

Analysis of Circulating microRNA During Early Gestation in Japanese Black Cattle

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Research

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Abstract

Background

Circulating microRNAs (miRNAs) are biomarkers for various diseases and physiological conditions in humans and mice; studies in domestic animals, particularly cattle, are limited. The importance of early pregnancy diagnosis (especially within the 21 day cow estrous cycle) in the livestock industry is extremely high. This study compared the circulating miRNAs in non-pregnant and pregnant Japanese Black cows, explored miRNAs as biomarkers for early pregnancy diagnosis, and established a measurement system that includes reference miRNA selection and the effect of hemolysis.

Methods

miRNA was extracted from the plasma of Japanese Black cows on day 21 after artificial insemination and subjected to a customized bovine oligonucleotide microarray for expression analysis. Differentially expressed miRNAs and reference miRNA candidates were selected and validated by real-time quantification PCR (RT-qPCR). Their stability was evaluated using NormFinder software. Hemolyzed samples were prepared using plasma from five cows in estrous cycle and subjected to RT-qPCR.

Results

A total of 124 miRNAs were detected in bovine plasma by microarray analysis in non-pregnant and pregnant cows. The levels of five circulating miRNAs were significantly higher in pregnant cows than in non-pregnant cows, and 24 miRNAs were detected only in the pregnant group. NormFinder analysis and RT-qPCR validation showed that miR-2455 was an appropriate reference miRNA in the plasma of non-pregnant and pregnant Japanese Black cows and miR-19b, miR-25, miR-29a, and miR-148a were significantly higher in the pregnant group. These four circulating miRNAs did not change during the estrous cycle and were less affected by hemolysis.

Conclusions

In the current study, we found high levels of four miRNAs in the plasma of pregnant Japanese Black cows. Since these miRNAs are less affected by hemolysis, they may potentially be used as markers for early pregnancy diagnosis in cattle.

Background

MicroRNAs (miRNAs) are single-stranded, non-coding RNAs as short as 18–25 bases and have been implicated in the post-transcriptional regulation of gene expression [1]. Serum, plasma, and mammalian tissues also contain miRNA, which is protected from RNA-degrading enzymes by inclusion within various

protein complexes, microvesicles, and exosomes which are consistently present [2–4]. Therefore, miRNAs in serum and plasma, which are easily obtainable by minimally invasive techniques, are expected to be useful as biomarkers for physiological conditions and various diseases. Although studies on the identification of circulating miRNAs in humans and mice have been carried out by many researchers [5–9], little is known about circulating miRNAs in domestic animals.

Early pregnancy diagnosis in cattle in the livestock industry is highly useful. Accurate early pregnancy diagnosis after artificial insemination can identify non-pregnant individuals and facilitate early re-insemination, leading to improved reproductive performance and productivity. Recently, in addition to conventional pregnancy diagnosis, molecular biology techniques using peripheral blood leukocyte gene expression as an indicator [10–12] have been proposed. Circulating miRNAs have been detected during early pregnancy in cattle [13–16], and their potential applicability for early pregnancy diagnosis in cattle has been suggested. However, there is limited information regarding this. In addition, since bovine breed-dependent miRNA expression has been confirmed [17, 18], verification is required for each breed. The purpose of the present study was to compare circulating miRNA levels in non-pregnant and pregnant Japanese Black cows, and to identify additional potential biomarkers for early pregnancy in cows. Furthermore, the internal reference miRNA of the measurement system and the effect of hemolysis on the quantification of miRNA were also examined for pregnancy diagnosis.

Methods

Animals and sample collection

The Iwate University Laboratory Animal Care and Use Committee approved the experimental and feeding conditions for the cattle used in this study (A201434, A201701). In the current study, we used 14 parous Japanese Black cattle from one experimental farm. Eleven Japanese Black (JB) cattle were subjected to controlled estrus following treatment with prostaglandin F2 (Regipron C, Aska Pharmaceutical Co., Ltd, Tokyo, Japan) and were artificially inseminated (AI) according to the AM-PM rule following estrus detection twice daily. Peripheral blood from a total of nine AI cows was collected on day 21 (day 0 being the date of AI) to compare circulating miRNA levels in four non-pregnant and five pregnant cows. Pregnancy status was also confirmed by ultrasonographic examination at approximately 30 d and 60 d post-pregnancy in AI cows. Additionally, five JB cows without AI that displayed a normal estrous cycle were used for collection on estrous cycle days 0, 3, 7, 14, 18, and 20 (behavioral detection of estrus was designated as day 0) to examine changes in circulating miRNA during the estrous cycle. Blood was obtained from the jugular vein and transferred into EDTA-containing vacutainers (Terumo, Tokyo, Japan). Plasma was collected by centrifugation at $2,000 \times g$ for 1 h at 4 °C.

The miRNA extraction and microarray analysis

Extraction of miRNA from plasma was performed using the miRNeasy Serum/Plasma Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The quality and quantity of the extracted miRNAs were evaluated using an Agilent Bioanalyzer with Small RNA Kit (Agilent Technologies, Santa

Clara, CA, USA) and Nanodrop (ND-1000, Thermo Fisher Scientific, Waltham, USA). A customized bovine oligonucleotide miRNA microarray (GPL30312, Agilent Technologies) was used to detect circulating miRNA profiles in non-pregnant or pregnant JB cows; it represented 744 mature bovine miRNA sequences based on miRBase (*Bos taurus* 19.0 release) (<http://www.mirbase.org>). The array was placed at 20 spots for each miRNA probe. The miRNA microarray experiment was performed using plasma collected from non-pregnant (n = 4) and pregnant (n = 4) JB cows on day 21 after AI. We performed one-color microarray analysis; fluorescence-labeled (Cy3) RNA was prepared from 50 ng of miRNA from each sample, and labeled RNA was then hybridized and washed using the miRNA Complete Labeling Reagent and Hyb Kit (Agilent Technologies) according to the manufacturer's instructions. After washing, the arrays were scanned using an Agilent SureScan Microarray Scanner and Feature Extraction ver. 9.5 (Agilent Technologies) for image analysis and data extraction. The microarray data from each sample were imported into the onboard GeneSpring 12.0 (Agilent Technologies) program for use in the software's normalization algorithm and for the detection of differentially expressed miRNAs. As a result of principal component analysis using GeneSpring for quality control of microarray data, however, we decided to use data from three non-pregnant and four pregnant groups in the next analysis, because of anomalies in the expression dynamics of one non-pregnant sample. Normalization was performed by dividing each measurement of each array by the median of all measurements in the array (per-chip normalization). The data were analyzed using Student's *t*-tests with the Benjamini–Hochberg false discovery rate (FDR) multiple testing correction (FDR corrected $P < 0.05$). Fold changes were calculated by comparing non-pregnant and pregnant cows. GEO accession numbers were as follows: platform, GPL30312 samples, GSM5400678 to GSM5400684; series, GSE178906.

Real-time quantitative RT-PCR (RT-qPCR)

Sixty nanograms of miRNA was reverse transcribed to complementary DNA (cDNA) using the Mir-X miRNA First-Strand Synthesis Kit (Takara, Shiga, Japan) according to the manufacturer's instructions. RT-qPCR was performed using KOD SYBR qPCR Mix (TOYOBO, Osaka, Japan) and the ABI7300 real-time PCR system (Thermo Fisher Scientific). The primers used are listed in Additional Table 1. In the cDNA synthesis system using the Mir-X miRNA First-Strand Synthesis Kit, an adapter sequence was added to the synthesized cDNA. The PCR reaction was performed with mature miRNA sequence as the forward primer and mRQ 3' primer, which corresponds to the adapter sequence, supplied in the kit as the reverse primer. The thermal cycling conditions included initial sample incubation at 98 °C for 2 min, followed by 40 cycles of 98 °C for 15 s, 60 °C for 10 s, and 68 °C for 30 s. To quantify the miRNA copy number, a standard curve was generated for each miRNA with a serial dilution of standard oligonucleotides containing the corresponding mature miRNA and the above adapter sequence. The standard oligonucleotides for RT-qPCR were synthesized by Eurofins Genomics (Tokyo, Japan) and are listed in Additional Table 2. The dissociation curve for the detection of the SYBR Green–based objective amplicon was confirmed, and the copy number of each miRNA was determined at each amplicon peak point.

Analysis of miRNA stability

A model-based method, the NormFinder algorithm, was used to rank candidate reference genes by stability value and consider the gene with the lowest value to be the most stable reference gene [19]. Analysis was performed according to the manufacturer's instructions. The normalized signal intensities from the miRNA microarray or raw Ct values of RT-qPCR for all reference miRNA candidates were converted to the required data format. Briefly, all microarray data in the present study were analyzed with the NormFinder program and high-ranked and abundant miRNA sequences in the plasma (normalized intensity signal value > 10 (\log_2 transformed values)) were selected as miRNA candidates. Secondly, the candidates from the stability analysis using microarray data and from previous reports [14,20] were validated by RT-qPCR and re-analyzed using the NormFinder program.

Preparation of hemolysis dilution

Approximately 0.5 ml of whole blood collected from the mid-stage of cows in estrous cycle (n = 5) was sonicated using an ultrasonic generator (UR-20P, TOMY SEIKO, Miyagi, Japan) at power control setting 5 for 30 s. Plasma was isolated by centrifugation from sonicated blood, as described above. This sample was classified as 100% hemolyzed plasma for this study [21]. In contrast, non-hemolyzed plasma was obtained by centrifugation of non-sonicated blood. A hemolysis dilution series comprising 0.032%, 0.16%, 0.8%, and 4% hemolyzed and non-hemolyzed samples (v/v) was prepared by serial dilution of the 100% hemolyzed sample with non-hemolyzed plasma. The degree of hemolysis in plasma was analyzed using a Nanodrop at a wavelength of 414 nm as free hemoglobin [21]. Hemolysis was also determined by the ratio of miR-451, an erythrocyte-derived miRNA, to miR-23a (delta Ct [miR-23a–miR-451]) using RT-qPCR. Extraction of miRNA from the hemolyzed sample, cDNA synthesis, and RT-qPCR was performed as described above.

Statistical analysis

Data from RT-qPCR and hemolysis experiments were analyzed using Student's *t*-test or one-way ANOVA followed by Dunnett's multiple comparison test using JMP version 7 software (SAS Institute Inc., Cary, NC).

Results

Analysis of circulating miRNA profiles using microarrays

Circulating miRNA profiles in the plasma of non-pregnant and pregnant cows on day 21 after AI were investigated using a bovine miRNA microarray. Of the 744 bovine miRNAs, a total of 124 miRNAs were detected in the plasma, and 98 miRNAs were commonly detected in non-pregnant and pregnant cows (Fig. 1 and Additional Table 3). The miRNAs detected in non-pregnant and pregnant cows were 2 and 24, respectively. Among the miRNAs expressed in both non-pregnant and pregnant cows, five miRNAs showed more than a 2-fold difference (Table 1). All five miRNAs were significantly elevated in pregnant cows.

Table 1

List of differentially expressed circulating miRNAs in microarray data of non-pregnant and pregnant Japanese Black cows

miRNAs	Fold Change ^a	P-value ^b
bta-miR-19b	3.0	0.042
bta-miR-25	2.5	0.028
bta-miR-27b	2.4	0.001
bta-miR-29a	2.5	0.022
bta-miR-30d	2.4	0.002
^a Fold change in miRNA quantities in the plasma of non-pregnant cows (n = 3) compared with pregnant cows (n = 4) on day 21 after artificial insemination. Values represent the pregnant/non-pregnant ratio. Data were obtained from microarray analysis.		
^b Corrected P-values after Benjamini–Hochberg false discovery rate correction.		

Selection of the reference miRNA in the plasma of non-pregnant and pregnant cows

To ensure quantification of bovine circulating miRNA by RT-qPCR, we examined reference miRNA candidates in the present study and from previous reports (let-7g and miR-128) [14, 20].

First, all microarray data were analyzed for stability using NormFinder (Additional Table 4), and high-ranked and abundant miRNAs in plasma were selected as reference miRNA candidates. Next, the levels of reference miRNA candidates (including let-7g and miR-128) in plasma were determined by RT-qPCR, and the expression stability of each miRNA was analyzed using the NormFinder program. The Ct values of each miRNA in plasma obtained by RT-qPCR were significantly different between the non-pregnant and pregnant cows in the five miRNAs (Table 2). Two miRNAs were not specifically amplified by the primers designed in the present study. NormFinder analysis revealed that the lowest stability value was 0.133 for miR-2455.

Table 2
Selection of reference miRNA in plasma from RT-qPCR data using NormFinder program.

miRNAs	Ct value		Stability value ^a	Rank ^b
	Non-pregnant	Pregnant		
bta-let-7g	31.6 ± 0.5	32.3 ± 0.5*	0.643	7
bta-miR-128	30.2 ± 0.4	31.9 ± 0.6**	0.668	8
bta-miR-1224	31.1 ± 0.3	31.2 ± 0.3	0.148	2
bta-miR-1835	28.4 ± 0.3	28.2 ± 0.2	0.284	4
bta-miR-2316	26.0 ± 0.4	26.4 ± 0.2	0.257	3
bta-miR-2348	N.D.	N.D.	–	–
bta-miR-2455	27.3 ± 0.6	27.2 ± 0.3	0.133	1
bta-miR-2478	20.1 ± 0.6	18.9 ± 0.4**	0.490	6
bta-miR-2888	30.8 ± 0.7	27.4 ± 0.3**	1.216	9
bta-miR-3141	N.D.	N.D.	–	–
bta-miR-6528	31.3 ± 1.0	31.8 ± 0.4	0.35	5

The levels of reference miRNA candidates in the plasma of non-pregnant and pregnant cows on day 21 after AI were analyzed by RT-qPCR. Ct value indicates the cycle threshold of RT-qPCR for each miRNA in the plasma of non-pregnant and pregnant cows. Data are shown as the mean ± SEM (n = 4). Statistical analysis was performed using Student's *t*-test. Asterisks indicate significant differences between the non-pregnant and pregnant groups (**P* < 0.05, ***P* < 0.01.). miRNAs using the NormFinder program. The values of gene stability were based on the expression variations of candidate reference genes, with a lower stability value indicating more stable reference genes.

^b Rank indicates order of stability value evaluated by NormFinder.

Validation of circulating miRNA levels in non-pregnant, pregnant, and cows in estrous cycle

The circulating miRNA levels in non-pregnant and pregnant cows on day 21 after AI as determined by RT-qPCR are shown in Fig. 2. We selected five miRNAs listed in Table 1 and five miRNAs detected only in pregnant cows, including miR-26b, miR-126-3p, miR-148a, miR-150, and miR-2382-3p (Additional Table 3). Target miRNA quantities were normalized to those of miR-2455. The levels of miR-19b, miR-25, miR-29a, and miR-148a were significantly higher in pregnant cows than in non-pregnant cows. Next, the dynamics of these circulating miRNAs in estrous cycle cows were examined by RT-qPCR. The miR-19b, miR-25, miR-29a, and miR-148a were detected in the plasma of cows in estrous but did not change significantly during the estrous cycle (Fig. 3).

Effect of hemolysis on the quantification of plasma miRNA levels

To investigate the effect of hemolysis on plasma miRNA levels, we prepared hemolytic plasma (0 to 4%) using an ultrasound generator. Absorption at 414 nm indicated that free hemoglobin concentration tended to increase with increasing hemolysis degree, with significant differences ($P < 0.001$) in 0.8% and 4% hemolyzed samples compared to non-hemolyzed samples (Fig. 4A). The ratio of miR-451 to miR-23a (ΔCt) was significantly increased in 0.16 to 4% hemolyzed samples compared to non-hemolyzed samples and tended to increase depending on the degree of hemolysis (Fig. 4B). The miRNA quantities of miR-19b, miR-25, and miR-148a were increased only in the 4% hemolyzed samples compared with the non-hemolyzed samples (Fig. 5). In contrast, miR-29a showed no differences among all hemolysis samples.

Discussion

This is the first report describing the analysis of circulating miRNA profiles in Japanese Black cattle during early gestation. Although circulating miRNAs have already been reported in pregnant Holstein-Friesian cows [13–16], the application of these miRNAs in Japanese Black cows remains unclear because breed-specific miRNA expression has been observed in some cattle breeds [17, 18]. Furthermore, it is necessary to clarify another miRNA candidate for early pregnancy diagnosis in cows and to investigate their potential for diagnosis. In the present study, we demonstrated that miR-19b, miR-25, miR-29a, and miR-148a levels in plasma were elevated in pregnant Japanese Black cows on day 21 after AI compared with the non-pregnant group. These circulating miRNA levels did not change during the estrous cycle in the early to late stages and were not affected by the degree of hemolyzed plasma.

Recently, remarkable advances in RNA-sequencing (RNA-seq) technology in gene expression analysis have also been applied to the expression analysis of miRNAs as small RNA-seq [22]. Many of the previously reported analyses of circulating miRNAs in cows have also used RNA-seq [1–3]. Oligonucleotide DNA microarray has been used for comprehensive analysis of gene expression, and we have also studied pregnancy diagnosis using this microarray to measure gene expression in bovine peripheral blood leukocytes [10]. In oligonucleotide DNA microarray, probes to be placed on array slides can be custom-designed, which has the advantage of not requiring the complicated analysis techniques necessary for RNA-seq. Here, we attempted a global expression analysis of bovine circulating miRNAs using oligonucleotide DNA microarray from the accumulation of our knowledge and techniques. In the present study, the microarray detected 124 miRNAs in bovine plasma, which is less than that of approximately 300 to 800 miRNAs in bovine plasma or serum, as previously reported [13–16]. This could have been inferior to RNA-seq in sensitivity detection using oligonucleotide DNA microarray. However, miR-19b [14], miR-25 [15], miR-26b [13, 14, 16], miR-27b [16], miR-29a [15], and miR-148a [16], which showed high values in the pregnant herds extracted from our microarray experiments, tended to be similar to the results of the previous RNA-seq or PCR array analysis. Therefore, these reports support the microarray results of the present study.

Appropriate normalization using reference genes is crucial for performing RT-qPCR and accurate miRNA expression analysis [23]. The selection of erroneous reference genes greatly influences the quantitative

results [24, 25]. NormFinder is a software used to assess gene expression stability and is useful for selecting endogenous controls [19]. Therefore, we searched for reference miRNAs in the plasma of Japanese Black cattle for more precise miRNA determination. In the present study, 11 reference miRNA candidates, including two reference miRNAs used in previous reports [14, 20], were validated by RT-qPCR. Validation experiments showed that miR-2348 and miR-3141 could not be amplified specifically by RT-qPCR with the prepared primer set in the present study and could not be accurately quantified. Comparing the Ct-values between the non-pregnant and pregnant groups for each miRNA, significant differences were observed in let-7g, miR-128, miR-2478, and miR-2888. These miRNAs may, however, be unsuitable as reference miRNAs for comparing miRNA expression in non-pregnant and pregnant Japanese Black cows. NormFinder analysis recommended the use of miR-2455 as the optimal reference miRNA. In addition, the reference miRNA candidates, except miR-2888, in the present study were more stable than the previously described candidates let-7g and miR-128. Therefore, the reference miRNA candidates selected in this study may be considered superior to previously reported reference genes. For the quantification of miRNA by RT-qPCR, standardization using the measurement results of the reference gene is critical, but no particular reference gene exists that can be applied to all tissues or plasma. [26]. Thus, the selection of appropriate reference genes requires validation between specific populations for comparison and types of samples such as tissues and plasma, and this study found appropriate reference genes for comparison between non-pregnant and pregnant groups using plasma. Although we have only validated the NormFinder algorithm for reference miRNA selection, RT-qPCR results showed that miR-2455 did not fluctuate between non-pregnant and pregnant females, making it reasonable to use miR-2455 as a reference miRNA.

The present results indicate that miR-19b, miR-25, miR-29a, and miR-148a may be useful indicators of pregnancy diagnosis in Japanese Black cows. Ioannidis and Donadeu (2017) reported the circulating miRNA profile of Holstein-Friesian heifers at days 0 and 60 post-AI using small RNA-seq and with RT-qPCR validation [14]. Although circulating miR-26b was only increased in pregnant cows validated by RT-qPCR, RNA-seq analysis showed elevated miR-19b levels on day 60 of pregnancy. Gebremedhn et al. (2018) reported 23 differentially expressed miRNAs, including miR-25 and -29a, in serum on day 24 of pregnancy in Holstein-Friesian cows [15]. In another report, the RNA-seq approach revealed that miR-148a was increased on day 30 of pregnancy [16]. Although we could not find new miRNAs for early pregnancy diagnosis as an indicator, it was confirmed that miR-19b, miR-25, miR-29a, and miR-148a were indicators for pregnancy diagnosis in Japanese Black cows. However, the sample size was small for both non-pregnant and pregnant cows in this study. Therefore, larger studies are needed to determine the proportion of non-pregnant or pregnant cows that can be identified using this technique.

Cows have their first estrus around 10 months of age and then repeat their estrus with a cycle of approximately 21 days. Hormones such as estradiol, progesterone, and luteinizing hormone are involved in the development of estrus, and cyclic variations in blood levels of these hormones have also been observed [27]. Circulating miRNAs have also been reported to change during the estrous cycle [28], and miRNAs used in pregnancy diagnosis should be unaffected by the estrous cycle. Therefore, four miRNAs that have been biomarker candidates for pregnancy diagnosis in the present study were examined for

changes in plasma from days 0 to 20 of the estrous cycle. No variation related to the estrous cycle was observed for any miRNA, indicating that these miRNAs were not affected by ovarian and pituitary hormones.

Circulating miRNAs are present in vesicular structures, such as exosomes, and are known to be relatively stable [29, 30]. Since blood can be collected relatively minimally invasively, there have been numerous studies aimed at using circulating miRNAs as biomarkers for various diseases. However, hemolysis may occur at the time of collection [30] and has been reported to affect plasma miRNA expression levels [30–32]. Therefore, in this study, the extent of hemolysis in miR-19b, miR-25, miR-29a, and miR-148a was determined. Hemolytic samples were quantified from free hemoglobin with 414 nm absorbance measurements and miR-23a and miR-451 content [21, 31]. The free hemoglobin and ratio of miR-451 to miR-23a in plasma increased with increasing hemolysis, and both showed significant differences compared with non-hemolyzed samples at 0.8% hemolysis or higher. The plasma appearance was also determined to be severe hemolysis, with hemolysis visible at 0.8% hemolysis or higher (pictures not shown). RT-qPCR showed that the content of miR-29a did not change significantly with hemolysis. Although miR-19b, miR-25, and miR-148a showed changes in content only in severely hemolyzed samples (4%), these miRNAs were largely unaffected by hemolysis, indicating that they may be useful as biomarker candidates in early pregnancy diagnosis.

Conclusions

The purpose of the present study was to compare circulating miRNA levels in non-pregnant and pregnant Japanese Black cows, and to identify markers for early pregnancy in cows. We demonstrated four circulating miRNAs with high levels in the plasma of pregnant Japanese Black cows. Since these miRNAs were less affected by the measurement system due to hemolysis, it was suggested that they may be used as markers for early pregnancy diagnosis in Japanese Black cows.

List Of Abbreviations

AI, artificial insemination; JB, Japanese-Black; miRNA, microRNA; NP, non-pregnant cows; P, pregnant cows; RT-qPCR, real-time quantitative RT-PCR

Declarations

Ethics approval and consent to participate

All animal procedures, including experimental and feeding conditions, were approved by the Iwate University Laboratory Animal Care and Use Committee.

Consent for publication

Not applicable.

Availability of data and materials

All miRNA microarray data are available at the Gene Expression Omnibus through the NCBI under Accession No. GSE178906. Other data that support the findings of this study are available within the article and its supplementary material.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

KO and SO performed the experiments and drafted the manuscript. CN, NT, and TIO executed data collection, experiments, and data analysis. TK, TT, and KI performed blood collection and participated in data interpretation. KK conducted and designed the experiments, analyzed, and wrote the manuscript. All authors read and approved the final manuscript.

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Figures

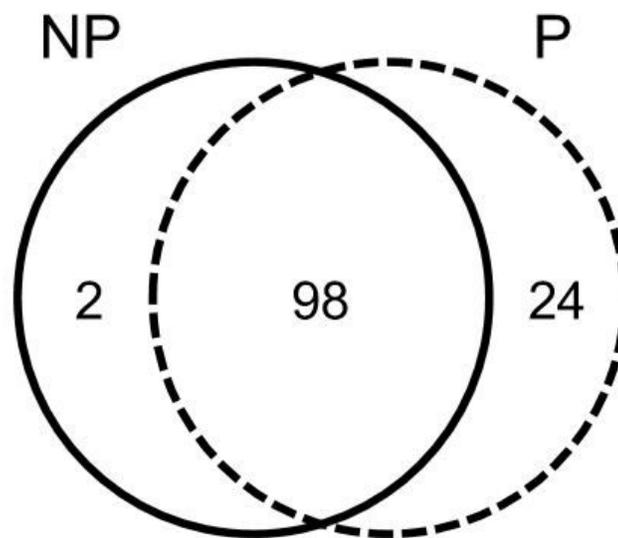


Figure 1

Venn diagram of circulating miRNA detected in non-pregnant and pregnant cows. Circulating miRNA from non-pregnant (NP) and pregnant (P) cows on day 21 after artificial insemination was analyzed by miRNA microarray. Each value represents the number of miRNAs that were detected.

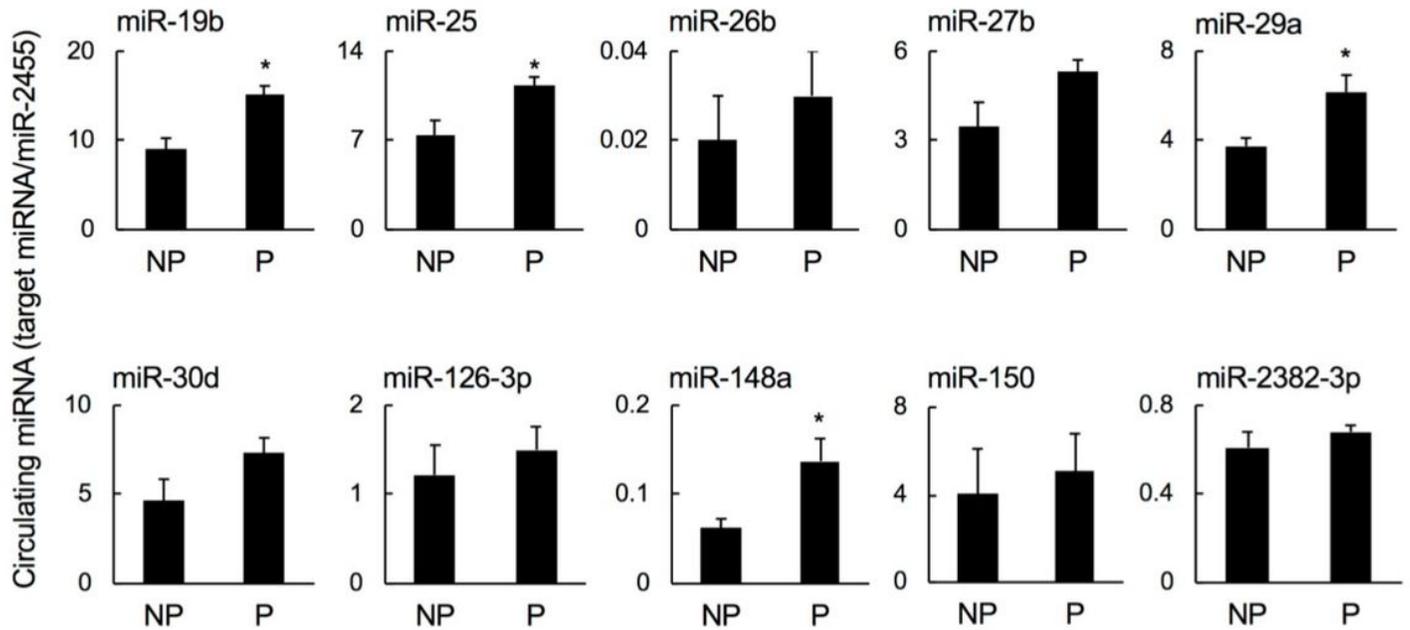


Figure 2

Circulating miRNA quantities in non-pregnant and pregnant cows on day 21 after artificial insemination. The levels of miR-19b, miR-25, miR-26b, miR-27b, miR-29a, miR-30d, miR-126-3p, miR-148a, miR-150, and miR-2382-3p in the plasma of non-pregnant (NP, n = 4) and pregnant (P, n = 5) cows on day 21 after AI were analyzed by RT-qPCR and normalized to miR-2455 levels. Data are shown as mean ± SEM. Statistical analysis was performed using Student's t-test. Asterisks indicate significant differences between NP and P groups (P < 0.05).

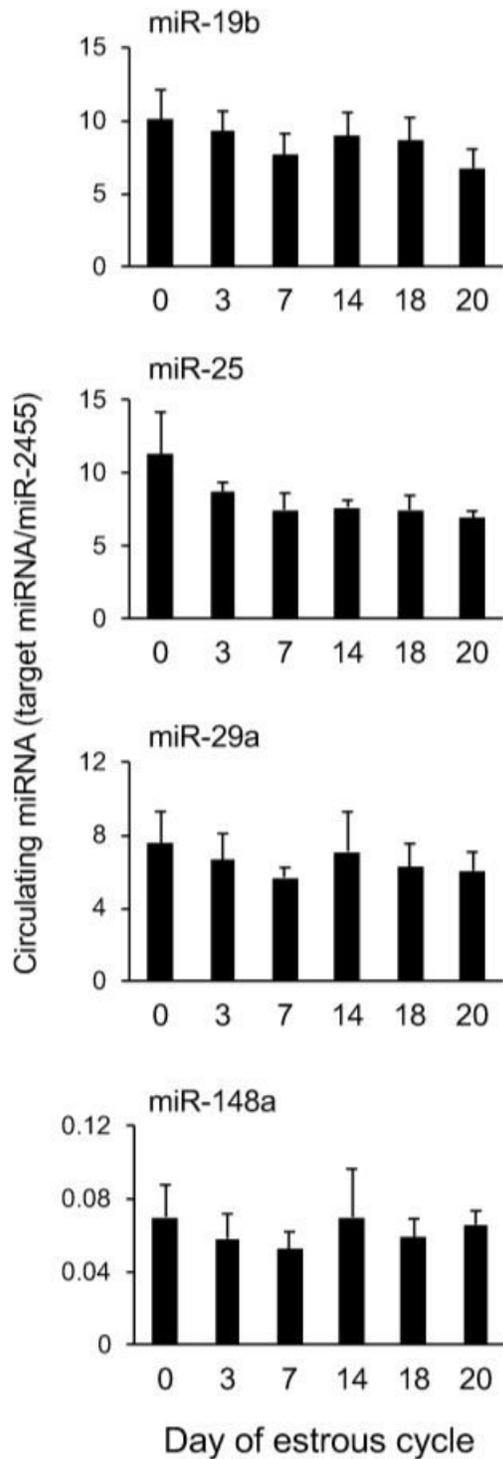


Figure 3

Circulating miRNA quantities in cows during the estrous cycle. The levels of miR-19b, miR-25, miR-29a, and miR-148a in plasma during estrous from days 0 to 20 were analyzed by RT-qPCR and normalized to miR-2455 levels. Data are shown as mean \pm SEM (n = 5).

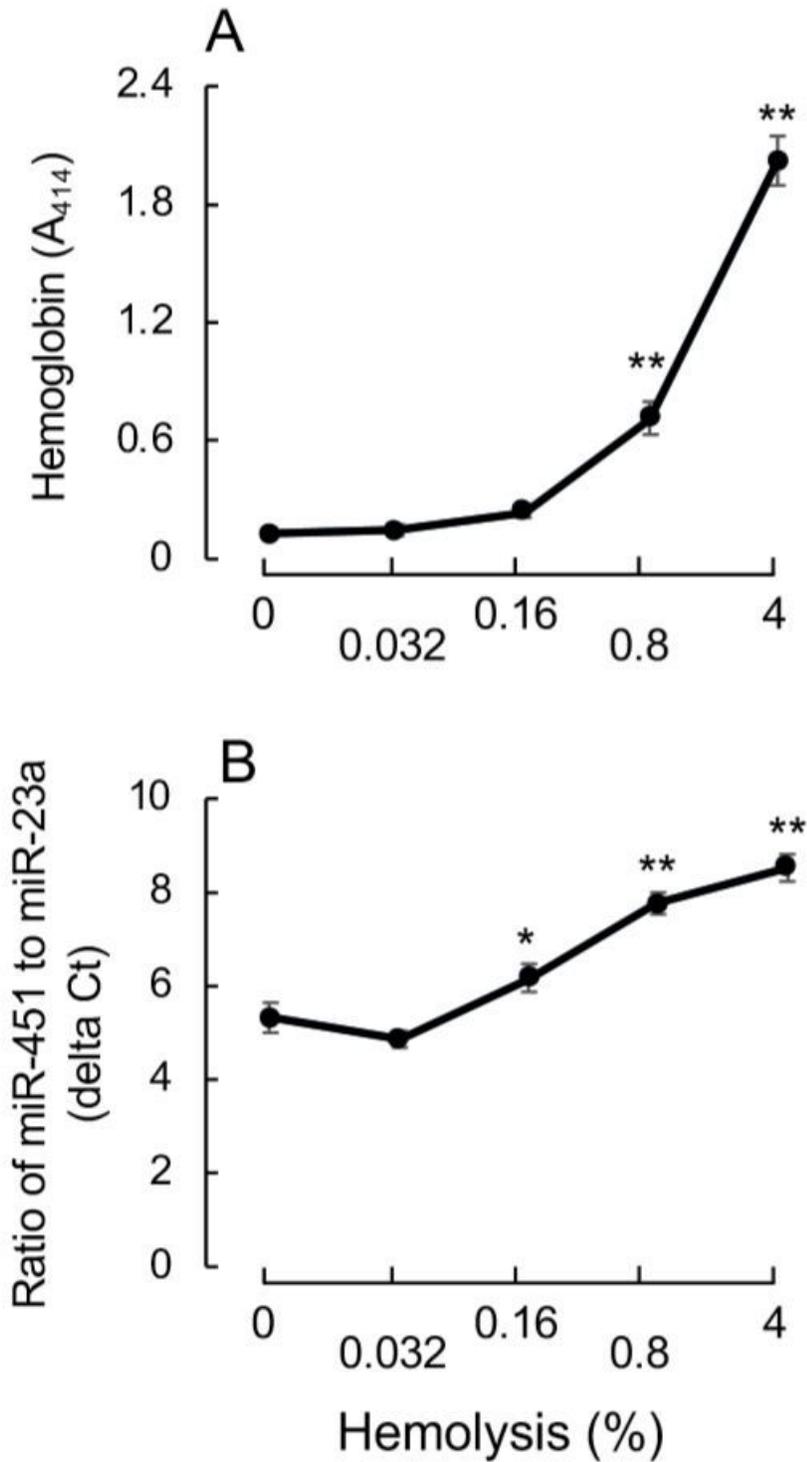


Figure 4

The free hemoglobin and erythrocyte-derived miRNA in hemolyzed plasma samples. Hemolyzed plasma samples were prepared by sonication of whole blood and evaluated by free hemoglobin concentration (A) and erythrocyte-derived miRNA amounts (B). Data are shown as mean \pm SEM (n = 4). Statistical analysis was performed using Dunnett's test. Values with significant differences from non-hemolyzed samples (0%) are indicated with asterisks (*P < 0.05, **P < 0.01)

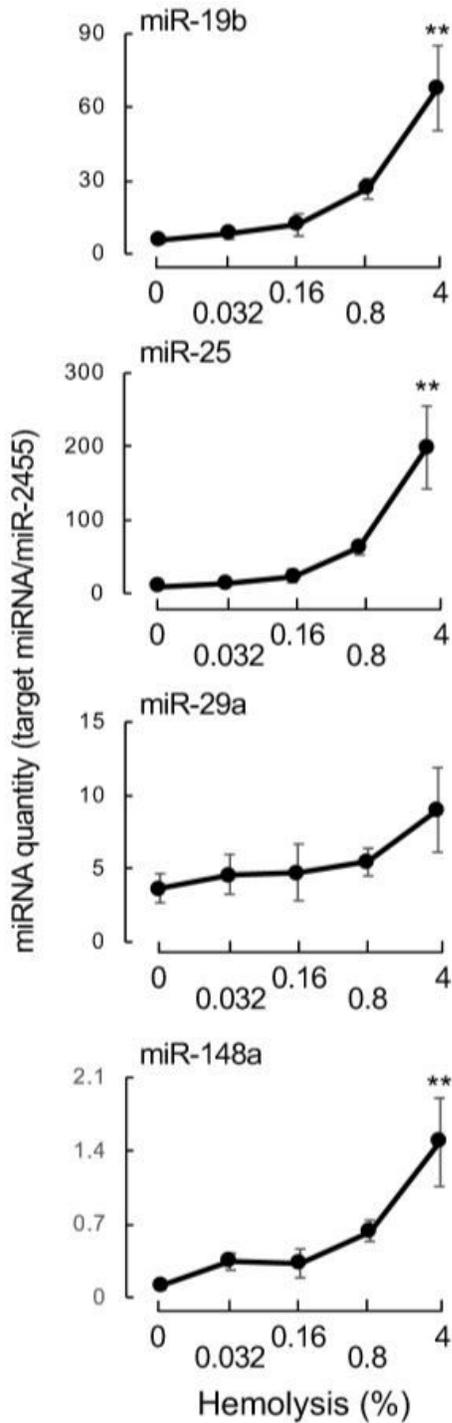


Figure 5

Changes in miRNA levels in hemolyzed plasma samples. The levels of miR-19b, miR-25, miR-29a, and miR-148a in hemolyzed plasma samples were analyzed by RT-qPCR and normalized to miR-2455 levels. Data are shown as mean \pm SEM (n = 4). Statistical analysis was performed using Dunnett's test. Values with significant differences from non-hemolyzed samples (0%) are indicated with asterisks (**P < 0.01).

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