial and stromal cells of the uterine lumen and exosomes derived from endometrial epithelium in dairy cows during the occurrence of endometritis, which to some extent promotes the development and progression of endometritis in dairy cows.

Key words: dairy cows; endometritis; *miR-433*; exosomes; inflammatory factor

Introduction

Endometritis is a common multiple reproductive system disease in dairy farming, which brings huge economic losses to dairy farming (HEIJNEN et al., 1999; MAGATA et al., 2015; PICCARDI et al., 2015; BI et al., 2017; WANG et al., 2019; KÄMPFER et al., 2000). Therefore, the correct understanding of the development of cow endometritis, and the active exploration of its prevention and control measures are important means to improve the economic benefits of livestock farms.

Endometritis is divided into clinical and subclinical endometritis on the basis of clinical symptoms (LEBLANC et al., 2002; DUBUC et al., 2010). Compared to clinical endometritis, dairy cows with subclinical endometritis have no obvious clinical symptoms, and have a normal estrous

*Corresponding author:
Xiangguo Wang, Animal Science and Technology College, Beijing University of Agriculture, Beijing 102206, China, email: xiangguo731@163.com

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cycle, but their conception rate is low and they are even repeatedly infertile (WAGENER et al., 2017). Subclinical endometritis is usually determined by the percentage of polymorphonuclear (*PMN*) cells in the collected endometrial cell samples. In the actual reproduction process, treatment of subclinical endometritis is overlooked and delayed because there are no obvious clinical symptoms, leading to prolonged vacancy and a decreased conception rate in dairy cows, which brings huge economic losses to the dairy industry. Therefore, it is important to achieve early diagnosis of subclinical endometritis.

Exosomes are membrane vesicles 30-150 nm in diameter and are secreted by a variety of cell types (HEIJNEN et al., 1999; STOORVOGEL et al., 2002; CABY et al., 2005; MATHIVANAN et al., 2010). These exosomes contain RNA molecules, including messenger RNA (mRNA) and microRNA (miRNA), which can be transferred between different cells, affecting the function of the recipient cells (KATAKOWSKI et al., 2010; PEGTEL et al., 2010; ZOMER et al., 2010). There is increasing evidence that exosomes play important roles in cell-to-cell communication (LOTVALL et al., 2007; SMALHEISER et al., 2007; VALADI et al., 2007; RECORD et al., 2011; XIN et al., 2012). There is evidence that miRNAs are key regulators of genes in many cellular processes, including inflammation (PANKRATZ et al., 2017; WANG et al., 2018). Related studies reported that exosomal miRNAs derived from cancer cells are associated with the tumor microenvironment and can be used for early diagnosis, prognosis prediction and treatment outcome assessment of cancers, such as miR-498, miR-183, miR-205 and miR-31(DAI et al., 2020). Endometrial epithelial cells (*EECs*) are located on the mucosal surface of the uterus, forming a physical barrier to protect the host from pathogen invasion (WIRA et al., 2005) and play key roles in local innate immunity (HERATH et al., 2006; SOBOLL et al., 2006; TURNER et al., 2014). In our previous study, we also found that exosome-derived uterine miRNAs, isolated from dairy cows with endometritis, impede the development of blastocysts (WANG et al., 2019). After analysis by exosome transcriptome sequencing, *miR-433* was found to be significantly differentially expressed in the exosomes derived from the peripheral blood of healthy and diseased dairy cows (WANG et al., 2020a). However, the mechanisms of *miR-433* derived from endometrial exosomes in the occurrence and development of endometritis are still unclear.

In this study, we investigated the function of exosomal miR-433 in regulating the immune response to endometritis, aiming to investigate the distribution and differential expression of miR-433 in the uterine tissues and luminal fluid of healthy and diseased cows, and to assess whether it can be a molecular marker for the early diagnosis of endometritis in dairy cows.

Materials and methods

Bovine uterus collection and exosomes isolation. This study was conducted in accordance with the guidelines of the Animal Ethics Committee of Beijing Agricultural University (Permit number: SYXK(JING)2015-0004). Bovine uteri were taken from the slaughterhouse. Before being slaughtered, postpartum Holstein cows from Beijing Shun Sunshine Farm were monitored by rectal temperature measurement and rectal examination for uterine rejuvenation on Days 1, 7, 14, 21 and 30 after delivery, as a part of health monitoring of dairy cows. The bovine (parity 2-4, body condition score 3.25-4.0) uteruses without other diseases, such as mastitis, hoof disease, dermatitis and postpartum paralysis, with a body temperature lower than 39.5°C, and with mucopurulent or purulent secretions secreted through vagina reaching the level 3 of vaginal mucus secretion in bovine endometritis, were collected at 21-30 days postpartum as the clinical endometritis group (WILLIAMS et al., 2005). In addition, healthy bovine uteruses without disease (vaginal endoscopy without mucopurulent discharge) but with normal body temperature, 21-30 days postpartum served as the control group. The cows in both the healthy and the endometritis group with no estrus, and the corpus luteum prominently protruding from the surface of the ovary within 21-30 days after delivery were diagnosed by rectal examination. Five uteruses from healthy cows and

five from those with endometritis were dissected to observe the inflammatory state, and then Diff rapid cell staining (Solarbio, G1540, Beijing, China) was used to count the proportion of polymorphonuclear cells (*PMN*) in the uterine cavity fluid (WANG et al., 2020b).

Exosomes were isolated from the uterine cavity fluid using the Exosome Extraction Kit-Body Fluids according to the manufacturer's recommendations (Bebo Bio, Shanghai, China). The specific operation was consistent with a previous paper published by the laboratory (WANG et al., 2020b).

Morphology of exosomes by electron microscopy. The sample of 10 μL was added to the copper grid to precipitate for 1 min, and the floating liquid was absorbed with filter paper. Then, 10 μL of uranyl acetate (phosphotungstic acid) was added to the copper grid to precipitate for 1 min, and the floating liquid was absorbed with filter paper. After this it was dried for 10 minutes at room temperature, and electron microscopy imaging was performed at 80 kV.

PKH26 staining for exosomes. The *PKH26* Red Fluorescent Cell Linker Kit (Sigma, Santa Clara, CA, USA) was used for labeling the lipid bilayers. The exosomes were first resuspended in 100 μL dilution C. The dye solution (4×10^{6} M) was prepared by adding 0.4 μL *PKH26* ethanol dye solution to 100 μL dilution C. The 100 μL exosome suspension was then mixed with the 100 μL dye solution. The exosomes were incubated and the dye suspension was mixed regularly for 1-5 min after the operation, and the staining was stopped by adding 200 μL serum and incubating for 1 min. Finally, the stained exosomes were washed twice with 1× PBS (Centrifugation at 4°C, 10,000 g for 60 min) and suspended in 10 μL PBS in a fresh sterile conical polypropylene tube. Afterwards, the internalization of the fluorescently labeled exosomes (10^{6} particles/cell) in the *EECs* after 12 h of incubation was examined by confocal microscopy.

Lipopolysaccharide (LPS) challenge of bovine endometrial epithelium cell lines (EECs). Bovine endometrial epithelial cells (*EECs*, *BEND cell* line purchased from ATCC Cell Bank, Beijing) were cultured in DMEM/F12 (Invitrogen) supplemented with 10% FCS (Gibco, New York, USA) streptomycin (Chengdu Huaxia Chemical Reagent Co. LTD, Chengdu, China) at 37°C in 5% CO_2. The BEND found to be greater than 90% pure were challenged with 100 μg/mL of *LPS* (*E. coli* 0111:B4, Invitrogen) for 24 h (similar to the uterine lumen concentration in endometritis) to induce the release of immune factors and expression of uterine receptivity-related genes in *LPS*-stimulated *EECs*.

Cell transfection. EECs or *LPS*-treated *EECs* were seeded into 6-well plates and transfected by use of Lipofectamine 2000 (Invitrogen, California, USA) and Opti-MEM (Gibco, New York, USA), according to the manufacturers' instructions. For miRNA upregulation and downregulation, a 100 pmol dose of *miR-433* mimics and nonsense control (NC) was used. In addition, *EECs* were harvested 24 h after transfection to isolate total RNA, total cell lysate, or assess the transfection efficiency. The *miR-433* mimics and NC sequences were designed and synthesized by Sangon Bioengineering (Shanghai, China) Co., Ltd. as follows: 5'-ATCATGATGGGCTCCTCGGTGT-3' (*miR-433* mimics, sense), 5'-GTGTAACACGT CTATACGCCCA-3' (NC, sense).

In situ hybridization. The uterine tissue was taken out of the -80°C freezer, thawed at 25°C, and stored in 4% paraformaldehyde for fixation, before the frozen sections were prepared. The frozen section samples were treated with a mixture of 30% H_2O_2 and methanol (1:9) at 25°C for 10 min. After being washed with DEPC (Beijing Baiolibo Technology Co., LTD, Beijing, China) 3 times, for about 1 min each time, the sections were placed in a wet box made with 5×SSC (pH: 7.5) (35 mL) mixed with formamide (35 mL), dropped onto the tissue with 0.25% hydrochloric acid, left at 25°C for 15 min, and washed twice with DEPC. Proteinase K was covered in tissue, hybridized in molecular hybridization apparatus at 37°C for 1 h, and protein K was stopped by washing with 0.2% glycine wash solution. The samples were first washed twice with PBS, and then twice with 5xSSC (pH: 7.5). The tissues were covered with prehybridization solution and prehybridized at 65°C for 1 h. Sections were

covered with a 500 ng/mL probe and left to react in the dark for 48 h in a hybridization device at 62-70°C. After washing once with 2×SSC (pH: 7.5), formamide was mixed 1:1 with 4×SSC (pH: 4.5) and the mixture was washed 3 times at 60°C, after that it was washed 5 times with PBS at 25°C, and left to react for at least 30 min at 25°C. Anti-digoxin antibodies from biotinylated mice were added dropwise for 2 h at 37°C and washed 3 times with PBS. The *FITC*-labeled antibody was added dropwise and the reaction was incubated for 1 h at 37°C in the dark and washed 3 times with PBS. *DAPI* was used to stain the nuclei of the cells and washed 3 times with PBS. Finally, anti-quenching agents were added dropwise, and the slices were sealed and observed by laser confocal microscopy (Olympus, Tokyo, Japan).

The *miR-433* gene probe was designed and synthesized by Suzhou Jima Gene Co., Ltd. as follows: 5'-ATCATGATGGGCTCCTCGGTGT-3'(*miR-433*) and 5'-GTGTAACACGTCTATACG CCCA-3'(NC).

Real-time detection of RT-PCR (qRT-PCR). Total RNA from exosomes and uterine tissue *EECs* was extracted with Trizol (Invitrogen Inc., Carlsbad, CA), and cDNA was synthesized with Prime Script RT Reagent Kit (TaKaRa Bio Inc., Dalian, China). The expressions of *miR-433* in healthy bovine uterus or endometritis, *miR-433* and GAPDH gene primers were designed and manufactured by Sangon Bioengineering (Shanghai, China) Co., Ltd. as follows: 5'-ATCATGATGGGCTCCTCG-3'(*miR-433*, sense), 5'-CAGTGCAGGGTCCGAGGT-3' (*miR-433*, anti-sense), 5'-GGCGTGAACCACGAG AAGTA-3' (GADPH, sense) and 5'-GGCGTGGAC AGTGGTCATAA-3' (GADPH, anti-sense).

Western blot detection. Western blot was used for detection of exosome markers. The exosomes obtained from the uterine cavity fluid were detected by Western blot for the purpose of the protein expressions of CD63 (Anti-CD63 antibody Abcam, Cambridge, UK, 1:1000 dilution, ab193349) and Calnexin (Anti-Calnexin Antibody, Abcam, Cambridge, UK, 1:1000 dilution, ab75801) protein in the exosomes. The next day, it was incubated with the corresponding secondary antibody conjugated to horseradish peroxidase (HRP, 1:2000; Zhongshan Golden Bridge Biotechnology, Nanjing, China) at room temperature for 1 h. Finally, immunoreactive bands were visualized with the Gel Image System (Tannon, Biotech, Shanghai, China).

ELISA detection. The concentrations of *IL-6*, *IL-8*, *TNF-α* and *IL-1β* in the culture media of *LPS* treated or *miR-433* mimics or NC transfection *EECs* were assayed with ELISA kits (BD Bioscience, San Jose, CA, USA), according to the manufacturer's recommendations. The optical densities at 450 nm of each well were determined by a micro-plate reader (Model 680, Bio-Rad, Hercules, CA, USA).

Statistical analysis. SPSS22.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis of data. One way ANOVA and Duncan's multiple comparison were performed. Data are presented as mean $\pm$ standard deviation (SD) of the mean. $P<0.05$ was used as the standard for significant differences.

Results

Anatomical observation and cytological identification of the uterus in dairy cows with endometritis. We first collected the uteruses of healthy dairy cows at 21-33 days postpartum, and the uteruses of dairy cows with endometritis for anatomical observation, as shown in Fig. 1A. The uterine tissue of healthy dairy cows was yellow, with no inflammation, a smooth uterine wall and a small amount of transparent or translucent fluid flowing out of the cervix. The uterine tissue of dairy cows with endometritis showed hyperemia edema, decreased elasticity, white mucopurulent secretions outside the cervix, and a large number of black and dark red nodules on the inner wall of the uterus. The uterine lavage fluid cytology smear of healthy dairy cows 21-33 day postpartum was mainly epithelial cells and no *PMN*s. A large amount of *PMN*s appeared in the cytology smears of the uterine lavage fluid of dairy cows with subclinical endometritis, the proportion of which was greater than 18% (Fig. 1B).

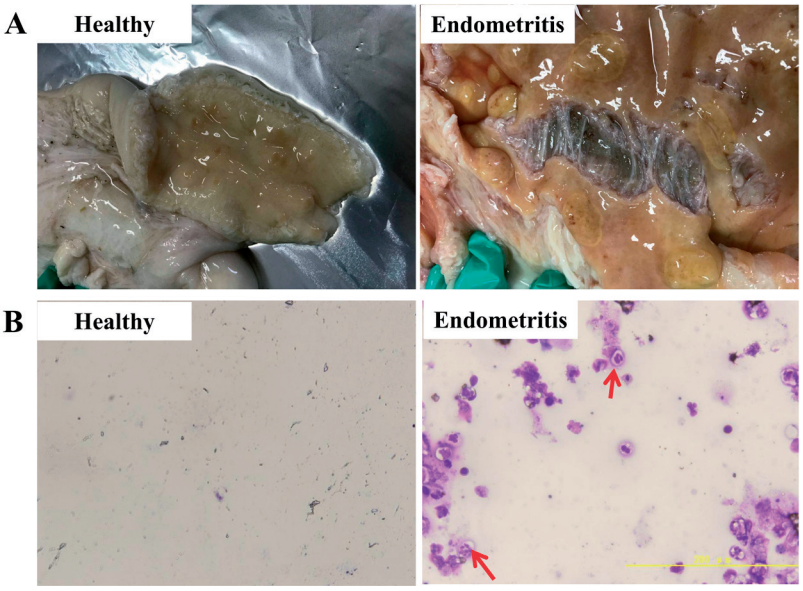

Fig. 1. Anatomical observation and cytological identification of the uterus in dairy cows with endometritis
(A) Dissection of uterine tissues from healthy and diseased dairy cows at 21-33 d postpartum. (B) Cytological smear of uterine fluid from healthy and diseased dairy cows at 21-33 d postpartum (neutrophils are indicated by arrows)

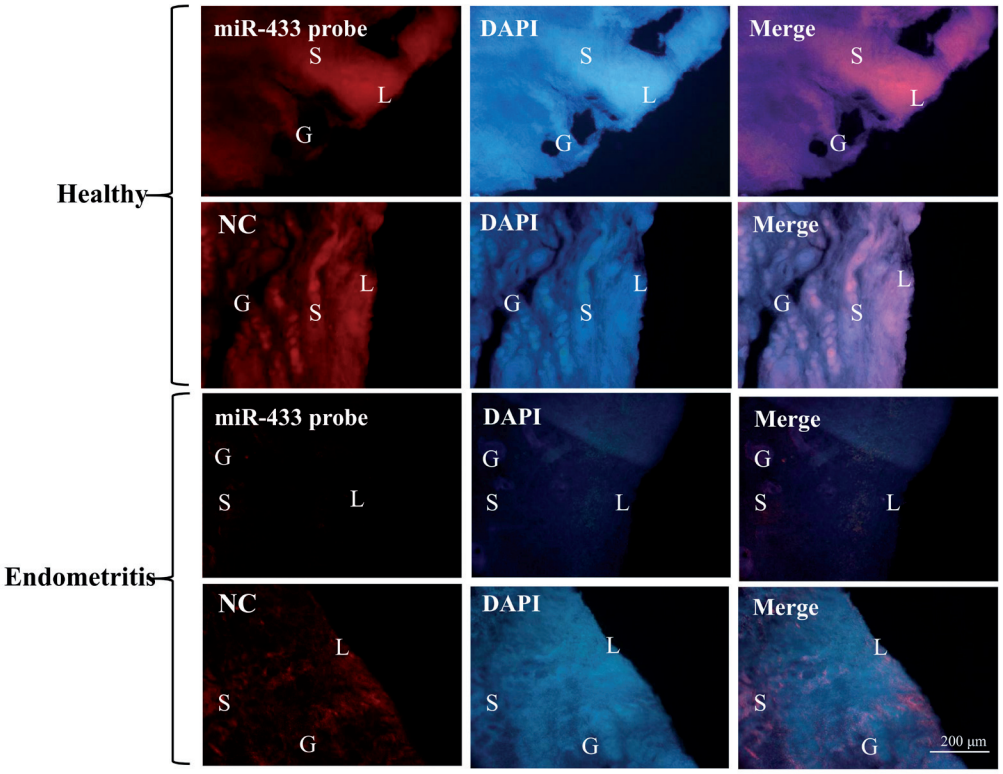

Fig. 2. Localization of *miR-433* in the uterine tissue of dairy cows with endometritis
Red: *miR-433* probe; *DAPI*: nuclear dye. S: matrix, G: gland, L: endometrial epithelium, Bars=200 μm. Data are representative of three independent experiments

Localization of miR-433 in the uterine tissue of dairy cows with endometritis. In situ hybridization results showed that *miR-433* was mainly located in the uterine stroma and luminal epithelium of healthy dairy cows, while the fluorescence intensity of *miR-433* in the stromal and luminal epithelial tissues was significantly weakened, and only low *miR-433* fluorescence intensity was observed in the stromal tissues in the uteruses of dairy cows with endometritis (Fig. 2).

Expression of miR-433 in the uterine tissue and epithelial cells of dairy cows with endometritis. The qRT-PCR results showed that the expression of *miR-433* in the uterine tissues of dairy cows with endometritis was significantly lower than in the uterine tissues of healthy dairy cows (Fig. 3A, $P<0.05$). Subsequently, we further found that the expression of *miR-433* in *LPS* treated *EECs* was also significantly lower than that in the normal cultured *EECs* (Fig. 3B, $P<0.05$).

Isolation and identification of exosomes derived from bovine uterine cavity fluid and miR-433 expression analysis. The exosomes derived from healthy dairy cows' uterine cavity fluid and the uterine cavity fluid of dairy cows with endometritis were extracted with an exosome extraction kit, and had a diameter of 30-150 nm and a cyst-like structure (Fig. 4A). Exosomes all express the marker protein CD63, but do not express Calnexin (Fig. 4B). The expression level of *miR-433* in exosomes derived from the uterine cavity fluid of dairy cows with endometritis was significantly lower than in exosomes derived from the uterine cavity fluid of healthy dairy cows (Fig. 3C, $P<0.05$).

MiR-433 transported between EECs and LPS treated EECs as vesicles fusion. The *PKH26* labeled (fluorescently red) exosomes derived from the uterine cavity fluid of healthy dairy cows were incubated with *LPS* treated *EECs* for 12 h (Fig. 5A). The expression of *miR-433* significantly increased after *LPS* treated *EECs* were co-incubated with exosomal vesicles derived from the uterine cavity fluid of healthy dairy cows (Fig. 5B, $P<0.05$). Meanwhile, *miR-433* mimics were transfected into the *LPS* treated *EECs,* with transfection efficiency higher than 98% (Fig. S1), and the *miR-433* expression significantly elevated in *LPS* treated *EECs* after *miR-433* mimics transfection (Fig. 5C. $P<0.05$).

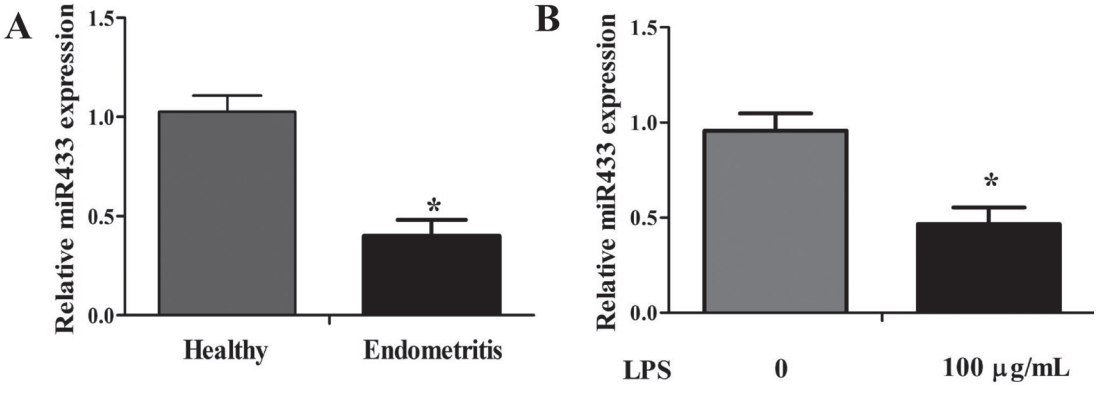

Fig. 3. The relative expression of *miR-433* in dairy cow uterine tissue and epithelial cells

The relative expression of *miR-433* in dairy cow uterine tissue. (B) The relative expression of *miR-433* in *LPS* treated *EECs* (100 μg/mL, 24 h). The values represent the mean ± standard deviations for three duplicates (*$P<0.05$)

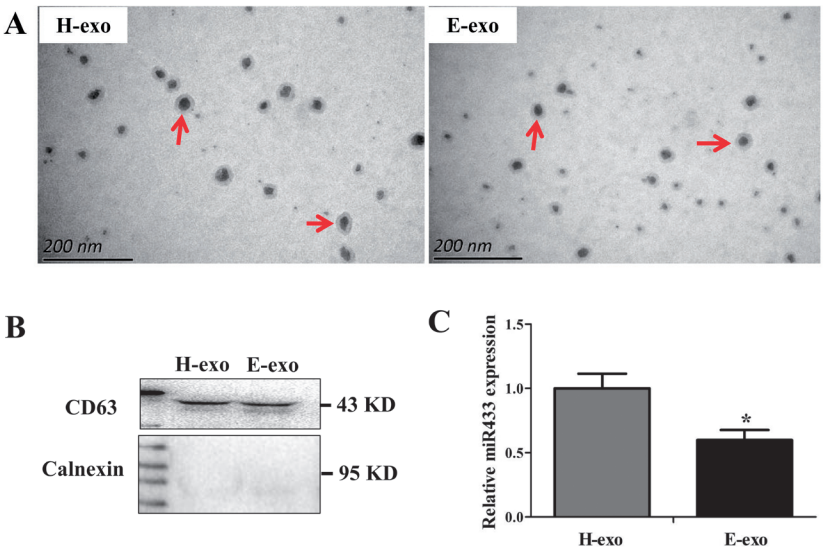

Fig. 4. Isolation and identification of exosomes from bovine uterine cavity fluid and *miR-433* expression analysis

(A) Electron microscopic detection of uterine cavity fluid exosomes derived from healthy and diseased dairy cows (Exosomes are indicated by arrows). (B) CD63 and Calnexin protein expression detection by Western blot. (C) Relative expression of *miR-433* in uterine cavity fluid exosomes derived from dairy cows. The values represent the mean±standard deviations for three duplicates (*P<0.05)

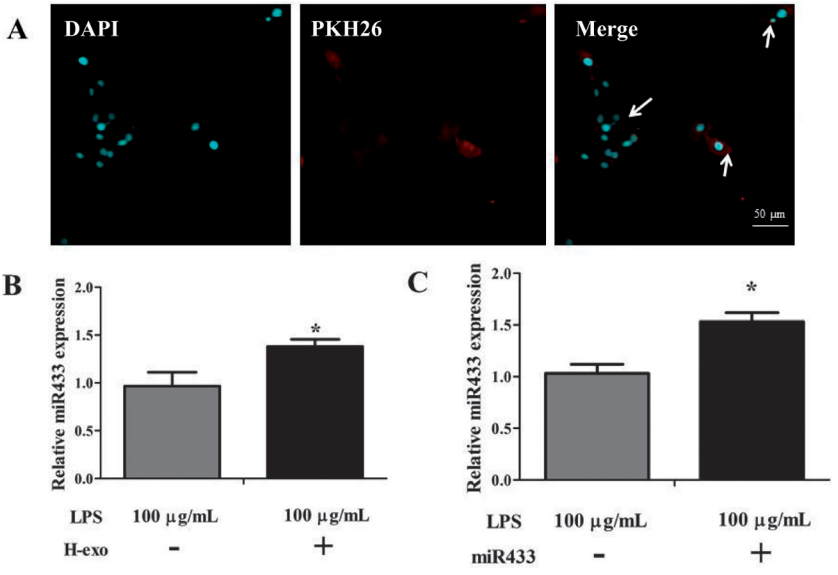

Fig. 5. *MiR-433* is transported between *EECs* and *LPS*-treated *EECs* with exosomal vesicle fusion

(A) Confocal microscopy of the internalization of *PKH26* labeled exosomes in *EECs* after 12 h incubation, Bars=50 μm. Data are representative of three independent experiments. The arrow points for a *PKH26* labeled exosome (red fluorescent). (B) The expression of *miR-433* in *LPS* treated *EECs* co-incubated with *PKH26* labeled exosome for 12 h and then detected with the qRT-PCR method. Data are presented with mean±standard deviation from three independent experiments, *P<0.05. (C) The expression of *miR-433* in *LPS* treated *EECs* after the transfection of *miR-433* mimics for 24 h and then detected using the qRT-PCR method. Data are presented with mean±standard deviation from five independent experiments, *P<0.05.

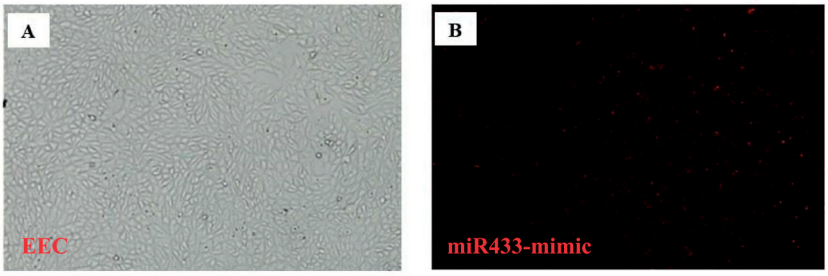

Fig. 6. Fluorescence imaging of *miR-433* mimics-transfected *EECs*
Data are representative of three independent experiments

Effect of miR-433 on the release of inflammatory factors and chemokines in LPS treated EECs. In this study, the secretions of *IL-6*, *TNF-α*, *IL-1β* and *IL-8* were all significantly inhibited in *LPS* treated *EECs* after *MiR-433* mimic transfection (Fig. 7A-D, P<0.01). The secretions of *TNF-α* and *IL-1β* were also significantly inhibited in *LPS* treated *EECs* after mimics-NC transfection (Fig. 7A-B, P<0.05). However, there was no differential change in the secretions of *IL-6* and *IL-8* when *LPS* treated *EECs* were transfected with mimics-NC (Fig. 7C and D, P>0.05). Certainly, the inhibitory ability of *miR-433* mimic on *IL-6*, *IL-1β* and *IL-8* secretions in *LPS* treated *EECs* is stronger than that in mimics-NC transfection group (Fig. 7B-D, P<0.05).

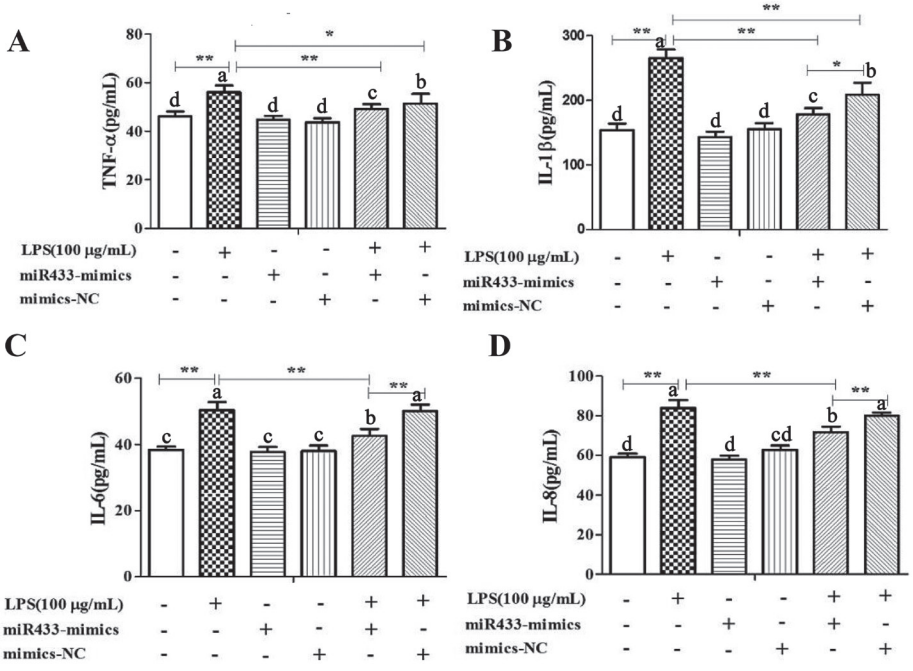

Fig. 7. The effect of *miR-433* mimics or NC transfection on the release of inflammatory factors and chemokines *TNF-α*(A), *IL-1β*(B), *IL-6*(C) and *IL-8*(D) induced by *LPS* administration

Data are presented with mean±standard deviation from five independent experiments, the same letters indicate no significant differences among then (P>0.05), different letters indicate a significant difference among them (P<0.05). In addition, "*P<0.05, **P<0.01" specifically indicates the difference between the two bar graphs

Discussion

In order to distinguish between clinical endometritis and subclinical endometritis, endometrial cytology is generally used to detect the percentage of *PMN*s in the sample (BARAŃSKI et al., 2012). The criteria for determining subclinical endometritis is *PMN*s>18% at 21-33 days postpartum or *PMN*s>10% at 34-47 days postpartum (KASIMANICKAM et al., 2004). It has been reported that *PMN*s>8% in the uterus at 28-41 days postpartum is a marker for the onset of occult endometritis in dairy cows (BARLUND et al., 2008). Other studies have reported that *PMN*s>18% in the fourth week of postpartum is the best threshold for diagnosing subclinical endometritis in dairy cows (BARAŃSKI et al., 2012). However, so far there have been no unified criteria for determining clinical endometritis and subclinical endometritis.

miRNAs are a class of broad-spectrum regulatory RNAs that regulate biological processes and play important regulatory roles in the inflammatory response (BI et al., 2017). miRNAs can directly target TLRs, or target related molecular proteins in the TLR signaling pathway to regulate the inflammatory response. In this study, we found that endometrial inflammation affected the expression of *miR-433* in the epithelial and interstitial tissues of the uterine cavity, which is consistent with our previous high-throughput sequencing results (WANG et al., 2020a). Coincidentally, the down-regulation of *miR-433* promotes the epithelial-mesenchymal transition and tumor metastasis of lung cancer (SHI et al., 2017). The proliferation of non-small cell lung cancer cells is also regulated by the *miR-433* (YANG et al., 2019). However, the uterus is a special organ, regulated by ovarian steroid hormones in different estrus cycles in mammals. Related studies have also found that ovarian hormones regulate the function of miRNAs and the immune system in the uterus (EDEY et al., 2018). Therefore, an *in vitro* model of endometritis was established by *LPS* treated *EECs*. At the same time, healthy dairy cows and diseased dairy cows with endometritis in the same ovarian state were used to eliminate the influence of estrus cycle on the *miR-433* expression and cytokine secretion (WANG et al., 2020a). The expression of *miR-433* decreased in the *LPS* treated *EECs*, which was consistent with the expression of *miR-433* in the uterine tissue of dairy cows with endometritis.

Here, we successfully obtained exosomes derived from the uterine fluid of healthy dairy cows and dairy cows with endometritis, and confirmed their morphology by electron microscopy. The expression of *miR-433* in exosomes derived from the dairy cows with endometritis was significantly lower than in exosomes derived from the uterine fluid of healthy cows, which is consistent with the expression of *miR-433* in the uterine tissue of dairy cows with endometritis and *LPS* treated *EECs*. Uterine cavity fluid exosomes derived from healthy dairy cows can fuse with *LPS* treated *EECs*, so *miR-433* can be transported to adjacent target cells. It has been reported that the functions of *miR-433* include inhibition of cell proliferation, migration, invasion and apoptosis through inactivation of the *MAPK* signaling pathway (ZHANG et al., 2018). In addition, *miR-433* is a tumor suppressor, and is frequently downregulated to inhibit *EMT* in *BCa* cells by regulating *c-Met/Akt/GSK-3β/Snail* signaling (XU et al., 2016). In this study, *miR-433* mimic transfection significantly reduced the secretions of *IL-6*, *IL-1β* and *IL-8* in the *LPS* treated *EECs*. *IL-6* and *IL-1β* are major inflammatory factors in many chronic inflammatory diseases, while *IL-8* is a major chemokine that regulates the inflammatory response and has a strong pro-angiogenic effect. Therefore, we inferred that *miR-433* can effectively inhibit the release of inflammatory factors *IL-1β*, *IL-6*, and *IL-8* in *EECs* induced by *LPS*.

Conclusions

In summary, *miR-433* showed a high expression in the tissues of healthy dairy cows, and significant downregulation in the tissues of dairy cows with endometritis. At the same time, the content of *miR-433* in exosomes derived from the uterine cavity fluid of healthy dairy cows and *EECs* was higher than in exosomes derived from the uterine cavity fluid of dairy cows with endometritis and *LPS* treated *EECs*. The secretion of inflammatory factors *IL-1β*, *IL-6*, and *IL-8* can be inhibited by exogenous

miR-433 in *EECs* induced by *LPS*. Compared with other inflammatory factors, the advantages of *miR-433* are mainly reflected in the early diagnosis of bacterial infection by its expression changes before the acute phase reactive protein, antibody reaction and cellular reaction, thus reducing antibiotic use and treatment costs.

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Authors' contributions

Naihan Yuan and Longfei Xiao contributed equally to this work. HY, LFX and XGW performed the experiments, collected and interpreted the data. MYY, XHS, and XLQ provided initial help with analysis. KX, CL and DC collected the samples. NHY and XGW conceptualized and wrote the manuscript. KJG reviewed the manuscript prior to publication.

Declaration of Competing Interest

None of the authors has any conflict of interest in publishing this study.

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SAŽETAK

Endometritis je česta poslijeporođajna bolest reproduktivnog sustava, koja uzrokuje poremećaje, pa čak i neplodnost u mliječnih krava. Sekvenciranje visoke propusnosti otkrilo je da je u perifernoj krvi mliječnih krava s endometritisom egzosomni *miR-433* diferencijalno eksprimiran. Još uvijek, međutim, nisu razjašnjene specifične uloge *miR-433* u nastanku i razvoju endometritisa u mliječnih krava. S obzirom na to, inicijalno je upotrijebljena sonda označena kao *miR-433* te je ustanovljeno da se *miR-433* uglavnom nalazi u epitelnim i stromalnim stanicama maternične šupljine. Ekspresija *miR-433* dobivena metodom fluorescencije iz epitela i stromalnog tkiva maternične šupljine mliječnih krava s endometritisom znakovito je manja nego u zdravim tkivima. Rezultati dobiveni qRT-PCR-om pokazali su da su ekspresija molekule *miR-433* u maternicnom tkivu mliječnih krava s endometritisom, endometrijske epitelne stanice (EEC) stimulirane lipopolisaharidom (LPS) i njihovi egzomi bili znakovito niži od onih u maternicnom tkivu zdravih mliječnih krava. Egzosomni *miR-433* dobiven iz EEC-a prevodi se u susjedne EEC-ove stimulirane LPS-om fuzijom egzosomskih vezikula, čime se regulira izlučivanje upalnih čimbenika u epitelu endometrija mliječnih krava s endometritisom, što dodatno utječe na razvoj endometritisa. Zaključno, intenzitet ekspresije *miR-433* smanjio se u epitelnim i stromalnim stanicama maternične šupljine i egzomima dobivenim iz epitela endometrija u mliječnih krava s endometritisom, što u određenoj mjeri potiče razvoj i progresiju endometritisa u mliječnih krava.

Ključne riječi: mliječne krave; endometritis; *miR-433*; egzosomi; upalni čimbenik