UNIVERSIDADE FEDERAL DE VIÇOSA

ANALYSIS OF MICRORNA EXPRESSION BETWEEN GESTATIONAL AGES IN NELLORE CATTLE.

Thaís Rodrigues Monteiro de Assis
Magister Scientiae

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Dissertation submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

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ABSTRACT

ASSIS, Thaís Rodrigues Monteiro de, M.Sc., Universidade Federal de Viçosa, September, 2024. **Analysis of microRNA expression between gestational ages in Nellore cattle.**. Adviser: Simone Eliza Facioni Guimaraes. Co-adviser: Daniele Botelho Diniz Marques.

Most pregnancy losses in high-yielding cows occur within the first three weeks after insemination. To better monitor embryonic development, some studies have identified numerous miRNAs expressed in the placenta, highlighting them as potential markers for tracking and supporting early pregnancy development. This study aimed to identify miRNA plasmatic levels in cows at days 7 and 21 of pregnancy, based on the hypothesis that the amount of plasmatic miRNAs may vary during embryonic development due to the constant changes in gene expression occurring throughout this period. Fifteen nulliparous and primiparous Nellore cows with an average body weight of 478.67 ± 13.17 kg were used for the study, estrus synchronized followed by artificial insemination (AI). Blood samples were collected from the jugular vein of each animal, with two samplings performed for miRNA extraction on days 7 and 21 after AI. Additionally, ultrasonography was used to detect embryonic stages and monitor pregnancy. Based on the literature, 6 microRNAs (bta-miR-433, bta-miR-99a-3p, bta-miR-301b, bta-miR-26a, bta-miR-1249, bta-miR-101) were selected for analysis in pregnant females at 7 and 21 days (N=15), evaluated by RT-qPCR. Only miR-1249 showed a significant difference at a = 0,05 between the days of pregnancy (7 days vs. 21 days, P = 0.003). Despite the involvement of the studied miRNAs in important functions during pregnancy, only one of them (miR-1249) showed significant differences. This enables the identification of plasma levels during days 7 and 21 of gestation and the identification of miRNAs that may be related to pregnancy and play important roles during embryonic development.

Keywords: ribonucleic acid; gestation; molecular markers; plasma.

RESUMO

ASSIS, Thaís Rodrigues Monteiro de, M.Sc., Universidade Federal de Viçosa, setembro de 2024. **Análise da expressão de microRNA entre idades gestacionais em bovinos Nelore.**. Orientadora: Simone Eliza Facioni Guimaraes. Coorientadora: Daniele Botelho Diniz Marques.

A maioria das perdas gestacionais em vacas de alto rendimento ocorre nas primeiras três semanas após a inseminação. Para monitorar melhor o desenvolvimento embrionário, alguns estudos identificaram inúmeros miRNAs expressos na placenta destacando-os como potenciais marcadores para acompanhar e apoiar o desenvolvimento precoce da gestação. Objetivou-se identificar os níveis plasmáticos de miRNAs em vacas nos dias 7 e 21 de gestação, baseando-se na hipótese de que a quantidade de miRNAs plasmáticos pode variar durante o desenvolvimento embrionário devido às constantes mudanças na expressão gênica que ocorrem ao longo deste período. Para o estudo foram utilizadas 15 vacas adultas nulíparas e primíparas da raça Nelore com peso corporal (PC) médio de 478,67 ± 13,17 Kg sincronizadas com o estro seguidas de inseminação artificial (IA). As amostras de sangue foram coletadas da veia jugular de cada animal, sendo realizadas 2 amostragens para extração dos miRNAs 7 e 21 dias após a IA. Além disso, foi utilizado o exame ultrassonográfico para detectar a fase embrionária e acompanhar a gestação. Com base na literatura foram escolhidos 6 microRNAs (bta-miR-433, bta-miR-99a-3p, bta-miR-301b, bta-miR-26a, bta-miR-1249, bta-miR-101) para análise em fêmeas gestantes de 7 e 21 dias (N=15) avaliadas por RT-qPCR. Apenas o miR-1249 apresentou diferença significativa a = 5 entre os dias de gestação (7 dias vs. 21 dias P = 0,003). Apesar dos miRNAs estudados estarem envolvidos e desempenharem funções importantes durante a gestação, apenas um deles, o miRNA-1249 apresentou diferenças significativas. Isso possibilita a identificação dos níveis plasmáticos durante os dias 7 e 21 de gestação e a identificação de miRNAs que podem estar relacionados à gestação e desempenhar papéis importantes durante o desenvolvimento embrionário.

Palavras-chave: ácido ribonucleico; gestação; marcadores moleculares; plasma.

SUMMARY

CHAPTER 1 - GENERAL CONSIDERATIONS

1 INTRODUCTION

Fertility constitutes a crucial factor in optimizing reproductive efficiency in bovine females, especially in those of the Zebu breed (*Bos indicus*), which exhibit a more extended period before reaching puberty compared to those of the Taurine breed (*Bos taurus*). Elevating reproductive rates means an increase in productivity throughout the animals' life cycle, providing a rise in the number of individuals fit for meat production, decreasing the need to retain a large number of replacement females and consequently, enhancing the production profitability as a whole (Oliveira Júnior et al., 2019).

The early identification of pregnancy plays a vital role in improving reproductive efficiency within the context of precision livestock farming. Notably, most gestational losses in high-yielding cows occur within the first three weeks after insemination. Failure to perform a re-insemination before the next estrous cycle, that is, within a 21-day period after the initial insemination, results in longer intervals between calvings (Gebremedhn et al., 2018).

According to DeCarlo et al. (2023), a high incidence of gestational losses in Angus cows was observed when employing assisted reproduction methods, such as in vitro fertilization (IVF) and somatic cell nuclear transfer, both procedures integrated into the embryo transfer technique. A considerable economy of financial resources and efforts could be achieved if we have effective ways to assess embryos for their developmental capability before their transfer. Although attempts have been made to identify indicators that could predict embryonic development competence and gestational success, efficient screening methods based on biomarkers have not yet been established. Recent research suggests that certain markers detectable in maternal blood may indicate the viability of embryonic development for subsequent fetal development (Ioannidis and Donadeu, 2016; Pohler et al., 2017).

miRNAs are small non-coding RNA sequences of 19-22 nucleotides (nt) that function as endogenous regulators of gene expression. They are transcribed as large primary miRNAs or pre-miRNAs by RNA polymerase II, and subsequently processed by the ribonucleases Drosha and Dicer to give rise to their mature forms. Soon after, these molecules are incorporated into the RNA-induced silencing complex (RISC), where they bind to the 3’- UTR complementary region of mRNAs, inducing their degradation or inhibiting its translation, resulting in gene silencing (Moreno-Moya et al., 2014).

miRNAs are essential for embryonic, cellular, and tissue development, regulating cell differentiation, proliferation, and apoptosis. Currently, the most widely used detection method is real-time polymerase chain reaction (RT-qPCR). miRNAs can be found in cells and in biological fluids such as serum, plasma, and saliva. After being secreted by cells, they are incorporated into microvesicles or associated with plasmatic proteins that protect them from RNAse degradation (nuclease that catalyzes the degradation of RNA into smaller components) so that they can remain intact for extended periods of time.

During embryonic development, crucial physiological events occur. On day 7 after AI, the embryo is at the blastocyst stage, a critical moment for maternal recognition of pregnancy. During this period, the embryo develops rapidly and prepares to signal the maternal organism, preventing luteolysis (degradation of the corpus luteum) and ensuring the continued production of progesterone, an essential hormone for maintaining pregnancy. This day is important for understanding how the initial signs of the embryo's presence can influence the maternal response and how markers such as miRNAs can reflect this early embryonic interaction.

On day 21, the embryo is already fully implanted in the uterus, and the corpus luteum must be fully functional to sustain the pregnancy. Hormonal and molecular changes intensify at this stage, including the production of interferon tau (IFNT), which is responsible for maternal recognition of pregnancy and prevention of luteolysis. Collecting blood on this day allows for capturing information on how the embryo is interacting with the uterine environment to establish and maintain the pregnancy, and how maternal physiological changes are reflected in blood markers.

In this way, we aimed to identify miRNA plasmatic levels in Nellore cows at days 7 and 21 of pregnancy. Embryonic development relies on crucial physiological events occurring during these early stages of bovine gestation. These stages are fundamental for maintaining pregnancy and ensuring reproductive success. Therefore, these two days represent distinct and critical moments in pregnancy: the first relating to embryonic development and the initial recognition of pregnancy, and the second relating to embryonic-maternal interaction and the maintenance of pregnancy. Evaluating these periods is crucial for understanding the dynamics of gestation, which could have practical implications for assisted reproduction in cattle, such as artificial insemination.

2 LITERATURE REVIEW

2.1 Embryology

Embryogenesis begins inside the oviduct, marked by the formation of the zygote, an event that encompasses the fusion of the male and female pronuclei. The zygote initiates a process of division through a series of mitoses, also referred to as cleavages, which are characterized by successive mitotic divisions, leading to an exponential increase in the number of cells, known as blastomeres. This process of zygote division begins approximately 30 hours after fertilization occurs. Notably, with each division by cleavage, the blastomeres reduce in size, a crucial aspect for subsequent embryonic development (Moore et al., 2016).

During oogenesis, the ovum is enriched with mRNAs and proteins of maternal origin, which are essential for initiating biosynthetic processes in the embryo, guiding the first mitotic divisions, and determining the initial cell fate and organization. As development progresses, the maternal-to-zygotic transition (MZT) occurs, marked by two critical events: the selective degradation of maternal mRNAs and the onset of transcription from the zygote's genome. Initially, the removal of maternal mRNAs is mediated by products generated by the mother. However, the activation of zygotic transcription catalyzes the production of proteins and miRNAs that enhance the destruction of maternal mRNAs and bolster zygotic transcription through newly synthesized transcriptional activators. This process marks the transfer of developmental control from the maternal genome to the zygotic (Tadros and Lipshitz, 2009).

Between the 8 to 16 cell stages, the bovine embryo undergoes a critical phase of reprogramming known as embryonic genome activation (EGA). This moment marks a fundamental transition, after which the embryo's development is no longer sustained by transcriptions originating from oocytes but is driven by *de novo* transcription. Following this transition, there is a significant change in the morphology of the blastomeres, which alter their shapes and begin to adhere more closely to each other. This compaction process facilitates enhanced cell interaction, establishing an essential condition for the segregation of internal cells that will form the inner cell mass, extremely important for embryonic development, the onset of cell differentiation, and the formation of the embryo's first tissues (Rodríguez-Alonso et al., 2020).

In cattle, the embryo transitions from the oviduct to the uterus occurs on the fourth day after estrus, reaching the 16-cell stage by this time. Once in the uterine environment, it continues its development, evolving into a structure known as morula, which houses between 12 to 32

blastomeres. The path to blastocyst formation, which occurs between the sixth and eighth day, is marked by significant events: the expansion of the blastocele, driven by sodium pumping activity in the cells of the outer layer of the morula, and the differentiation into two distinct cell populations. These populations are the trophoblast, which will give rise to structures that nourish and protect the embryo (embryonic placenta), and the embryoblast, which will develop into the embryo (Rodríguez-Alonso et al., 2020).

Between days 9 and 11 after fertilization, the expansion of the blastocyst, coupled with the action of proteolytic enzymes secreted by the trophoblast, induces the hatching of the blastocyst from the zona pellucida. This process results in the formation of a fluid-filled cavity (blastocystic cavity) within the compact structure of the morula. Following hatching, the trophoblast enters a phase of elongation, a phenomenon that begins between days 12 and 14. In ruminants, the trophoblast elongates exponentially, reaching dimensions greater than 150 mm even before implantation in the uterus. Research has uncovered a series of genes active during this elongation process, which are associated with molecules involved in early interactions between the fetus and mother prior to implantation, as well as essential functions for this differentiation. Studies suggest that molecules such as interferons (IFNT), prostaglandin synthases (PGHS-2), protease inhibitors (TKDP family), and pregnancy-associated proteins (PAG family) exhibit an increase in transcription or secretion as the bovine trophoblast elongates (MacLean et al., 2003; Green et al., 2000). During the initial phases of elongation, characteristics such as overall metabolism, protein trafficking, and cellular proliferation are evident. In the final stages, there is an increase in cellular interactions, cell-to-cell signaling, and cell adhesion. Around the 18th day of gestation, the conceptus extends into the uterine horn. This elongation occurs through trophoblastic hyperplasia and coincides with the synthesis of estrogen by the embryo (Degrelle et al., 2005).

The conceptus sends a signal to prevent endometrial luteolytic activity and the regression of the corpus luteum in order to sustain progesterone production. The maintenance of the corpus luteum is essential for the establishment of pregnancy. Maternal recognition of pregnancy occurs between days 16 and 19, with the trophoblast of an elongated conceptus synthesizing and secreting interferon tau (IFNT), a protein molecule which belongs to the family of type 1 interferon proteins, that serves as a signal for recognition and implantation. The endometrium of pregnant cows produces a prostaglandin synthesis inhibitor (PSI) that reduces its synthesis to prevent endometrial luteolytic activity and the regression of the corpus luteum. IFNT is believed to perform multiple additional functions, reinforcing its importance

as one of the key embryonic factors for the successful establishment of pregnancy in ruminants (Gebremedhn et al., 2018; Hafez, 2004).

In a study conducted by Yun et al. (2021), the existence of factors that, in addition to IFNT, contribute to the regulation of endometrial functions in cattle was explored. This study highlights IFNT as the most extensively investigated cytokine, revealing its central role in embryo maternal modulation. Specifically, IFNT influences maternal recognition of pregnancy by regulating both the abundance of mRNA transcripts and gene expression in the endometrium. Cytokines, including IFNT, play overlapping critical roles in cell growth and differentiation and have a significant impact during the period of embryonic implantation. These factors are considered potential regulators of endometrial cells at this critical stage of embryonic development. IFNT acts to inhibit luteolysis and promote the continuation of progesterone production by the corpus luteum, by suppressing the synthesis of PGF2α in the endometrial epithelium. Therefore, the study suggests that IFNT plays a role in pregnancy recognition, influencing gene expression in the endometrium and the maintenance of pregnancy.

In cattle, interferon-tau (IFNT) plays a fundamental role as an indicator of pregnancy. As the embryo develops and elongates, there is an increase in the secretion of IFNT, reaching its peak around the 18th day of development. However, evidence indicates that the embryo signals its presence even more early on, through the release of extracellular vesicles (EVs) that carry a variety of molecules, including miRNAs (Machtinger et al., 2021; Giacomini et al., 2017). These molecules have the potential to prepare the maternal endometrium for the recognition and subsequent implantation of the embryo. Therefore, the release of EVs constitutes an essential physiological mechanism during the initial stages of embryonic development. This process reflects the complexity and dynamism of the molecular and cellular events characterizing the pre-implantational period, highlighting the importance of intercellular communication in the successful orchestration of pregnancy (Melo-Báez et al., 2023).

In a study conducted by Kusama et al. (2021), which explored the factors originating from both the embryo and the endometrium and their role in modulating the uterine immune environment on the seventh day of pregnancy in cattle, it was observed that bovine embryos at the pre-hatching stage (D7) already begin communication with the uterus through the innate immune system. This mechanism of interaction suggests the possibility of establishing immunological tolerance to the semi-allogeneic embryo, creating the necessary conditions for the success of bovine pregnancy. However, the exact role played by neutrophils, as an element

of the uterus's innate immune functions, in promoting pregnancy in cattle still requires further studies, especially during the embryo's pre-hatching stage.

Embryonic development that occurs after hatching and before implantation is regulated by maternal factors. In ruminants, this pre-implantation period is marked by a prolonged implantation phase, characterized by the intense proliferation of the trophoectoderm cells. This process is dependent on histotrophic nutrients present in the uterine lumen, which are secreted by the endometrium, especially by the uterine glands, playing a vital role in the sustenance and development of the embryo. This dependence is evidenced by the fact that post-hatching embryonic elongation does not occur in *in vitro* environments, and the absence of uterine glands inhibits the elongation of blastocysts, preventing proper development. The implantation phase of the blastocyst is usually completed during the second week of development, and by around the tenth day, the embryo is fully implanted in the endometrium (Lonergan and Forde, 2014).

Following the implantation of the embryo, the third week of embryonic development begins, a period of intense morphological activity marked by the rapid development of a trilaminar embryonic disc that starts with the emergence of the primitive streak, followed by the development of the notochord and the differentiation of the three germ layers (ectoderm, endoderm, and mesoderm). Gastrulation represents the transformation of the bilaminar embryonic disc into a trilaminar one and marks the beginning of morphogenesis. The formation of the primitive streak initiates the process of neurulation, which involves the formation of the neural plate. Around the 18th day, the neural plate undergoes invagination, forming a neural groove. By the end of the third week, these neural folds begin to approach each other and eventually fuse, transforming the neural plate into a neural tube. The completion of neurulation occurs during the fourth week. This is a critical period, as between the fourth and eighth weeks, all the embryo's major external and internal structures are established. By the end of this phase, the major organ systems are already in the process of development, marking a crucial stage in the formation of the organism (Moore et al., 2016).

2.2 Role of microRNA in embryonic development

The reproductive cycle of the female exhibits cyclic patterns of ovarian follicular growth aimed at ovulation and by the degeneration of non-ovulatory follicles. The mechanism of ovarian follicle degeneration during atresia is called apoptosis. Some research using genomic tools aims to determine the expression profiles of mRNAs and miRNAs in dominant and

subordinate bovine ovarian follicles at different stages of the follicular wave. These studies indicate that there are molecular factors associated with ovarian follicular development (Zielak-Steciwko and Evans, 2016). The bta-miR-301b expressed in theca cells is involved in the development of the dominant follicle. The CYP19 and TBC1D1 genes were more highly expressed in dominant follicles. The former encodes the aromatase enzyme (P450), which is responsible for estrogen production by converting testosterone into estradiol. The latter is involved in the cellular differentiation and growth of the follicle (Zielak et al., 2007; Zielak et al., 2008).

Many studies demonstrate the various regulatory functions of specific miRNAs in ovarian follicles, including their involvement in bovine steroidogenesis (Xu et al., 2011; Donadeu et al., 2017). The bta-miR-222 was found in bovine fetal ovaries, and LHCGR (luteinizing hormone receptor) was identified as a potential target, participating in signaling pathways involved in follicular cell proliferation, steroidogenesis, and oocyte maturation through the modulation of the target gene SPRED1 (Hossain et al., 2009; Sontakke et al., 2014). Furthermore, it is proven that miRNAs belong to the main molecules involved in the regulation of gene expression at the post-transcriptional and translational levels through mRNA cleavage and/or translation repression. Through these mechanisms, miRNAs are important regulators of development, cell proliferation, apoptosis, and are involved in various biological and physiological processes of follicular and embryonic development (Santos et al., 2018).

The observation of a progressive increase in gene expression during the early cleavage stages and in the formation of morulae in mouse embryos highlights the fundamental importance of gene expression dynamics for successful embryonic development. This phenomenon underlines not only the relevance of changes in gene expression over time and space but also the essential transitions from maternal to embryonic transcription. For example, in the early stages of bovine conceptus development, significant variations in the levels of certain miRNAs, such as miR-496 and miR-125a, are observed. miR-125a showed lower expression in the oocyte and higher expression at the 4-cell stage. In contrast, miR-496 exhibited low expression at the 2-cell stage and higher expression in the morula. (Tesfaye et al., 2009). This variation suggests that they may influence the transcriptional control transition from the mother to the zygote. Furthermore, other miRNAs, such as miR-27a and miR-92b, exhibit differential expression during placental development and are associated with processes like trophoblastic differentiation and vascularization (Ioannidis and Donadeu, 2016).

Melo-Baez et al. (2020) reported that the release of EVs, as well as their morphological characteristics and composition, change in response to critical moments of pre-implantational development, particularly during the activation of the embryonic genome (EGA) and the blastulation phase. In bovine embryos, EGA occurs at the eight-cell stage, characterized by active transcription of the embryonic genome, which occurs at the culmination of the maternal-to-embryonic transition (MET). This process involves the elimination of maternal mRNA and the introduction of new embryonic transcripts, with the degradation of maternal transcripts mediated by various mechanisms, including miRNAs, expressed by the embryonic genome. Based on this understanding, variations in the miRNA content in embryonic cells during EGA may reflect in the secreted EVs, which, in turn, show variations according to the embryo's competence.

In bovine embryos, IGF1R (Insulin-like Growth Factor 1 Receptor) is crucial for growth and development prior to implantation. It is activated by binding to IGF-1 (Insulin-like Growth Factor 1), promoting intracellular signaling pathways important for cell growth. IGF1R can influence the developmental competence of embryos, impacting embryo quality, cell division rate, and implantation capacity, as IGF-1 is also associated with stimulating steroidogenesis and improving oocyte and embryo survival (McCarthy et al., 2012). The downregulation of miR-140 in embryos is essential for implantation, as it reduces the number of implantation sites in the uterus by negatively regulating IGF1R expression in endometrial epithelial cells (Sirohi et al., 2018).

The study by Gross et al. (2017) with mouse embryos revealed that vesicle-like structures play a crucial role in communication between the inner cell mass (ICM) and the trophectoderm (TE) in the blastocyst. Both ICM and TE cells secrete distinct extracellular vesicles (EVs), which are vehicles for the transport of morphogens and miRNAs responsible for regulating gene expression and determining cell fate. Interestingly, Desrochers et al. (2016) observed that EVs originating from stem cells derived from the ICM carry molecules capable of promoting the migration of TE cells, and the injection of these vesicles into the blastocele enhances the implantation of the blastocyst. These findings highlight the fundamental importance of extracellular vesicles in embryonic development, acting as essential mediators in intercellular communication (Melo-Báez et al., 2023).

A diversity of miRNAs can be found in the extracellular environment and in various biological fluids. These miRNAs can be found protected through association with high-density lipoproteins, Argonaut protein (Ago2), or within extracellular vesicles, exosomes, and

microvesicles. EVs protect the miRNAs and enable their transport between different cells and tissues as mediators of intracellular communication. These characteristics confer on miRNAs the ability to assess the functional state of various cells and tissues, making them a better source to be used as biomarkers. Moreover, there are various studies on the potential hormonal role of miRNAs present in plasma/serum and the effects of these molecules at distant sites in the body. The miRNAs present in serum are stable and are not degraded when exposed to high temperatures, high or low pHs, or freeze-thaw cycles (Gad et al., 2020).

In bovine embryos, the levels of some miRNAs vary throughout embryonic development. miR-496 and miR-125a are associated with the transcriptional transition from the mother to the zygote (Tesfaye et al., 2009). The miRNAs miR-27a and miR-92b are differentially expressed during placental development, playing roles related to vascularization and trophoblastic differentiation (Su et al., 2010). Meanwhile, let-7 and miR-125b regulate the development and lactation of the mammary gland (Zhou et al., 2014).

Ioannidis and Donadeu (2016) aimed to map the miRNA profiles in the plasma of Holstein cows between days 16 and 24 of gestation, with the intent of identifying specific miRNA signatures that could serve as biomarkers for early pregnancy diagnosis. Using advanced RNA sequencing techniques and qPCR analysis, the researchers were able to detect, for the first time, variations in miRNA levels in plasma during the early stages of gestation. Remarkably, a significant increase, of up to two-fold, in the levels of miR-26a was observed during the period between days 16 and 24 of gestation. This variation suggests changes in miRNA expression in one or more body tissues, which could have crucial implications for the process of establishing pregnancy.

Continuing with the investigations initiated in previous studies, Ioannidis and Donadeu (2017) directed their focus toward a more advanced stage of gestation, specifically day 60, starting from the hypothesis that variations in plasma miRNA levels between pregnant and non-pregnant Holstein heifers would be more pronounced at this stage compared to the differences observed between days 16 and 24 of gestation. The detection of changes in circulating miRNA levels as early as the eighth day of gestation is an unprecedented finding, suggesting an innovative role for miR-26a, possibly related to its immunomodulatory properties, at the beginning of the gestation process. Although miR-26a has been associated with various functions, some of which seemingly unrelated to pregnancy, the significant increase (threefold) in plasma levels of miR-26a between days 8 and 60 of gestation indicates its potential as embryonic development biomarker.

From the previous discussions, it becomes evident that plasma miRNAs, protected by exosomes, extracellular vesicles, high-density lipoproteins, or Argonaute proteins, play a crucial role in transporting information pertinent to the state of the embryo. This ability to carry information, which can be collected and analyzed without the need for invasive procedures such as cell biopsies, positions plasma miRNAs as promising markers in the field of assisted reproduction. Thus, they emerge as valuable tools for the classification and selection of embryos, offering a non-invasive method to assess the viability and developmental potential of the embryo.

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CHAPTER 2 - ANALYSIS OF MICRORNA EXPRESSION BETWEEN GESTATIONAL AGES IN NELLORE CATTLE

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ABSTRACT

The profiles of circulating miRNAs, which are associated with the gestational state, become more evident as pregnancy progresses. This study suggests that the composition of plasmatic miRNAs varies throughout embryonic development, reflecting changes in gene expression during this period. The research is based on the possibility of using circulating plasmatic miRNAs as biomarkers to differentiate stages of gestation in Nellore cows on days 7 and 21 after conception. The main objective is to identify the levels of miRNAs in the plasma of these cows on the mentioned days. The 15 animals used were nulliparous and primiparous Nellore cows with an average body weight of 478.67 ± 13.17 kg, estrus synchronized followed by artificial insemination (AI). Blood samples were collected from the jugular vein of each animal, with two collections made for the extraction of miRNAs at 7 and 21 days after artificial insemination. Based on the literature, 6 microRNAs (bta-miR-433, bta-miR-99a-3p, bta-miR-301b, bta-miR-26a, bta-miR-1249, bta-miR-101) were selected for analysis in pregnant females at 7 and 21 days (N=15), evaluated by RT-qPCR. Only miR-1249 showed a significant difference at $\alpha = 0,05$ between the days of pregnancy (7 days vs. 21 days, $P = 0.003$). Despite the involvement of the studied miRNAs in important functions during pregnancy, only one of them (miR-1249) showed significant differences. This enables the identification of plasma levels during days 7 and 21 of gestation and the identification of miRNAs that may be related to pregnancy and play important roles during embryonic development.

Keywords: Extracellular vesicles; Gestation; Molecular markers; Ribonucleic acid

1 INTRODUCTION

Embryonic mortality is identified as the primary cause of reproductive failure in cattle, leading to a reduction in the number of calves born, delayed genetic progress, and significant financial losses for the livestock sector. This phenomenon describes the losses that occur from fertilization until the end of the embryo differentiation phase (Dunne et al., 2000). The primary issue related to low fertility in cows lies in the difficulty to identify early pregnancy, which hinders the immediate re-insemination of animals that do not conceive at the first service, resulting in prolonged intervals between calvings. Current methods, such as transrectal ultrasonography, palpation, and measurements of progesterone in milk, are only accurate after the first 3 weeks of pregnancy, missing the critical opportunity for re-insemination after conception failures. Recently, it has been discovered that circulating markers in the maternal bloodstream can diagnose pregnancy before the 28th day, providing insights into the competence of embryonic development. Detecting pregnancy at this early stage benefits producers by allowing for quick financial decisions, as conventional recognition only occurs around the 30th day of gestation (DeCarlo et al., 2023; Ioannidis and Donadeu, 2017).

MicroRNAs (miRNAs) are small non-coding RNA molecules that play a crucial role in the post-transcriptional regulation of gene expression. Studies have highlighted the importance of miRNAs in various aspects of the reproductive system, including the development of ovarian follicles, functionality of the corpus luteum, uterine cyclicity, establishment of pregnancy, and embryonic development (Ioannidis and Donadeu, 2016; Tesfaye et al., 2009).

It has been discovered that miRNAs are excreted from cells into body fluids through various mechanisms, including the release of microvesicles, exosomes, apoptotic bodies, or associated with proteins. Numerous studies suggest that miRNAs can serve as effective biomarkers for monitoring pregnancy and distinguishing between normal and abnormal embryonic development, which may indicate pregnancies at risk of complications. Furthermore, studies indicate that miRNAs contained in extracellular vesicles (EVs) can play crucial roles in intercellular communication and the regulation of fundamental biological processes. For example, specific placental miRNAs are released into the maternal circulatory system and can be detected during pregnancy (DeCarlo et al., 2023; Pohler et al., 2017).

The main goal of the current study is to identify the levels of miRNAs in the plasma of Nellore cows at 7 and 21 days after AI. Embryonic development relies on crucial

physiological events occurring during these early stages of bovine gestation. These stages are fundamental for maintaining pregnancy and ensuring reproductive success. Therefore, both days represent distinct and critical moments in pregnancy: the first relating to embryonic development and the initial recognition of pregnancy, and the second relating to embryonic-maternal interaction and the maintenance of pregnancy. Evaluating these periods is crucial for understanding the dynamics of early gestation, which might have practical implications for assisted reproduction in cattle, such as artificial insemination or embryo transfer. To our knowledge this is the first study to access this subject in a Zebu breed.

2 MATERIAL AND METHODS

2.1 Ethics

This research was conducted in accordance with the recommendations of Brazilian Legislation, following review and approval by the Ethics Committee on the Use of Production Animals of the Federal University of Viçosa (CEUAP/UFV) (process number 020/2024). It adhered to the Code of Conduct for the Use of Animals in Education, Research, and Extension of the Department of Animal Science at the Federal University of Viçosa.

2.2 Local and animals

The experiment was conducted at the Boa Vista Farm located in the municipality of Coimbra, MG, where blood collection took place. Subsequent molecular analyses were performed at the Animal Biotechnology Laboratory (LABTEC) of the Department of Animal Science at UFV. The 15 animals used were nulliparous or primiparous Nellore cows with an average body weight (BW) of 478.67 ± 13,17 kg. Animals were estrus synchronized followed by artificial insemination (AI), as described later. Blood samples from the jugular vein were collected for the extraction of miRNAs at 7 and 21 days after artificial insemination. Additionally, ultrasonographic examination was used at 30 days of pregnancy to detect the embryonic phase and monitor pregnancy. The cows were kept in a grazing system with *Brachiaria decumbens*, provided with a water trough and feed bunk, following the protocols of Boa Vista Farm and maintained under identical conditions throughout the study.

2.3 Fixed-Time Artificial Insemination Protocol (FTAI)

During November-January 2023-2024, Nellore cows were estrus synchronized (Day 0) using a single-dose intravaginal implant (Primer®, Tecnopec, Brazil) containing 1.9 g of

progesterone for 8 days (CIRD-B®, Pfizer Animal Health, Brazil) along with 2 mL of estradiol benzoate (RIC-BE®, Tecnopec, Brazil) for 8 days. After eight days from the start of the IATF (Day 8), the implant was removed and 1.5 mL of equine chorionic gonadotropin (Norvomon®, ZOETIS, Brazil), 2 mL of prostaglandin F2 alpha (Estron®, Agener União, Brazil), and 0.5 mL of estradiol cypionate (E.C.P.®, ZOETIS, Brazil) were administered to each cow. The diameter of the pre-ovulatory follicle was measured by ultrasound (DP-2200Vet® with a 7.5 MHz linear array transrectal transducer; Mindray) 48 hours after the removal of the implant (Figure 1). Cows that presented pre-ovulatory follicles with a diameter of 11 to 20 mm were artificially inseminated. On the 22nd day of the protocol's initiation, ovarian follicles and the presence of the corpus luteum in the cows were assessed using Doppler ultrasonography. The examination was performed to evaluate the vascularization of the corpus luteum, providing information about its functional activity. The vascularization assessment was based on the visualization of blood flow, allowing the analysis of the corpus luteum's perfusion.

Conception and pregnancy rates were ultrasound evaluated 30 days after artificial insemination, with each animal being diagnosed as pregnant or not pregnant. Pregnancy was visually verified with the presence of the conceptus and amniotic fluid. The pregnancy rate in our study was 100%

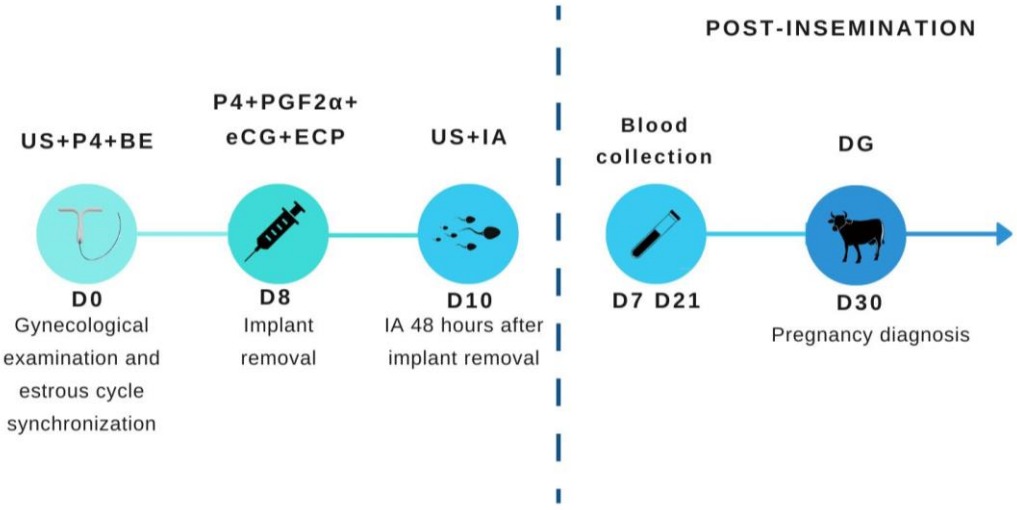


Figure 1. Diagram of the Artificial Insemination Protocol. US: ultrasound; P4: intravaginal implant of progesterone; BE: estradiol benzoate; PGF2α: prostaglandin F2 alpha; eCG: equine chorionic gonadotropin; ECP: estradiol cypionate; IA: artificial insemination; DG: pregnancy diagnosis; D0: day 0; D7: day 7; D8: day 8; D10: day 10; D21: day 21; D30: day 30.

2.4 Blood sample collection

Blood was collected 7 and 21 days after artificial insemination from 15 animals into eight K3 EDTA 4 mL tubes (32 mL per animal on each collection day) (Vacutainer Descarpack;

São Paulo, BR) via jugular venipuncture, placed on ice, and transported to the laboratory within 2 hours after collection. The samples were centrifuged at 1,900 x g at 4°C for 10 minutes. The plasma was then transferred to clean 2.0 mL tubes and centrifuged for 15 minutes at 10,000 x g at 4°C to sediment any remaining blood cells and contaminating platelets. The clarified plasma was transferred to new 2.0 mL tubes and immediately stored at -80°C to later perform hemolysis tests (Tzelos et al., 2023).

2.5 Hemolysis Determination

For greater accuracy and reliability of the data, before the extraction of total RNA, the plasma was thawed, subjected to centrifugation at 10,000 x g for 15 minutes at 4°C, and spectrophotometry to determine the degree of hemolysis (rupture of erythrocytes), as hemolysis releases miRNA from red blood cells, which can produce misleading results. For the plate assembly, 150 µL of PBS (pH: 7.4) was used as a calibrator and 150 µL of RNase-free water as a blank, with a dilution of 1:10 (15 µL of sample to 135 µL of PBS). For the reading, the Soret Band (414 nm) was used at spectrophotometry to assess the characteristic of free hemoglobin. For screening at the wavelength of 414 nm, the samples must not exceed 0.300 nm (Blondal et al., 2013). Therefore, out of the eight blood samples collected from each animal on both day 7 and day 21, the three with the lowest hemolysis values were selected to create a pooled sample for each animal for extraction, with 1 mL collected from each sample. For each animal on the respective collection days, a total volume of 3 mL of plasma was used for extraction, with this volume coming from the three samples with the lowest hemolysis levels. Samples that could not have the miRNAs extracted on the same day were stored at -80°C.

The level of hemolysis was assessed using the miRNA ratio approach, where the ratio between the red blood cell-enriched miRNA, miR-451, and a stably expressed miRNA in circulation, miR-23a, was used to determine the extent of hemolysis in plasma (Table 1). When the miR-451/miR-23a ratio is below 7, it is considered indicative of no measurable hemolysis. In this experiment, the miRNA ratio test resulted in average values of 1.260 ± 1.195 and 0.021 ± 0.835 (7 and 21 days of collection, respectively). These values are below the threshold level, indicating that the enrichment of blood cell-derived miRNAs is not due to hemolysis; any bias that could occur due to hemolysis is insignificant (Gebremedhn et al., 2018).

Table 1. Selected miRNAs to assess the degree of hemolysis.

ID miRBase	miRBase Accession	MATURE miRNA sequence	GeneGlobe ID
bta-miR-451	MIMAT0003775	5' AAACCGUUACCAUUACUGAGUUU	YP00204036
bta-miR-23a	MIMAT0003827	5' AUCACAUUGCCAGGGAUUUCCA	YP02102718

2.6 Total miRNA extraction

For our experiment, a custom miRNA extraction protocol was developed, adapted from methodologies established in previous experiments (Ioannidis and Donadeu, 2016; Ioannidis and Donadeu, 2017; Tzelos et al., 2023). The creation of a specific protocol was necessary due to the particularities of the study, such as the collection of samples at different gestational stages (days 7 and 21 of pregnancy) and the target tissue used (blood plasma from Nellore cows). Existing miRNA extraction protocols were adjusted to maximize extraction efficiency, ensuring the integrity of the miRNA molecules, which are small and sensitive to degradation, in addition to adapting the process to the characteristics of the biological material used. The importance of this study is related to the creation of this miRNA extraction protocol, which could serve as a basis for future studies in animals with similar characteristics. Moreover, this protocol may contribute to advancing research focused on identifying plasma levels of miRNAs in cattle during embryonic development.

Before extraction, the samples stored at -80°C were thawed and centrifuged at 10,000 x g for 15 minutes at 4°C. Total miRNA was extracted from 3.0 mL of plasma (a pool of samples with the lowest hemolysis levels, as described at item 2.5) using 9.0 mL of TRIzol LS reagent (Life Technologies, USA), which lyses cells while preserving the integrity of miRNA, according to the following protocol.

In a 50 mL Falcon tube, 9.0 mL of TRIzol LS was added to the 3.0 mL of each sample. The mixture was vortexed and then incubated for 5 minutes at 4°C. Subsequently, 3.0 mL of chloroform was added (maintaining a 1:1 ratio, with 3.0 mL of sample to 3.0 mL of chloroform), and the solution was vortexed again and incubated for 3 minutes at 4°C. The solution was then centrifuged at 20,000 x g for 15 minutes at 4°C. After centrifugation, the supernatant was carefully recovered and transferred to five 2.0 mL Eppendorf microtubes, with 1.0 mL in each tube. One mL of isopropanol (1:1 ratio) was added to each tube, vortexed, and incubated for 10 minutes at 4°C. The tubes were then centrifuged at 12,000 x g for 10 minutes at 4°C. All supernatant was discarded, and 2.0 mL of 75% ethanol was added to each tube, followed by centrifugation at 7,500 x g for 5 minutes at 4°C. The supernatant was discarded again, and the

microtubes were inverted and dried at room temperature to allow any residual ethanol to evaporate completely. After drying, the pellet was resuspended in 16 µL of RNase-free water in the first tube vortexed, and then centrifuged in a microcentrifuge. The pellet was transferred to the next tube, and the process was repeated for all five tubes to efficiently recover the entire content for each animal in a final volume of 16 µL.

After extraction, the RNA concentration and purity were determined using the Invitrogen™ Qubit™ 3 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and the Qubit™ microRNA Assay Kit (Invitrogen™, Thermo Fisher Scientific, Eugene, USA, Cat. Nº. Q32880) according to the manufacturer's recommendations. The concentration values should be at least 1.5 ng/µL to allow cDNA synthesis and RTqPCR. The samples were frozen at -80°C until cDNA synthesis.

2.7 cDNA synthesis

The cDNA was synthesized using the Mircury LNA RT Kit (Qiagen®, Hilden, Germany, Nº. 339340) along with the RNA Spike-in Kit (Qiagen®, Hilden, Germany, Nº. 339390) in an TC-412 Thermal Cycler (Techne, Keison Products, Chelmsford, England), following the manufacturer's recommendations, as detailed below.

The UniSp6 RNA from the miRCURY LNA RT kit was resuspended by adding 80 µL of RNase-free water, vortexed, briefly centrifuged, and left on ice for 20-30 minutes to dissolve properly. In the same solution, the endogenous control cel-miR-39-3p was resuspended by adding 80 µL of the previously resuspended UniSp6, vortexed again, centrifuged, and left on ice for 20-30 minutes to dissolve. The solution was stored in aliquots at -15 to -30°C. For setting up the reverse transcription reactions, 0.5 µL of this aliquot was added per 10 µL of RT reaction.

Before preparing the reagents, the concentrations of each sample were calculated to determine the amount of RNA in each dilution. In 200 µL PCR tubes, the samples were standardized to 10 ng per sample and diluted with RNase-free water to complete the volume of 6.5 µL. After the standardization and dilution of the samples, 0.5 µL of UniSp6 RNA Spike-in (resuspended with cel-miR-39-3p that was previously prepared and stored in the aliquot), 2 µL of 5x miRCURY SYBR® Green RT Reaction Buffer, and 1 µL of 10x miRCURY RT Enzyme Mix were added in each of the 200 µL tubes, vortexed, and briefly spun down. After the entire process, the reverse transcription reactions were incubated at 42°C for 60 minutes, followed by heat inactivation at 95°C for 5 minutes.

2.8 Target selection for gene expression analysis

The miRNAs involved in embryonic development and their possible interactions with the fetal implantation process were selected based on the literature. Primers were obtained based on the mature sequence of the target miRNAs (Table 2): bta-miR-433, bta-miR-99a-3p, bta-miR-301b, bta-miR-26a, bta-miR-1249, bta-miR-101, and the reaction controls UniSp6 miRCURY LNA™ miRNA PCR Assay as reaction control and Cel-miR-39-3p as endogenous control. The ratio between bta-miR-451 and bta-miR-23a was used as an indicator of hemolysis. The mature sequence of the bovine miRNA was obtained from the miRBase database (https://www.mirbase.org/. Accessed: 24/03/2024). The customized LNA assays for each one of the miRNAs were builded by Qiagen (Qiagen®, Hilden, Germany).

Table 2. Selected miRNAs for gene expression and endogenous control.

ID miRBase	miRBase Accession	MATURE miRNA sequence	GeneGlobe ID
bta-miR-433	MIMAT0001627	5' AUCAUGAUGGGCUCCUCGGUGU	YP00204036
bta-miR-99a-3p	MIMAT0012533	5' CAAGCUCGCUUCUAUGGGU	YP02102718
bta-miR-301b	MIMAT0009277	5' CAGUGCAAUGAUAUUGUCAAAGCAU	YP02113417
bta-miR-26a	MIMAT0000082	5' UUCAAGUAAUCCAGGAUAGGCU	YP00206023
bta-miR-1249	MIMAT0005901	5' ACGCCCUUCCCCCCCUUCUUCA	YP00204122
bta-miR-101	MIMAT0000099	5' UACAGUACUGUGAUAACUGAA	YP00204786
cel-miR-39-3p	MIMAT0000010	5' UCACCGGGUGUAAAUCAGCUUG	YP00203952

2.9 Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

The analysis of miRNA expression using customized miRCURY LNA miRNA PCR assays followed the manufacturer's recommendations. The miRCURY LNA SYBR® Green PCR Kit (Qiagen®, Hilden, Germany, cat. no. 339345) was used for RTqPCR. 220 µL of RNase-free water was added to each customized primer assay, then briefly vortexed and centrifuged in a spin, and left at room temperature for 20 minutes.

Plasmatic cDNA from each cow was diluted (1:15) with RNase-free water immediately before use, using 3 µL of cDNA and 42 µL of water in 1.5 mL tubes. For the RTqPCR on the 10 assays: cel-miR-39-3p (endogenous control), UniSp6 (reaction control), bta-miR-451 and bta-miR-23a (hemolysis controls), bta-miR-433, bta-miR-99a-3p, bta-miR-301b, bta-miR-26a, bta-miR-1249, and bta-miR-101, 1 µL of RNase-free water, 1 µL of PCR primer mix, and 5 µL of 2x miRCURY SYBR® Green Master Mix were added. The reactions were homogenized and briefly centrifuged before being distributed onto 96 wells PCR plates. After setup, the plate was placed in the Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad

Laboratories, Hercules, CA, USA) for amplification, following the temperature recommendations (Table 3), followed by 40 amplification cycles.

Table 3. PCR cycling conditions for miRCURY LNA miRNA PCR Assays.

Step	Time	Temperature	Ramp rate	Additional comments
PCR initial heat activation	2 minutes	95 °C	Maximal/fast mode	
2-step cycling:				
Denaturation	10 seconds	95 °C	Maximal/fast mode	
Combined annealing/extension	60 seconds	56 °C	Maximal/fast mode	Perform fluorescence data collection (SybrGreen)
Number of cycles	40*			
Melting curve analysis		60-95 °C		

(Adapted from miRCURY LNA miRNA PCR – Exosomes, Serum/Plasma and Other Biofluid Samples Handbook 09/2019)

2.10 Statistical analysis

The Ct (Threshold Cycles) data obtained were normalized using cel-miR-39-3p as the endogenous control. After normalization, values were analyzed using the comparative ΔCT method, and relative expression levels were assessed through the $2^{(-\Delta Ct)}$ values (Livak and Schmittgen, 2001).

Statistical analyses were performed using Statistical Analysis System (SAS,6 SAS Institute Inc., Cary, NC, USA) software. Treatment (days of gestation 7 or 21) expression means were compared by two-sample t-tests using the TTEST procedure of SAS, with significance level $\alpha = 0.05$.

3 RESULTS AND DISCUSSION

A recently identified source of significant variation in miRNA profiles in serum/plasma samples is hemolysis, which occurs when there is rupture of erythrocytes. This phenomenon can happen at various stages of sampling and handling procedures, until the serum/plasma is separated from other circulatory components. However, it is possible to monitor the occurrence of hemolysis in serum and plasma samples using methods that measure the levels of free hemoglobin. A simple and accessible approach involves the use of a spectrophotometer to measure the absorbance of oxyhemoglobin at $\lambda = 414$ nm. The detection

of distinct absorbance peaks allows for the disqualification of samples with hemolysis, thus avoiding the lengthy and costly processing of the sample (Blondal et al., 2013).

The assessment of the relative expression of erythrocyte-specific miRNAs, such as miR-451, in conjunction with the stable miR-23a in the bloodstream, can serve as a reliable indicator of hemolysis in RNA samples or miRNA profiling data in serum and plasma samples. As evidenced in the study conducted by Blondal et al. (2013), which investigated the quality of samples and miRNA profiles in serum and plasma, miR-23a demonstrated relative stability in these fluids and was not impacted by hemolysis.

RT-qPCR analyses were performed on the 15 Nellore cows on both blood collection days (days 7 and 21). However, for statistical analysis, the sample size (N) varied according to the PCR amplification results, as some miRNAs did not amplify on both collection days. For bta-miR-26a, the sample size was reduced to 6, for bta-miR-101 (n = 10), bta-miR-1249 (n = 10), bta-miR-301b (n = 8), bta-miR-433 (n = 7), and bta-miR-99a-3p (n = 4). The results of the statistical analysis for the six miRNAs are presented in Table 4.

Table 4. Differences in the Expression, in arbitrary units, of Plasma miRNAs Between Days 7 and 21 of Gestation Obtained by RT-qPCR.

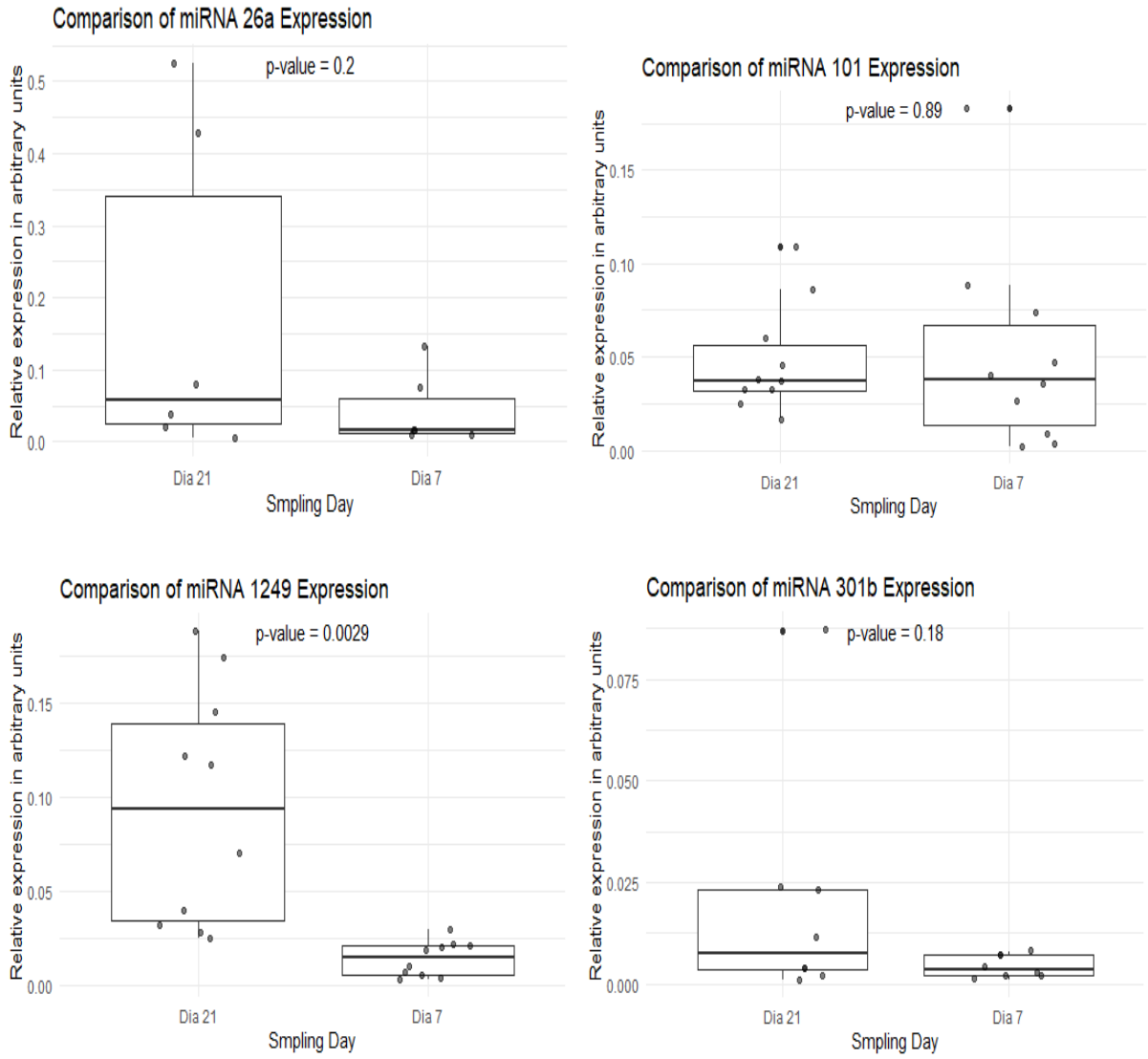
Day of pregnancy	Mean	SEM	P value	N° animals
			bta-miR-26a	
Day 7	0.043	0.021	0.202	N = 6
Day 21	0.183	0.094		
			bta-miR-101	
Day 7	0.051	0.017	0.887	N = 10
Day 21	0.048	0.009		
			bta-miR-1249	
Day 7	0.014	0.003	0.003	N = 10
Day 21	0.094	0.020		
			bta-miR-301b	
Day 7	0.004	0.001	0.178	N = 8
Day 21	0.020	0.010		
			bta-miR-433	
Day 7	0.023	0.009	0.926	N = 7
Day 21	0.021	0.008		
			bta-miR-99a-3p	
Day 7	0.011	0.003	0.482	N = 4
Day 21	0.007	0.004		

Relative expression of plasma miRNAs on days 7 and 21 of gestation based on Qiagen PCR array expression data. SEM: Standard error of the mean. Significance level (p-value) < 0.05.

Only one miRNA (bta-miR-1249) showed a significant difference between the gestation days at a significance level of $\alpha = 5\%$. Two possibilities can explain the lack of

significance in the results for the other micro RNAs: 1) biological variability, as miRNA expression can vary between different individuals. The available literature refers to miRNA expression in *Bos taurus*, and research on *Bos indicus* is limited, requiring more studies on the expression of these specific miRNAs during gestation; 2) due to the variability found in the Nellore cows, in the next studies to validate our results, we suggest a larger sample size.

The mean relative expression of each miRNA on the gestation days is illustrated in Figure 2. Only miR-1249 showed a significant difference between gestation days (7 days vs. 21 days, P = 0.003).



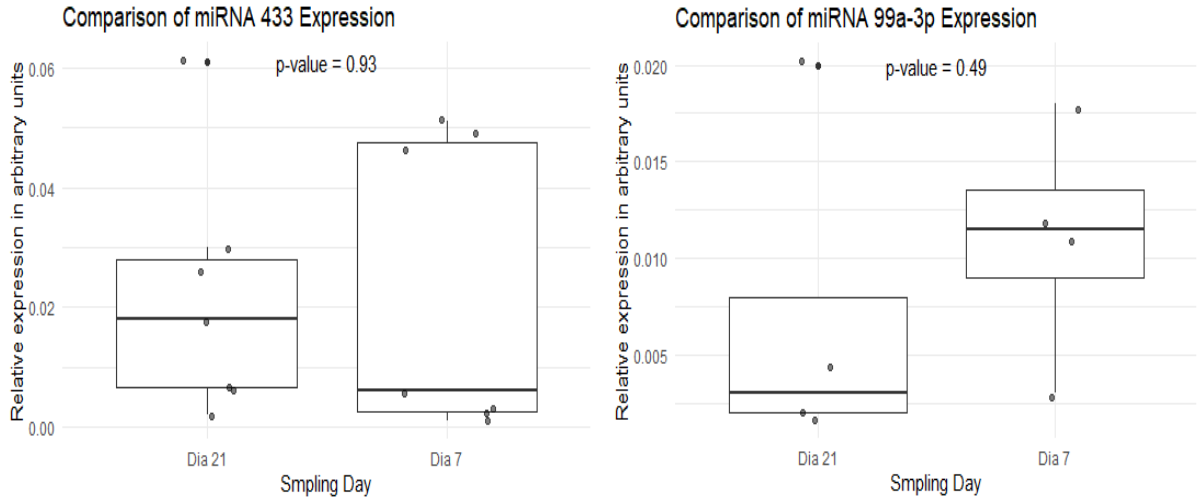


Figure 2. Expression of miRNAs during early pregnancy as determined by RT-qPCR. The box plot illustrates the relative expression levels (mean ± SEM, normalized to the mean expression of cel-miR-39-3p) of plasma miRNAs on days 7 and 21 of pregnancy.

The results found in our study for miR-26a indicate that the expression of miR-26a on day 21 was approximately 4.26-fold greater than on day 7 (n = 6); however, this difference did not reach statistical significance. These results are consistent with those of Ioannidis and Donadeu (2016), who quantified 15 individual miRNAs by RT-qPCR in plasma samples collected from a group of Holstein heifers during early pregnancy. Plasma levels of miR-26a were higher on day 24 of gestation (P = 0.027) compared to pre-insemination levels. Expression was also elevated on day 16, though not significant (P = 0.118).

In our study, changes in miR-26a expression during the early stages of pregnancy were relatively small. However, several studies report the presence of this miRNA in the embryo, ovary, and in tissues and cells related to the immune system, such as the thymus and B/T cells in various species (Coutinho et al., 2007; Huang et al., 2011). Kw et al. (2010), demonstrated that miR-26a is directly involved in the negative regulation of Interferon beta (IFN-β), which is involved in immune responses. In their research, Tzelos et al. (2023) demonstrated that miRNAs from the miR-26 family are expressed by bovine embryos from Holstein cows in intrauterine extracellular vesicles and are involved in regulating early conceptus development and/or the negative regulation of immune-related genes to facilitate implantation. Furthermore, miR-26a has been shown to regulate immunity through macrophages and T cells, which explains its role in establishing maternal immunotolerance, a crucial function during early pregnancy. These findings indicate that miR-26a is involved in pregnancy, exerting immunomodulatory effects (Ioannidis and Donadeu, 2016).

Additionally, miR-26a is involved in the expression of genes such as IGF1 (insulin-like growth factor 1) and PGR (progesterone receptor), which are important in regulating the early development of the conceptus. PGR is typically expressed in the isthmic epithelium of cyclic and pregnant cows during the post-ovulatory period (Pranomphon et al., 2024; Schmaltz-Panneau et al., 2015). The embryo expresses IGF1 receptors as it reaches the morula and blastocyst stages, exerting its effects on the embryo's competence to develop to the blastocyst stage after entering the uterus. Transcripts for IGF1 are also found in the endometrium, and alterations in maternal physiology may modify the regulation of the embryo by this growth factor and increase cellular resistance to heat shock (Block et al., 2007; Hansen and Tríbulo, 2019).

For miR-101, we did not identify differences in expression levels (Day 21 vs. Day 7, 0.94 fold, $P = 0.887$; $n = 10$). These results contrast with Ioannidis and Donadeu (2017), who reported a significant effect of the day of pregnancy in Holstein cows ($P<0.05$) for this miRNA. According to these authors, miR-101 levels tended to increase (2.3-fold, $P = 0.069$) on day 16 compared to day 8, with no additional elevation in average levels of this miRNA until day 60. However, they observed a significant higher expression in plasma levels of this miRNA ($P<0.05$) on day 60 compared to day 0. This miRNA may play an important role in regulating maternal metabolic changes in early pregnancy, such as insulin signaling and lipid metabolism, as well as in the expression of the genes PGRMC2 (progesterone receptor membrane component 2) and GHR (growth hormone receptor).

Progesterone can modulate oviductal functions to promote early embryonic development in cattle through PGRMC2, ensuring a successful pregnancy (Saint-Dizier et al., 2012; Takahashi et al., 2016). Growth hormone (GH) is known to stimulate postnatal development. The mRNA that encodes GHR was expressed at the early embryonic age of 8 days in the inner cell mass of the blastocyst and plays an important role in the implantation process. Its presence in the vascular and nervous systems and in muscle tissue implies that GH is involved in the embryo's differentiation processes (Kölle et al., 1997; Kölle et al., 1998; Kölle et al., 2001).

miR-1249 showed highly significant expression on day 21 (6.71 fold, $P = 0.003$; $n = 10$) compared to day 7. Ioannidis and Donadeu (2016) also identified an increase in miR-1249 levels on day 16 of pregnancy, though it was not statistically significant ($P = 0.081$). Li et al. (2015) reported the expression of miR-1249 in bovine mammary cells from Holstein cows infected with *Staphylococcus aureus*, indicating its relationship with the processes of

proliferation, differentiation, and cell apoptosis. The regulation of the expression profile of this miRNA is associated with mastitis, providing an experimental basis to reveal the cause and regulatory mechanism of mastitis. These authors also suggest the potential of miRNAs to serve as biomarkers for diagnosis.

Salilew-Wondim et al. (2014) demonstrated that miR-1249 levels in granulosa cells vary during follicular development in Simmental heifers, being more highly expressed on day 7 of the estrous cycle in granulosa cells of dominant follicles. According to Zhao et al. (2016), this miRNA was differentially more expressed in exosomes than in serum of Holstein cows. Exosomes exclusively express miRNAs packaged in exosomes, which are directed towards tissue development and lipid metabolism functions. Furthermore, it is involved in the gene expression of homeobox A13 (HOXA13). These homeobox gene family plays a crucial role in the regulation, patterning, and axial morphogenesis of the developing embryo. In their study, Ponsuksili et al. (2001) reported the transcription of Hoxa1 and Hoxc9 in the embryo at the 8-cell stage. The regulation of the expression of the factor affecting the viability of pre-implantation stage embryos depends on the maternal expression of HOXA10 in the distal epithelium of the oviduct and uterus.

The mean relative expression of miR-301b on day 21 was 5-fold higher than on day 7 ($P = 0.178$; $n = 8$), but they did not differ statistically. In contrast, a study published by Gebremedhn et al. (2018), which aimed to determine the expression of circulating miRNAs in serum samples from pregnant Holstein cows on days 19 and 24 post-insemination, found that miR-301b was significantly elevated from day 16 of gestation. miR-301b is related to PTGES3 (prostaglandin E synthase 3) and IRF1 (interferon regulatory factor 1). PTGES3 is the gene that encodes the enzyme prostaglandin E synthase 3 (cytosolic), which is involved in the conversion of prostaglandin H2 (PGH2) to prostaglandin E2 (PGE2). Prostaglandins are biologically active lipid mediators that play crucial roles in the regulation of reproductive events, embryonic implantation, and the establishment of pregnancy (Marei et al., 2014). Signal transducer and activator of transcription (STAT) proteins are essential for the regulation of biological processes. In cattle, the STAT1 gene is differentially expressed in the endometrium during the pre-implantation period through a highly specific mechanism involving the IFNT and STAT1 signaling pathways during pregnancy up to implantation (Vitorino Carvalho et al., 2016).

For miR-433, we did not identify differences in expression levels (Day 21 vs. Day 7, 0.91 fold, $P = 0.926$; $n = 7$). However, some studies have reported significant differential expression in serum samples from pregnant Holstein cows on day 19 post-insemination. The

expression level of miR-433 was upregulated compared to non-pregnant animals. It has been reported that miR-433 is associated with pregnancy, showing elevated abundance in the first trimester and decreasing as gestation progresses to the third trimester (Gebremedhn et al., 2018). This miRNA targets the CSHL1 gene (chorionic somatomammotropin hormone-like 1), which belongs to the chorionic somatomammotropin (CSH) gene family, involved in placental development and regulation of fetal growth. This gene encodes a protein similar to somatomammotropin, a hormone produced by the placenta during pregnancy that performs functions akin to prolactin and growth hormone. CSH1 acts on the uterine endometrial glands, stimulating their development and the expression of genes encoding proteins (Spencer et al., 2008).

The mean relative expression of miR-99a-3p on day 21 was 0.64-fold lower than on day 7 ($P = 0.482$; $n = 4$), not differing statistically. However, in the study by Gebremedhn et al. (2018), this miRNA was abundantly expressed in the plasma of pregnant Holstein cows on day 19. Reports indicate that this miRNA was significantly expressed in the plasma of pregnant women and in the endometrium of sows harboring healthy concepts compared to non-pregnant endometrium. Additionally, this miRNA was among the predominantly abundant miRNAs in an ovarian library of pregnant goats (Kotlabova et al., 2013; Wessels et al., 2013; Zhang and He, 2013). One of the target genes of this miRNA is IRF1 (interferon regulatory factor 1), an interferon-stimulated gene induced by type I interferon, including IFN-τ. According to studies by Ma et al. (2024), IFN-τ can induce the expression of IRF1 in endometrial epithelial cells of Holstein cows, positively regulating the expression of LIFR (leukemia inhibitory factor receptor) by binding to the LIFR promoter, thereby increasing endometrial receptivity. This mechanism is a key factor in the regulation of embryonic implantation.

Given the results and previously published studies, the importance of miRNAs in all stages of embryonic development is evident. After the onset of embryogenesis, the synthesis of all classes of RNA begins. At ovulation, the oocytes contain rRNA, mRNA, tRNA, and ribosomes, all necessary components for protein synthesis. After fertilization, the zygote undergoes cleavage and blastocyst formation, along with active transcription and accumulation of zygotic genomic mRNA. The embryo becomes dependent on its transcription after the first cleavage division, with increased protein synthesis already at the two-cell stage (Hafez, 2004). In ruminants, placental attachment (implantation) occurs with the appearance of binucleated cells, which arise from the mononucleated trophoblast cells by day 17. According to Yun et al.

(2021), before implantation, IFNT secretion by the elongating embryos is important for regulating gene expression in the endometrium during pregnancy recognition.

Therefore, pregnancy is a complex process involving many physiological and hormonal changes. miRNA expression can be influenced by many factors beyond the presence or absence of pregnancy. Further research is needed in this area, particularly in *Bos indicus*, to identify miRNAs involved in embryonic development.

4 CONCLUSION

In conclusion, our results for miR-1249, confirmed the initial hypothesis that the expression of plasmatic miRNAs may vary during embryonic development due to the constant changes in gene expression that occurs throughout this period. This condition allows the identification of miRNAs plasma levels during days 7 and 21 of gestation that may be related to pregnancy and play important roles during embryonic development. Although the studied miRNAs are involved during pregnancy development, only the expression of miR-1249 showed significant differences. As future perspectives, this microRNA will be tested earlier in a higher number of Bos indicus cows in different ages of gestation to validate its potential as a marker for pregnancy. Additionally, miRNA expression will be evaluated in different estrus phases in non-pregnant animals and also during different gestation periods close to pregnancy recognition, implantation, and placental formation.

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