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Pregnancy effects in the bovine oviduct during early embryonic development *in vivo*:
modulation of the microRNA profile in small extracellular vesicles of the oviductal fluid
and oviductal epithelial cells

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Pregnancy effects in the bovine oviduct during early embryonic development *in vivo*: modulation of the microRNA profile in small extracellular vesicles of the oviductal fluid and oviductal epithelial cells

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RESUMO

MAZZARELLA, R., **Efeitos da prenhez no oviduto bovino durante o desenvolvimento embrionário inicial *in vivo*: modulação do perfil de micro NA em vesículas extracelulares pequena do fluido oviductal e em células epiteliais do oviduto.** 2021. 117 f. Dissertação (Mestrado) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2021.

O oviduto fornece o microambiente ideal para desenvolvimento embrionário inicial. Este ambiente é formado por células epiteliais do oviduto (CEOs) e pelo fluido oviductal (FO) produzido por essas células. O FO possui vesículas extracelulares (VEs) pequenas, que por meio de seu conteúdo de biomoléculas, incluindo microRNAs (miRNAs), podem promover o ambiente adequado para a fertilização e o desenvolvimento inicial do embrião. Entretanto, a modulação do perfil de miRNAs em VEs pequenas do FO e em CEOs em vacas prenhes ainda é desconhecida. Assim, o objetivo deste projeto foi caracterizar o perfil de 383 miRNAs em VEs pequenas provenientes do FO e de CEOs de vacas prenhes em comparação a não prenhes. Os resultados indicam que a passagem de gametas e a presença do embrião modulam os conteúdos de miRNAs tanto de VEs quanto de CEOs. Em VEs, 8 miRNAs (bta-miR-126-5p, bta-miR-129, bta-miR-140, bta-miR-188, bta-miR-219, bta-miR-345-3p, bta-miR-4523 e bta-miR-760-3p) foram regulados positivamente em vacas prenhas e um (bta-miR-331-5p) foi regulado positivamente em vacas não prenhas. Enquanto em CEOs, 6 miRNAs (bta-miR-133b, bta-miR-205, bta-miR-584, bta-miR-551a, bta-miR-1193 e bta-miR-1225-3p) estão elevados apenas no grupo não prenhe. O enriquecimento funcional destes miRNAs destaca vias envolvidas em processos cruciais tanto para o trato reprodutivo quanto para o desenvolvimento embrionário, como inflamação, proliferação celular e resposta imune. Além disso, demonstramos a modulação dos níveis transcricionais de genes selecionados em CEOs de vacas prenhes. Estes resultados sugerem que a comunicação materna-embrionária potencialmente começa durante o desenvolvimento embrionário inicial no oviduto e através da modulação de miRNAs em CEOs e VEs, aumentando a receptividade oviductal, permitindo o desenvolvimento embrionário adequado e assim levando a uma prenhez de sucesso.

Palavras-chave: comunicação materno-embrionária, inflamação, proliferação celular, resposta imune, receptividade oviductal, trato reprodutivo bovino.

ABSTRACT

MAZZARELLA, R. **Pregnancy effects in the bovine oviduct during early embryonic development *in vivo*: Modulation of the microRNA profile in small extracellular vesicles of the oviductal fluid and oviductal epithelial cells.** 2021. 117 f. M.Sc. Dissertation – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2021.

The oviduct provides the ideal microenvironment for early embryonic development. This environment is formed by the oviductal epithelial cells (OECs) and the oviductal fluid produced by these cells. Among the contents of OF are the small extracellular vesicles (sEVs), which through their content, including microRNAs (miRNAs), can ensure proper cell communication between the mother and the embryo. However, the miRNA profile modulation in sEVs of the oviductal fluid and OECs in pregnant cows is still unknown. Thus, this project aimed to characterize 383 miRNAs in sEVs from the oviductal flushing and OECs in pregnant (pregnant group) compared to non-pregnant cows (non-pregnant group). Our results indicate that the passage of gametes and the embryo presence modulate miRNAs contents of sEVs and OECs. In OF-sEVs, 8 miRNAs (bta-miR-126-5p, bta-miR-129, bta-miR-140, bta-miR-188, bta-miR-219, bta-miR-345-3p, bta-miR-4523 and bta-miR-760-3p) were up-regulated in pregnant and one miRNA (bta-miR-331-5p) was up-regulated in non-pregnant cows. In OECs, 6 miRNAs (bta-miR-133b, bta-miR-205, bta-miR-584, bta-miR-551a, bta-miR-1193 and bta-miR-1225-3p) were up-regulated in non-pregnant and none was up-regulated in pregnant cows. The functional enrichment of miRNAs highlights pathways involved in physiological processes such as inflammation, cell proliferation, and immune response crucial for both the reproductive tract and embryo development. Additionally, we demonstrated the modulation of the transcriptional levels of selected genes in OECs in pregnant cows. These results suggest that embryonic-maternal crosstalk potentially begins during early embryonic development in the oviduct through the modulation of miRNAs in OECs and sEVs in pregnant cows, increasing oviductal receptivity, allowing proper embryo development leading to a successful pregnancy.

Keywords: Embryonic-maternal crosstalk, inflammation, cell proliferation, immune response, oviductal receptivity, bovine reproductive tract.

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1. INTRODUCTION

The oviduct provides the ideal microenvironment for early embryonic development (AVILÉS; COY; RIZOS, 2015; BESENFELDER; HAVLICEK; BREM, 2012). The bovine embryo develops in the oviduct for four days, and through it's zona pellucida, stays in contact with oviductal epithelial cells (OECs) as well as its secretions, the oviductal fluid (KÖLLE et al., 2009). The oviductal epithelium is composed of ciliated cells and secretory cells that produce the oviductal fluid (LEESE et al., 2008). The oviductal fluid comprises simple and complex carbohydrates, proteins, ions, inorganic salts, lipids, phospholipids, small metabolites, and vesicles (AVILÉS; GUTIÉRREZ-ADÁN; COY, 2010; LEESE et al., 2008). Changes in this fluid and the oviductal cells are regulated by different factors such as the oviduct region (ampulla or isthmus), the ipsilateral or contralateral side to the corpus luteum (BAUERSACHS et al., 2003; RODRÍGUEZ-ALONSO et al., 2020a; ULBRICH; KETTLER; EINSPANIER, 2003), and the concentration of steroid hormones (BINELLI et al., 2018; BUHI, 2002).

The oviductal fluid and OECs are also affected by the presence of gametes and embryos. Different studies have demonstrated changes in gene expression patterns of bovine OECs co-cultured with embryos (MAILLO et al., 2016a; SCHMALTZ-PANNEAU et al., 2014). *In vivo*, despite no differences in OECs gene expression were found using microarray analysis in the presence of a single embryo in artificially inseminated heifers compared to cyclic heifers by Maillo et al. (2015) or using target genes analysis by Rodríguez-Alonso et al. (2019), the same group identified differentially expressed genes by RNA-seq when multiples embryos were transferred (MAILLO et al., 2015). In the oviductal fluid, different proteins were described, indicating that the fluid composition could be influenced by gametes' presence (GEORGIU et al., 2005). Recently, Rodríguez-Alonso et al. (RODRÍGUEZ-ALONSO et al., 2020b) demonstrated that the oviductal fluid presents a different composition of carbohydrates, proteins, and amino acids in pregnant cows (artificially inseminated) when compared to cyclic cows (non-breed). Thus, it is possible that other components within the oviductal environment, such as extracellular vesicles present in the oviductal fluid, can also be modulated in pregnant animals.

Extracellular vesicles (EVs) are nanoparticles secreted by cells in the extracellular environment (RAPOSO; STORVOGEL, 2013) and contain bioactive materials such as proteins (SIMPSON; JENSEN; LIM, 2008), lipids (SUBRA et al., 2007), mRNAs, and non-coding RNAs, including microRNAs (miRNAs) (JEPPESEN et al., 2019; VALADI et al., 2007). EVs are classified as exosomes, microvesicles, and apoptotic bodies according to their size, biogenesis, and secretion (GYÖRGY et al., 2011). However, due to the difficulty to establish the specific origin of these EVs, they can be classified according to their size as small EVs (sEVs; < 200nm) or large EVs (lEVs; > 200nm) (THÉRY et al., 2018). In the oviduct, EVs have been identified in the oviductal fluid and are potentially related to the maternal crosstalk with gametes and embryos (AL-DOSSARY; MARTIN-DELEON, 2016; ALMIÑANA; BAUERSACHS, 2019; PAVANI et al., 2017; SAADELDIN, ISLAM M., HYUN JU OH, 2015). Functionally, both sEVs originating from the oviductal fluid and bovine OECs conditioned culture medium are internalized by bovine embryos and improve *in vitro* embryo development and quality (ALMIÑANA et al., 2017; LOPERA-VASQUEZ et al., 2016, 2017a). However, the protein profile of sEVs that originated from OECs *in vivo* is different from the sEVs produced by OECs *in vitro* (ALMIÑANA et al., 2017). Lopera-Vasquez et al. (2017a) demonstrated that sEVs from the isthmus have a better effect on *in vitro* embryo quality (cryotolerance and the expression of genes related to metabolism and epigenetics) than sEVs from the ampulla. Although they have not studied the content of these sEVs, it can be inferred that the contents from the isthmus are somehow different and better for the embryo. The molecular contents of oviductal sEVs (RNA, protein, and small ncRNA, including miRNAs) are also regulated by steroid hormones across the bovine estrous cycle (ALMIÑANA et al., 2018). To our knowledge, there are no studies on how the sEVs miRNA contents are modulated in pregnant cows.

MiRNAs are small non-coding molecules (~22 nucleotides) that act in post-transcriptional regulation (HA; KIM, 2014; LAU, 2001). MiRNAs are transcribed as primary miRNAs (pri-miRNAs), which are processed in precursor miRNA (pre-miRNA) and then transferred to the cytoplasm for further processing into mature miRNAs sequences (LEE, 2002). In mammals, the partial complementary base pairing miRNA-mRNA can induce transcription inhibition, thus regulating protein levels (GUO et al., 2010). MiRNAs are involved in different reproductive events, such as spermatogenesis

(KOTAJA, 2014), follicular development (DA SILVEIRA et al., 2015; TESFAYE et al., 2018), oocyte maturation (ANDRADE et al., 2017; TESFAYE et al., 2009), and embryo development (MONDOU et al., 2012a). To our knowledge, there are no studies on miRNAs regulation and function in OECs from pregnant cows. MiRNAs are also involved in cell-to-cell communication through sEVs (KOSAKA et al., 2013). MiRNAs within sEVs may play a role in modulating embryo-maternal communication during early pregnancy (HWANG; MENDELL, 2006; TESFAYE et al., 2017). EVs can mediate the delivery of its miRNAs contents into embryos, a phenomenon already implicated in embryo development and maternal-embryonic communication (BARKALINA et al., 2015; HOSSAIN et al., 2012; SALILEW-WONDIM et al., 2020; TESFAYE et al., 2017). However, there is no evidence of the modulation of the miRNA's content within oviductal sEVs obtained from pregnant cows during the first embryo cleavages.

Therefore, miRNAs' modulation and function within oviductal epithelial cells and EVs from the oviductal fluid in early pregnant cows is still elusive. Thus, we hypothesize that the miRNAs contents in small extracellular vesicles from the oviductal flushing (OF-sEVs) and in oviductal epithelial cells (OECs) undergo differential modulations in pregnant cows compared to non-pregnant. The present study evaluated the profile of 383 miRNAs in OF-sEVs and OECs in artificially inseminated (pregnant group) and in sham-inseminated (non-pregnant group) cows. Additionally, we access in OECs genes known to influence the immune system, inflammation, and pregnancy recognition. To our knowledge, this is the first study to characterize the changes in miRNA profile from both OF-sEVs and OECs obtained from pregnant and non-pregnant cows. Additionally, functional enrichment analysis was performed to evaluate the predicted pathways related to differentially expressed miRNAs between pregnant and non-pregnant cows.

The results presented here were published on *Frontiers in Veterinary Science*, section Animal Reproduction – Theriogenology entitled “Changes in Oviductal Cells and Small Extracellular Vesicles MiRNAs in Pregnant Cows” (Appendix A).

2. THE OVIDUCT

The use of assisted reproduction techniques (ARTs) is an alternative to increase reproductive efficiency and to solve reproductive problems in domestic species and humans. Besides, it is a relevant tool to accelerate the selection of high genetic value animals, increasing the number of descendants of genetically superior animals. According to data from the International Embryo Transfer Society (IETS), the number of embryos produced *in vitro* in the world has continuously increased since 2012. In 2019, Brazil produced *in vitro* and transferred more than 275 thousand embryos (VIANA, 2019). Much of this growth is due to advances in knowledge about the bovine reproductive physiology. However, there are still challenges in *in vitro* embryo production (IVP) that the industry must overcome; thus the search for new information and a better understanding of all the processes are extremely important.

One of the most significant barriers in the production of embryos is to ensure an *in vitro* environment as efficient as the physiological one. For reproductive success, gametes and embryos undergo many physiological processes. Together, these events support adequate oocyte and sperm competence and, thus, embryonic development. *In vitro*, these processes will occur in controlled and static environments that seek to mimic the environment *in vivo*. However, compared to embryos produced *in vivo*, bovine embryos produced *in vitro* have different gene expression (LONERGAN et al., 2003), lower quality parameters (RIZOS et al., 2002), and lower pregnancy rates (PONTES et al., 2009). Therefore, it is essential to decipher the physiological processes occurring in the bovine reproductive tract and from this, to improve the *in vitro* environment. More specifically, the oviduct, the place where fertilization and the initial embryonic development occur.

The oviduct is a tubular organ connecting the ovary to the uterus. Described by Gabriele Fallopius (1523-1562), it was considered for a long time as just a duct for gametes and embryo transport (LEESE, 1988). Although its contribution to reproductive events is not as studied as, for example, the ovaries and uterus, the oviduct has significant biophysical, biochemical, and nutritional functions (LI; WINUTHAYANON, 2017). Recent studies have shown that the oviduct and its fluid provide a suitable microenvironment for the transport of oocytes and sperm to the site of fertilization, for sperm selection and capacitation, fertilization, and early embryonic

development (CABALLERO et al., 2014; PÉREZ-CEREZALES et al., 2018; RODRIGUEZ-MARTINEZ, 2007).

Anatomically, the oviduct is divided into 4 longitudinal regions: the infundibulum, ampulla, isthmus, and tubal uterus junction (Figure 1). The cumus-oocyte-complex (COC) is captured after ovulation by adhesion at the cilia of by the infundibulum; in the ampulla fertilization occurs; in the isthmus, the sperm are capacitated and stored as well as the activation of the embryonic genome and the passage of the embryo into the uterus through the tubal uterus junction (HAMDÍ et al., 2018; HUNTER; FLÉCHON; FLÉCHON, 1991; KÖLLE et al., 2009). The oviduct's vascular support derives from branches of the ovarian and uterine arteries and possibly also has a counter-current mechanism that allows the transport of molecules from the ovary to the oviduct (HUNTER; COOK; POYSER, 1983; LEESE, 1988). The oviduct has a rich lymphatic supply, which is expanded during estrus due to the increase in the volume of liquid in the oviduct (OTSUKI et al., 1989).

Figure 1 - Anatomical division of the oviduct (infundibulum, ampulla, isthmus, and tubal uterus junction) and functions of each region.

Source: own authorship.

Histologically, the oviduct is composed of a serous layer (serous tunic), followed by a layer of muscle fibers and blood vessels (sub-serous) and the muscular tunic,

more prominent in the isthmus portion. The oviduct epithelium is formed mainly by ciliated cells and secretory cells (Figure 2). The infundibulum has a large number of ciliated cells, and the proportion of ciliated cells decreases as the number of secretory cells increases along the oviduct towards the isthmus, where secretory cells become the more abundant ones (KANAGAWA et al., 1972). The number of oviductal secretory cells varies during the estrous cycle (ERIKSEN et al., 1994; LEESE, 1988).

Figure 2 - Scanning electron microscopy of ipsilateral bovine OECs on day 3 of pregnancy. A. ampulla, bar = 9 μ m. B. Isthmus, bar = 7 μ m. C. Isthmus and eight-cell stage embryo, bars = 9 μ m and 75 μ m (with the embryo inserted in c).

Source: adapted from KÖLLE, S. et al. Ciliary Transport, Gamete Interaction, and Effects of the Early Embryo in the Oviduct: Ex Vivo Analyses Using a New Digital Videomicroscopic System in the Cow. **Biology of Reproduction**, v. 81, n. 2, p. 267–274, 1 ago. 2009.

Different factors can affect the oviduct epithelium. Studies have shown that gene expression of oviduct cells is influenced by the presence of gametes as well as with spermatozoa X or Y in pigs (MARTINEZ et al., 2014), seminal fluid in mice (SCHJENKEN; ROBERTSON, 2015), by embryos (SCHMALTZ-PANNEAU et al., 2014), dependent on the region (ampulla or isthmus) and the stage of the estrous cycle (ULBRICH; KETTLER; EINSPANIER, 2003). Different transcriptional profiles were also found in the ipsilateral or contralateral oviduct of the ovulatory corpus luteum (BAUERSACHS et al., 2003). Through proteomics, different proteins were described in the oviductal fluid, indicating that the oviduct was influenced by the presence of gametes or embryos (BANLIAT et al., 2020; GEORGIU et al., 2005). Besides, there

are variations in gene expression between ipsi or contra-lateral side to the corpus luteum (BAUERSACHS et al., 2003) and also between the ampulla and the isthmus according to the size of the ovulatory follicle and the steroid hormones concentration (GONELLA-DIAZA et al., 2015).

Throughout the estrous cycle, the oviduct's functions and its cells are controlled by steroid sex hormones: progesterone (P4) and estrogen (E2). As reviewed by Binelli et al. (2018), steroid hormones modulate gene expression and protein expression in the oviduct. Additionally, these hormones regulate morphological, biochemical, and physiological changes in the oviduct (BUHI, 2002), and consequently modulate motility through contraction of smooth muscle, ciliary beat, secretion, and transport of gametes (CRISMAN; MCDONALD; THOMPSON, 1980; HUNTER; WILMUT, 1983; RUCKEBUSCH; BAYARD, 1975). Briefly, in the follicular phase, E2 induces secretory activity, transport speed, and cell proliferation. While in the luteal phase, increasing levels of P4 decrease the speed of transport and secretion and subsequently modulate the flaking and renewal of the oviductal epithelium, preparing the oviduct for the next estrous cycle (Reviewed by Binelli et al. 2018). The main events that occur in the oviduct in association with changes in the concentration of sex hormones are summarized in Figure 3.

Figure 3 - Gametes and embryos passage along the oviduct in association with changes in the concentration of sex hormones (bar graph).

Day zero (estrus): maximum concentration of E2, high secretion in the ampulla and isthmus, a higher proportion of ciliated cells, increased ciliary beat, and sperm accumulation in the caudal portion, linked to ciliated cells. Day 1 (ovulation and fertilization): decreasing concentrations of E2, cumulus-oocyte complex (CCO) in the infundibulum going towards the ampulla for fertilization and sperm released from the isthmus, beginning hyperactivation and early acrosomal reaction. Day 2 (after fertilization): lower concentrations of E2 and beginning of the increase in P4, decreasing the speed of transport of the embryo. Day 3: initial embryonic development in contact with the follicular fluid, maternal-embryonic cross-communication. Day 4 (3.5 days after fertilization): increasing concentrations of P4 and transport of the embryo to the uterus. Days 10-15 (diestrus): a high concentration of P4 inhibits secretion and transport activities in the oviduct, decreased amount of secretory and ciliated cells with evidence of apoptosis and less vascularization. Source: adapted from BINELLI, M. et al. Sex Steroid-Mediated Control of Oviductal Function in Cattle. **Biology**, v. 7, n. 1, p. 15, 2 fev. 2018.

Additionally, under the control of these hormones, the oviduct produces the follicular fluid, a complex mixture derived from serum transudate and products from epithelial secretory cells (BUHI; ALVAREZ; KOUBA, 2000; LEESE et al., 2001). During the last few years, several studies have recognized the importance of this fluid in reproduction.

2.1 The oviduct: Oviductal fluid

The oviductal fluid is a complex mixture of constituents originated by the transudation of plasma from the systemic circulation and the active biosynthesis of specific proteins in the oviduct epithelial secretory cells (LEESE, 1988). The oviductal fluid is composed of simple and complex carbohydrates, proteins, glycosaminoglycans, ions, inorganic salts, lipids, phospholipids, and small metabolites and vesicles (AVILÉS; GUTIÉRREZ-ADÁN; COY, 2010; LEESE et al., 2008). The small metabolites correspond to different substrates, such as lactate, pyruvate, amino acids, and glucose (HUGENTOBLE et al., 2007). Recently, extracellular vesicles originating from the oviduct epithelium have been identified as one of the main components of the oviductal fluid (ALMIÑANA; BAUERSACHS, 2019; PAVANI et al., 2017). This fluid's composition throughout the estrous cycle is variable (ACUÑA et al., 2017; CERNY et al., 2015). Recently, Rodríguez-Alonso et al. (2020a) demonstrated that the oviductal fluid presents a different composition of carbohydrates, proteins, and amino acids in pregnant cows (artificially inseminated) when compared to cyclic cows (non-breed). These changes in the oviductal fluid's physiological characteristics can cooperate for the success of fertilization and embryonic development (PÉREZ-CEREZALES et al., 2018).

Both oviductal fluid itself and some of its molecules have already been studied and used to improve IVP. During IVM, oviductal fluid supplementation favors early embryonic development and improves blastocysts' quality, increasing their cryotolerance and enhance antioxidant activity (HAMDÍ et al., 2018). Supplementation with oviductal fluid during IVC increases TE cells' number and modifies the expression of essential genes for development (LOPERA-VASQUEZ et al., 2017a). Considering the supplementation only with isolated components of the oviductal fluid, the use of essential and non-essential amino acids in the same concentration present in the oviductal fluid improves embryonic development (SANTANA et al., 2014). Supplementation of the IVF medium with oviductin, an oviduct-specific glycoprotein, decreases polyspermy in cattle and pigs (COY et al., 2008). Also, supplementation with growth factors found in the oviductal fluid is efficient for improving embryonic development, increasing the number of cells and the survival of the transferred embryo (BLOCK et al., 2011; SIMINTIRAS et al., 2017). Additionally, the use of extracellular

vesicles isolated from both the oviductal fluid (LOPERA-VASQUEZ et al., 2017b) and the culture medium conditioned by oviduct epithelial cells (LOPERA-VASQUEZ et al., 2016) have shown promising results. Their use will be discussed in a specific topic regarding EVs.

2.2 The oviduct: Roles in fertilization

The ovulated cumulus-oocyte complex (COC), containing the oocyte and numerous cumulus cells wrapped in an extensive extracellular matrix, is captured by the oviduct infundibulum. The infundibulum has fimbriae, which captures the COC, and its epithelium is composed predominantly of ciliated cells that conduct the COC into the ampulla (SUAREZ, 2015). Although these mechanisms have not been completely elucidated, the oocyte uptake seems to depend on the adhesion between the COC extracellular matrix and the glycocalyx of the crown of the ciliated cells of the infundibulum (LAM et al., 2000; TALBOT; SHUR; MYLES, 2003). In humans, failure in this uptake process can lead to infertility and ectopic pregnancy (SUAREZ, 2015). Once captured, the COC is transported to the place where fertilization will occur.

Fertilization requires sperm to survive in the hostile environment of the female reproductive tract and reach the COC. The ejaculate has a heterogeneous population of sperm (RAMÓN et al., 2014). Not all of them are suitable for fertilization, and sperm with different epigenetic patterns and DNA fragmentation levels coexist (JENKINS et al., 2015). Only a small fraction of high-quality sperm will overcome the physical and anatomical barriers in females' reproductive tract and reach the oviduct (WILLIAMS et al., 1993). In the oviduct, they will also go through different selection mechanisms.

The isthmus portion of the oviduct is involved in sperm capacitation and transport of the sperm cells to the ampulla. Additionally, the caudal portion of the isthmus also serves as a reservoir for sperm cells in several species, such as cattle (HUNTER; WILMUT, 1984). In this reservoir occurs the binding of sperm cells to the oviductal epithelium (Figure 4) and releasing of the sperm for fertilization at the time of ovulation (HUNTER; WILMUT, 1984). In bovine, the sperm surface contains a protein called Binder of Sperm (BSP) (GWATHMEY et al., 2006). In the oviduct, the BSP binds to its oviductal receptor, known as the annexin (ANXA) proteins family (ANXA1,

ANXA2, ANXA4, and ANXA5) (IGNOTZ; CHO; SUAREZ, 2007). Several mechanisms are associated with sperm detachment from the oviductal epithelium, such as sperm capacitation in bulls (LEFEBVRE; SUAREZ, 1996) and motility hyperactivation in mice (DEMOTT; SUAREZ, 1992). Additionally, the interactions between sperm and the oviduct epithelium modify sperm membrane proteins. One of these interactions is responsible for inducing hyperactivation by activating a membrane cation channel of sperm (CatSper), leading to the influx of calcium into the sperm flagella and triggering the flagellar beat (LI; WINUTHAYANON, 2017; REN et al., 2001).

Figure 4 - Scanning electron microscopy of the interaction of bull sperm with *in vivo* bovine oviductal cells from the ipsilateral oviductal side on day 3 of pregnancy, X 5000.

Source: adapted from LEFEBVRE, R. et al. Characterization of the Oviductal Sperm Reservoir in Cattle. **Biology of Reproduction**, v. 53, n. 5, p. 1066–1074, 1 nov. 1995.

Once the utero-tubal junction is reached, the oviduct facilitates sperm hyperactivation and guides the sperm to the site of fertilization by mechanisms such as rheotaxis, thermotaxis, and chemotaxis. Rheotaxis is the mechanism by which sperm moves against the fluid flow from the oviduct generated by ciliated cells and tubal contractions (LEESE, 1988). This current will also act in the transport of oocytes and embryos. In the isthmus, the head of the sperm binds to the oviduct epithelial cells. This physical interaction has already been observed in cattle, horses, and mice (CHANG; SUAREZ, 2012; DOBRINSKI et al., 1997; MORALES et al., 1996; POLLARD et al., 1991) and also creates a reservoir of sperm in the oviduct.

Thermotaxis is the ability that allows the sperm to be guided according to the different temperature gradients in the oviduct. Although not as crucial as rheotaxis, it has already been described in pigs, cattle, and humans (BAHAT; EISENBACH, 2006; HUNTER, 2012). Chemotaxis is due to the release of substances by the cumulus-oocyte complex (COC) that attract sperm. Chemotactic substances are also present in the follicular fluid and attract the sperm to the site of fertilization. The COC, in humans, secretes P4 as a chemotactic for sperm (OREN-BENAROYA et al., 2008; TEVES et al., 2006), and different chemokine receptors, such as CCR1, CCR5, and CCR6, have been identified in human sperm (CABALLERO-CAMPO et al., 2013).

2.3 The oviduct: Roles in early embryo development

In vivo, the initial embryonic development occurs in the oviduct. Although much remains to be elucidated, there is evidence that the embryo interacts with the oviductal microenvironment (MAILLO et al., 2015). The bovine embryo develops in this location until the 8-16 cell stage, remaining from 3 to 4 days in direct contact through its pellucid zone with the oviductal epithelium cells and secretions, the oviductal fluid (HACKETT et al., 1993; KÖLLE et al., 2009). In this environment, occurs the first mitotic division, metabolic alterations and, the activation of the embryonic genome around the 8 to 16 cell stage (GRAF et al., 2014; LONERGAN; FORDE, 2014). After fertilization, the embryo's development and cleavages are supported by factors produced by the oviduct. The oviduct epithelium and the oviductal fluid provide to the embryo the necessary physiological and biochemical environment for initial development (LI; WINUTHAYANON, 2017). Among the oviduct functions in early embryonic development are nutrition, support of embryo cleavage and development, protection, epigenetic regulation, and transport (Figure 5).

Figure 5 - Cross-section of the oviduct and the early-stage embryo and the main interactions between the oviduct and the embryo.

Source: LI, S.; WINUTHAYANON, W. Oviduct: roles in fertilization and early embryo development. **Journal of Endocrinology**, v. 232, n. 1, p. R1–R26, jan. 2017.

Different concentrations of nutrients are needed as the embryo develops within the oviduct. Such as during the transition from oxidative to glycolic metabolism during the early embryonic development (LEESE, 1995; LOPES; DESMARAIS; MURPHY, 2004). Right after fertilization, embryos acquire energy through oxidative metabolism, using pyruvate and lactate as the primary energy sources. As development progresses and as embryonic mitochondria become active, glycolytic metabolism gradually becomes the primary energy source, using single-chain sugars and glucose to generate ATPs for embryonic development (GARDNER et al., 1996; GARDNER; LEESE, 1990). Additionally, there is a change in oxygen tension to support oxidative metabolism (ABSALÓN-MEDINA; BUTLER; GILBERT, 2014).

The oviduct plays an important role in cleavage and early embryonic development. The oviductal fluid has growth factors such as EGF (epidermal growth factor), TGF (growth transforming factor), FGF (fibroblast growth factor), and IGF (insulin-like growth factor) that are related to embryonic growth (LI; WINUTHAYANON, 2017). Factors such as IGF-I and IGF-II in mice, for example, are one of the main determinants of embryonic growth (LOUVI; ACCILI; EFSTRATIADIS, 1997). *In vitro*, IGF-I's addition in the culture medium improves cleavage rates and increases the pregnancy rate after embryo transfer (BLOCK et al., 2003; KAYA et al., 2017). Thus, the embryotrophic factors of the oviductal fluid can have a positive impact on early embryonic development.

The oviduct assists in the defense against oxidative agents and participates in the modulation of the immune system. For protection against oxidative agents, the oviduct fluid contains substances, such as glutathione, taurine, and hypotaurine. These substances protect the embryo against reactive oxygen species that could induce blockages and developmental delays (GUERIN, 2001). Regarding the immune system, although maternal recognition of pregnancy occurs in the uterus, studies have shown that early-stage embryos induce signaling pathways that are recognized in the oviduct (PÉREZ-CEREZALES et al., 2018).

The early-stage embryo's effects on the bovine oviduct's immune response has not yet been fully elucidated. In rats, during days 1 and 2 of pregnancy, E2 and its receptors are necessary to suppress the mother's immune system (W. et al., 2015). In cattle, it has been reported that the presence of multiple embryos in the oviduct negatively regulates genes associated with the inflammatory response, such as NFKB2 (MAILLO et al., 2015). *In vitro*, bovine embryos presence also generate an anti-inflammatory response in OECs, positively regulating the expression of PGES and the secretion of PGE2 (anti-inflammatory action) and decreasing NFKB (pro-inflammatory action) (TALUKDER et al., 2018). Thus, the embryo's presence can decrease the expression of genes related to the immune system (ALMIÑANA et al., 2012), modulating this system so that the embryo is not recognized as foreign to the maternal environment.

The oviduct can also act in the regulation of epigenetic changes during the initial embryonic development. Genome activation is the most critical event of pre-implantation embryonic development in cattle, and its peak is evident in embryos in the

8 to 16 cell stage (GRAF et al., 2014). Therefore, the greatest epigenetic reprogramming during development occurs in the oviduct, and changes in this environment can generate aberrations during genome de novo methylation (CHASON et al., 2011). The effect of the oviduct on the epigenetic marks of the embryo has been proven in studies that show that embryos produced *in vitro* supplemented with oviductal fluid have patterns of global methylation and methylation, of genes essential for development, similar to the methylation found in blastocysts produced *in vivo* (BARRERA et al., 2017; CANOVAS et al., 2017).

Another function of the oviduct is to transport the embryo to the uterus. The embryo transport occurs during the entire time that the embryo is in the oviduct and varies from 1 to 10 days depending on the species (SUAREZ, 2015). As previously mentioned, in cattle, this period is 3 to 4 days (HACKETT et al., 1993; KÖLLE et al., 2009). Embryo transport within the oviduct depends on beating of the epithelium's ciliated cells, on the secretion and flow of the oviductal fluid, and muscle contractions (LI; WINUTHAYANON, 2017). The best-known example of how the embryo can modulate its transport in the oviduct is the mechanism observed in horses, where only embryos, when producing PGE₂, are transported to the uterus while unfertilized oocytes remain retained (WEBER et al., 1991).

In vitro, although the IVP is a reality and allows to overcome the oviduct, it is clear that the oviduct is supporting the initial embryonic development. Bovine embryos produced *in vitro* have different gene expression (LONERGAN et al., 2003), lower quality parameters (RIZOS et al., 2002), and lower pregnancy rates (PONTES et al., 2009). Thus, it has been shown that changes in the *in vitro* culture environment that mimic the interactions that occur in the oviduct can improve the quality of the produced blastocysts (LOPERA-VASQUEZ et al., 2016; MAILLO et al., 2016b; PAVANI et al., 2017). Moreover, one of the possible strategies for this is to supplement the culture medium with oviductal contents and among them are extracellular vesicles, which can be separated from the oviductal fluid.

3. EXTRACELLULAR VESICLES

Extracellular vesicles (EVs) are nanoparticles formed by a lipid bilayer secreted by cells in the extracellular environment in response to physiological or pathological specific stimuli (RAPOSO; STOORVOGEL, 2013). EVs are classified according to their size, biosynthesis, protein content in exosomes, microvesicles, and apoptotic bodies. Exosomes have between 30 and 150 nm and are formed inside by the budding of intraluminal vesicles (ILV), forming multivesicular bodies (MVB). The exosomes are released into the extracellular environment by fusing the MVBs with the cell's plasma membrane. Microvesicles are between 100 and 1000 nm and emerge directly from the plasma membrane. Organelles and fragments released by cells in apoptosis form the apoptotic bodies, which have from 1 to 5 μm (TANNETTA et al., 2014). EVs can also be classified according to the fluid from which they were isolated, such as, for example, the oviductosomes that come from the oviduct (AL-DOSSARY; STREHLER; MARTIN-DELEON, 2013). It is important to note that due to their heterogeneity, overlapping size, and difficulty to track each subtype's exact origin, it is recommendable to use the generic term extracellular vesicles (LÖTVALL et al., 2014). Therefore, in this work, EVs will be classified according to their size into two types: small EVs (<200nm) or large EVs (> 200nm), as recommended by THÉRY et al., 2018.

Through their bioactive content, EVs have the function of modulating recipient cells, mediating communication between cell-to-cell. EVs' contents are similar to that of the source cell, indicating that these EVs have specialized functions (SIMPSON; JENSEN; LIM, 2008). EVs have bioactive materials such as RNA (mRNAs, miRNAs, and other non-coding RNAs), proteins, and lipids (JEPPESEN et al., 2019). Although the presence of markers for each EV subtype is not well established, the proteins commonly found on EV membranes include tetraspanins (CD63, CD9, CD81) and heat shock proteins (HSP70) (JEPPESEN et al., 2019). These surface molecules will mediate the interaction with the recipient cells. When the EV attaches to the target cell membrane, it may fuse directly with the plasma membrane or undergo endocytosis and fuse with the membrane of an endosome of the target cell (Figure 6). In all cases, the result is the communication between the source cell and the target cell.

Figure 6 - Extracellular vesicles mechanisms of action. Vesicles can bind to the plasma membrane of a cell (1), can fuse directly with the plasma membrane (2) or undergo endocytosis (3) and subsequently fuse with the membrane of an endosome in the target cell (4).

Source: RAPOSO, G.; STOOORVOGEL, W. Extracellular vesicles: Exosomes, microvesicles, and friends. **The Journal of Cell Biology**, v. 200, n. 4, p. 373–383, 18 fev. 2013.

In reproduction, extracellular vesicles can contribute to gamete maturation, fertilization, polyspermia prevention, early embryonic development, and embryo implantation (MACHTINGER; LAURENT; BACCARELLI, 2015). EVs have been described in several fluids, such as in follicular fluid (DA SILVEIRA et al., 2012), in seminal plasma (PIEHL et al. 2013), in oviduct fluid (AL-DOSSARY; STREHLER; MARTIN-DELEON, 2013), in the uterine fluid (BURNS et al. 2014), in the endometrium (NG et al., 2013) and secreted by embryos and gametes (BARKALINA et al., 2015; MELLISHO et al., 2017).

It is worth mentioning that EVs can also act in an autocrine way, such as in the OEC-OEC and embryo–embryo communication. PAVANI et al. (2017) demonstrated the oviductal EVs (exosomes) uptake by bovine OEC during in vitro culture. PAVANI et al. (2019) demonstrated that sEVs from medium conditioned by a group of bovine

embryos were uptake by embryonic cells and improved the development of blastocyst individually cultured, thus acting as embryotropins in embryo–embryo communication. Importantly, sEVs have emerged as key components of embryo-maternal communication in the oviduct during early embryo development. In this study, the sEVs present in the oviductal fluid and the embryo-maternal cross-talk through these vesicles are the main focus.

3.1 Oviductal Extracellular Vesicles

In the oviduct, oviductal EVs, or oviductosomes, are components of the oviductal fluid and favor oocyte and embryonic quality (LEESE et al., 2008). Oviductal EVs were reported for the first time in mice, and their uptake by sperm cells during storage in the oviduct was involved in sperm viability, capacitation, and acrosomal reaction (AL-DOSSARY; STREHLER; MARTIN-DELEON, 2013). In bovines, oviductal sEVs have been studied by ALMIÑANA et al. (2017, 2018), LOPERA-VASQUEZ et al. (2016, 2017), GATIEN et al. (2019), and BAUERSACHS; MERMILLOD; ALMIÑANA (2020). With the growing interest in the oviductal functions and the advancements in omic sciences, several studies have characterized these oviductal EVs.

The proteomic analysis revealed in sEVs the presence of essential proteins for exosomes biogenesis, traffic, membrane fusion, and intracellular vesicle release in addition to several proteins, such as heat shock proteins (HSP), which are crucial for fertilization and initial embryonic development (ALMIÑANA et al., 2017). The functional enrichment of the sEVs mRNA content indicated their involvement with chromatin changes, thus the authors suggests that the chromatin changes occurring in early embryonic development could potentially be influenced by maternal transcripts delivered through these sEVs (ALMIÑANA; BAUERSACHS, 2019). The same study showed, through functional enrichment, that these sEVs mRNAs are related to extracellular vesicles, cilia expression, embryonic development, and transcripts that encode ribosomal proteins, thus suggesting theses as roles of sEVs in oviductal functions and maternal-embryonic communication (ALMIÑANA; BAUERSACHS, 2019).

The content of these EVs may be differently modulated along the different

regions of the oviduct and across the estrous cycle. LOPERA-VASQUEZ et al. (2017) demonstrated that EVs from different portions of the oviduct (ampulla or isthmus) had different effects when used in supplementation. ALMIÑANA et al. (2018) demonstrate that the hormonal variation throughout the estrous cycle influences the profile of mRNA, miRNAs, and EV proteins of the oviduct. Additionally, GATIEN et al. (2019) analyzed the metabolomic profile of oviductal sEVs across the estrous cycle and showed that the metabolite content is under hormonal control. Based on that information, to evaluate changes in miRNA profile in the presence of a single embryo, it is essential to guarantee that tissues are not under the influence of different hormonal levels.

The internalization of oviductal sEVs by bovine embryos and the communication involving these vesicles from the oviduct has already been demonstrated. *In vitro*, the supplementation of the culture medium with oviductal sEVs has shown positive effects and helped to understand the role of EVs in embryonic development (ALMIÑANA et al., 2017). LOPERA-VÁSQUEZ et al. (2016) reported that conditioned medium containing sEVs improved the embryonic quality in a way equivalent to embryo-OECs co-culture. Additionally, embryos treated with EVs originated from the isthmus have better quality and are more resistant to vitrification than those treated with EVs from the ampulla (LOPERA-VASQUEZ et al., 2017). Recently, BAUERSACHS; MERMILLOD; ALMIÑANA (2020) also showed that the up-take of oviductal sEVs in vitro is associated with changes in embryonic gene expression. However, these methodologies have limitations related to contamination, the presence of different cell types, risk of disease transmission (THOMPSON; PETERSON, 2000), and also further studies of validation of the functional mechanisms at the molecular level are needed.

3.1.1. Oviductal Extracellular Vesicles: Interaction with Gametes

As previously mentioned, the oviductal EVs have been described and characterized for the first time in mice (AL-DOSSARY; STREHLER; MARTIN-DELEON, 2013). Al-Dossary et al. (2013) find that Plasma Membrane Ca ATPase 4a (PMCA4a) in oviductal EVs are uptake by the sperm and are related to sperm viability during storage in the oviduct, capacitation, and acrosomal reaction. Fereshteh, Z. et

al. (2018) identified that oviductal EVs donate miRNAs to sperm cell in mice. The sperm could then deliver these oviductal miRNAs to the zygotes and, based on miRNAs targets enrichment, modulate cell division, cellular growth, and embryonic development (FERESHTEH et al., 2018). Bathala et al. (2018) demonstrated in mice that after the interaction with oviductal EVs, there is an up-regulation of PMCA1 and PMCA4, proteins related to sperm motility and male infertility. Recently, A. Franchi et al. (2020) demonstrate in bovine the *in vitro* uptake of oviductal EVs from the isthmus and ampulla by frozen-thawed spermatozoa stimulates and its effects on sperm capacitation and acrosome reaction, thus improving sperm competence.

The effects of oviduct EVs on the COC are less known and have been described in canines (LEE; SAADELDIN, 2020), possibly because the canine oocyte undergoes the maturation process within the oviduct (CHASTANT-MAILLARD et al., 2011), while in other species, this process occurs in the ovary. Lange-Consiglio, A. et al. (2017) demonstrated *in vitro* that oviductal EVs (microvesicles), through their miRNA content, contribute to the canine oocyte maturation. Lee, Seok Hee, et al. (LEE et al., 2020) showed *in vitro* that oviductal sEVs (exosomes) are internalized, both by cumulus cells and by the oocyte, and increased cumulus expansion and proliferation as well as the maturation rate of canine COC via EGFR/MAPK pathway. In other species, the functional effects of oviductal EVs are still elusive.

3.1.2. Oviductal Extracellular Vesicles: Early Embryo-Maternal Cross-Talk

The embryo-maternal cross-talk is imperative for a successful pregnancy. The importance of this communication is already well known between the conceptus and the uterus and has a crucial role in the pregnancy establishment in cattle (MAMO et al., 2012). For example, controlled by the circulating P4 levels, the endometrium stimulates embryo elongation (FORDE et al., 2009). On the other hand, the conceptus produces bioactive molecules, known as embryotropins (reviewed by WYDOOGHE et al., 2017). One of these biomolecules is the interferon tau (IFNT) secreted by trophoblastic cells in domestic ruminant since the pre-hatching embryo stage, and increasing its secretion after elongation (KUBISCH; LARSON; ROBERTS, 1998). The

IFNT acts in the maternal pregnancy recognition, modulating prostaglandin synthesis, blocking luteolytic pulse, supporting prostaglandin E₂ (PGE₂) secretion, and thus preserving the CL and improving uterine receptivity (AROSH; BANU; MCCRACKEN, 2016). Different studies showed that EVs could participate in this process. For example, Nakamura et al. (2016) showed that ovine embryonic sEVs (exosomes) contain IFNT and induces IFNT-Stimulated Genes in endometrial cells. BURNS et al. (2016) demonstrated in sheep the internalization of EVs from the uterine luminal fluid by the conceptus trophoctoderm and uterine epithelia. The same authors also showed that EVs mediate the communication between the endometrium and the conceptus once EVs from the uterine luminal fluid transport RNAs from the endogenous beta retroviruses (enJSRVs) that are crucial for conceptus elongation in sheep (BURNS et al., 2014).

In the oviduct, although the number of studies on maternal embryo communication has been increasing, its importance has long been forgotten since the oviduct is by-passed in the *in vitro* embryo production (IVP). However, there is a higher incidence of pregnancy loss before implantation in bovine, i.e. 47.9 % in beef cattle (REESE et al., 2020), which is enhanced by communication failures between the oviduct and the embryo (DISKIN; MORRIS, 2008). Additionally, despite advances in the field of bovine IVP, only 40-50% of oocytes matured and fertilized *in vitro* reach the blastocyst stage, while *in vivo*, this proportion is 70% (LONERGAN et al., 2006; RIZOS et al., 2002). Thus, it is imperative to understand the physiological oviductal milieu in which early embryo development takes place.

One of the missing factors *in vitro* is the embryo-maternal cross-talk that through sEVs could exchange molecules between the oviduct and the embryo. As an example, ALMIÑANA et al. (2017) studied the protein content of oviductal sEVs and showed that during *in vitro* culture, the bovine embryos internalize these sEVs and consequently their protein cargo. Importantly, studies also showed that the bovine embryo secretes EVs containing mRNAs and miRNAs (ANDRADE et al., 2019; MELLISHO et al., 2017, 2019; QU et al., 2017; TAQI et al., 2019). Regarding the internalization of bovine embryonic EVs by the BOEC, more studies are needed in order to demonstrate the maternal internalization of these EVs into the oviductal cells, as already shown in human endometrial epithelial cells *in vitro* (GIACOMINI et al., 2017).

Under *in vitro* conditions, different studies showed positive effects of EVs in

early embryo development. So far, the functional effect of oviductal EVs on the embryo has been demonstrated in bovine (ALMIÑANA et al., 2017; BAUERSACHS; MERMILLOD; ALMIÑANA, 2020; LOPERA-VASQUEZ et al., 2016, 2017a) and in murine (QU et al., 2019). LOPERA-VASQUEZ et al. (2016) evaluated the effect of oviductal EVs from bovine OECs *in vitro* cultured on *in vitro* produced bovine embryos development and quality. In this study, the oviductal EVs improved embryo quality and cryosurvival. Although no difference was found in the cleavage rate, blastocyst yield on Days 7, 8, and 9 between the groups, blastocysts cultured fresh sEVs and frozen sEVs had a significantly increased number of cells. Additionally, the survival rate after vitrification was higher in embryos treated with fresh sEVs. ALMIÑANA et al. (2017) evaluated the protein contents of *in vivo* (from oviductal flush) and *in vitro* (from bovine OECs culture) derived oviductal sEVs. In Addition, these authors studied the effects of sEVs from oviductal flush (fresh and frozed) on bovine embryos *in vitro* produced. The authors demonstrated differences in the sEVs protein content between *in vivo* and *in vitro* origin. Interestingly, the functional analysis showed that some crucial proteins related to sperm binding, fertilization, and embryo development were present only in sEVs from oviductal flush (ALMIÑANA et al., 2017). Regarding the sEVs effects on embryos *in vitro* produced, sEVs improved blastocyst rate, survival, and quality. Although no difference was found in the cleavage rate between the groups, at days 7 and 8, frozen sEVs increased blastocyst rates (ALMIÑANA et al., 2017). Frozen EVs also increased the hatching rate on day 9 and the total number o blastocyst cells (ALMIÑANA et al., 2017). Additionally, ALMIÑANA et al. (2017) showed that during *in vitro* culture, the bovine embryos internalizes these sEVs.

LOPERA-VASQUEZ et al. (2017) studied the effect of oviductal EVs from the ampulla and isthmus on the bovine embryos *in vitro* produced. No differences were found in cleavage and blastocyst rates at days 8 and 9 between the groups (SOF+BSA/C-, SOF+FCS/C+, EVs from the ampulla and EVs from the isthmus at 10k or 100k). Although the blastocyst rate at day 7 was higher in the control group, embryos produced with isthmic EVs (100k) had a significantly higher survival rate 24h after vitrification and warming (LOPERA-VASQUEZ et al., 2017). Additionally, differences in gene expression related to membrane traffic and epigenetics were also observed on embryos produced with isthmic EVs (100k) (LOPERA-VASQUEZ et al., 2017). Embryos produced with isthmus EVs (100k) presented higher expression of

epigenetics (DNMT3A and SNRPN) and membrane trafficking (AQP3) genes compared with C+ (LOPERA-VASQUEZ et al., 2017).

QU et al. (2019) evaluated in mice the effect of oviductal EVs derived from donors oviduct in birth rates after embryo transfer (ET). The authors showed that the EVs concentration from the donor (EDOF) is higher than the EVs derived from recipient oviduct fluid (EROF). Subsequently, embryo transfer medium (ETM) was supplemented with EDOF from 10 donors and EROF from 10 recipients. The ETM supplemented with EVs derived from donor oviduct improved ET efficiency, as the blastocyst quality and birth rates were enhanced in this group. Additionally, in QU et al. (2019) the EDOF group showed better resistance to apoptosis, as indicated by the lower apoptotic index and higher cell differentiation, as indicated by the lower inner cell mass/ trophoctoderm ratio. Overall, these studies showed that early embryo maternal cross-talk initiates in the oviduct and is potentially mediated by EVs.

3.2. MiRNAs Content within Extracellular Vesicles

MiRNAs are small non-coding molecules (~22 nucleotides) that act in post-transcriptional regulation (HA; KIM, 2014; LAU et al., 2001). For miRNAs canonical biogenesis (Figure 7), genes encoding miRNAs are transcribed by RNA polymerase II, and originates the primary miRNAs (pri-miRNAs) containing hundreds of nucleotides, a "7-methyl guanosine cap" structure and a poly-A tail in the 5' and 3' regions (LEE et al., 2004; O'BRIEN et al., 2018). Pri-miRNAs are then processed by the enzyme RNase III (Drosha) and the DGCR8 cofactor still in the nucleus, forming the precursor miRNA (pre-miRNA), a hairpin-shaped structure of approximately 70 nucleotides (BARTEL, 2004; WINTER et al., 2009). The pre-miRNA is transported by the Exportina-5 transmembrane protein to the cytoplasm, where it is processed by the RNase III enzyme (Dicer), originating the mature double-stranded miRNA (miRNA: miRNA) of approximately 22 nucleotides, which could be one or both mature miRNA (LEE et al., 2003, 2004). To form the RISC silencing complex (RNA-induced silencing complex) that will bind to the 3' UTR region and act on the repression or degradation of target RNAs, one of the mature miRNA molecule binds to the argonaut protein, forming the silencing complex RISC (KIM, 2005). In mammals, the partial complementary base pairing miRNA-mRNA results in transcription inhibition, thus regulating protein levels (GUO et al., 2010).

Figure 7 - Canonical and non-canonical miRNA biogenesis.

In the canonical miRNA biogenesis, initially, MiRNAs are transcribed as primary miRNAs (pri-miRNAs), processed Drosha/DGCR8 in precursor miRNA (pre-miRNA), and then transferred to the cytoplasm by Exportin5/RanGTP-dependent manner for further processing into mature miRNAs duplex. The 5p or the 3p mature miRNAs is loaded into the AGO family of proteins, forming the miRNA-induced silencing complex (RISC). In the non-canonical pathways, small hairpin RNAs (shRNAs) are processed by Drosha/DGCR8, exported to the cytoplasm via Exportin5/RanGTP, where they are cleavage via AGO2-dependent, but Dicer-independent while mirtrons and (m⁷G)-pre-miRNA are dependent on Dicer to complete their cytoplasmic maturation and are transported to the cytoplasm by Exportin5/RanGTP and Exportin1, respectively. Both pathways lead to the formation of the RISC complex, that binds to the 3' UTR region inducing the degradation of target RNAs. Source: O'BRIEN, J. et al. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. **Frontiers in Endocrinology**, v. 9, n. AUG, p. 402. 2018.

MiRNAs are involved in the physiological and pathological processes. As reviewed by SALILEW-WONDIM et al. (2020) in reproductive events, miRNAs are involved in process such as spermatogenesis (KOTAJA, 2014), oogenesis (LEI et al., 2010), follicular development (DA SILVEIRA et al., 2015; TESFAYE et al., 2018), oocyte maturation (ANDRADE et al., 2017; TESFAYE et al., 2009), regulation of the bovine CL (MA et al., 2011), and embryo development (MONDOU et al., 2012). Importantly, changes in the miRNA profile have already been described as a result of ARTs. These altered miRNAs can impact gametogenesis, endometrial receptivity, implantation, and embryonic development (SIRISTATIDIS et al., 2015). MiRNAs are also crucial for the development of the female reproductive system (NOTHNICK, 2012). As an example, the deletion of DICER, important for miRNA processing, in mice results in abnormal differentiation of the oviduct and consequently infertility (GONZALEZ; BEHRINGER, 2009; NAGARAJA et al., 2008).

MiRNAs are involved in the modulation of physiological processes occurring in OECs. As previous discussed, GONELLA-DIAZA et al. (2017) showed that the endocrine milieu modifies the oviductal environment. Recently, GONELLA-DIAZA et al.(2021) also showed that sex-steroid hormones (P4 and E2) affects the miRNA expression and the processing machinery of miRNAs in bovine OECs, allowing proper sperm capacitation, fertilization, and early embryo development. Moreover, miRNAs are involved in processes occurring within the oviductal milieu, such as the maternal-fetal transition. The initial embryonic development is controlled by transcripts and maternal proteins produced and stored during oogenesis (TADROS; LIPSHITZ, 2009). During the embryonic maternal transition, maternal transcripts are depleted by degradation or translation and replaced by new embryonic origin transcripts (SIRARD, 2011). In this process, miRNAs can act on maternal transcripts' degradation (MONDOU et al., 2012b; TANG et al., 2007). Thus, it regulates the degradation of mRNAs and proteins of maternal origin and also the activation of the embryonic genome (LI; ZHENG; DEAN, 2010). Additionally, miRNAs within sEVs are also involved in cell-to-cell communication (KOSAKA et al., 2013) and may play a role in modulating embryo-maternal communication during early pregnancy (HWANG; MENDELL, 2006; TESFAYE et al., 2017).

As reviewed by GROOT; LEE (2020) and JANAS et al. (2015), miRNAs can be selectively loaded into EVs. The sorting of miRNAs is controlled by RNA-binding

proteins (RBPs, Figure 8), such as hnRNPA2B1 (VILLARROYA-BELTRI et al., 2013) and membranous proteins. Although these molecular mechanisms are still largely unknown, this group of proteins binds specific miRNAs and selectively facilitates their transport and transfer into EVs. In bovines, the presence of miRNAs within EVs have been reported in many fluid, such as the follicular fluid (FF) (DA SILVEIRA et al., 2017; DE ÁVILA et al., 2020), oviductal fluid (ALMIÑANA et al., 2018), uterine fluid (NAKAMURA et al., 2019), and also in the culture medium obtained after in vitro embryo production (ANDRADE et al., 2019; MELLISHO et al., 2019).

Figure 8 - RNA-binding proteins and membrane proteins controlling miRNA sorting into EVs.

Source: VILLARROYA-BELTRI, C. et al. Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs. **Nature Communications**, 2013.

Importantly, EVs can mediate the delivery of its miRNAs contents into embryos, a phenomenon already implicated in embryo development and maternal-embryonic

communication in the oviduct (BARKALINA et al., 2015; HOSSAIN et al., 2012; SALILEW-WONDIM et al., 2020; TESFAYE et al., 2017). However, there is no evidence of the modulation of the miRNAs profile within oviductal sEVs from pregnant cows during the first embryo cleavages at the oviduct. Thus, miRNAs are candidates with great potential for understanding the molecular activities that occur during early embryonic development while still in the oviduct.

4. HYPOTHESIS AND OBJECTIVES.

Hypothesis:

Pregnancy modulates the miRNA profile of small extracellular vesicles from the oviductal flushing and the profile of miRNAs and mRNAs of oviduct cells during the initial embryonic development within the oviduct.

Objective:

To evaluate the miRNA profile of oviductal sEVs from the oviductal flushing and epithelial cells from oviducts of pregnant cows (pregnant cows) compared to non-pregnant (non-pregnant cows).

Specific objectives:

- Obtain oviductal flushing and epithelial cells from oviducts from pregnant and non-pregnant cows.
- Separate and characterize sEVs obtained by the flushing of oviducts from pregnant group and non-pregnant group, regarding the size, concentration and profile of miRNAs.
- Assess the miRNA profile and the levels of selected mRNAs in epithelial cells from pregnant group and non-pregnant group.
- Investigate the biological pathways modulated by miRNAs differently expressed between the pregnant and non-pregnant groups in sEVs and OECs.

5. MATERIAL AND METHODS

All the reagents were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO) unless otherwise stated. The present study was approved by the University of São Paulo Research Ethics Committee (protocol number: 4909010817).

5.1. Animal model

This study was conducted at the University of São Paulo (campus of Pirassununga – SP, Brazil) with Nelore (*Bos indicus*) multiparous cows with a body condition score 4 (0-emaciated; 5-obese) according to Ayres et al. (2009). Cows were fed with corn silage, supplemented with mineral salt, and free access to potable water. In order to obtain the oviducts exposed or not exposed to embryos, 25 cows were submitted to estrous synchronization protocol. As described by Gonella-Diaza (2015) with minor modifications: on the first day, all the cows received 2 mg of estradiol benzoate (2 ml, i.m., Sincrodiol, Ourofino Saúde Animal, Brazil), 0.53 mg of sodium cloprostenol (2 ml, i.m., Sincrocio, Ourofino Saúde Animal, Brazil) and an intravaginal P4-releasing device (1g; Sincrogest®, Ourofino Saúde Animal, Brazil). On day 7, the P4 device was removed, and cows received 0.53 mg of sodium cloprostenol (2 ml, i.m., Sincrocio, Ourofino Saúde Animal, Brazil). On day 9, cows received 0.01 mg of gonadotrophin releasing hormone (GnRH) analog (2.5 ml, i.m., Sincroforte, Ourofino Saúde Animal, Brazil). Twelve hours after GnRH analog application, cows were randomly assigned into two groups: fixed-time artificial inseminated (FTAI, n=14) or sham-inseminated (control, n = 11) (Figure 9). In the group FTAI, cows were artificially inseminated with frozen-thawed semen from the same bull of proved fertility. In the control group, cows were sham-inseminated with deposition of depleted semen extender in the uterine tract. For sperm depletion, straws from the same bull used in the FTAI group were thawed, centrifuged at 4000xg for 15 minutes, and the supernatant was filled into a new straw. The ovulation time was controlled by ultrasound (MyLab Delta, Esaote, Italy) and only animals that ovulated with more than 24 hours and less than 36 hours after GnRH analog application were kept in this study. All cows were slaughtered about 120 hours after ovulation induction with GnRH at the local slaughterhouse within the campus at the University of São Paulo (campus of Pirassununga/SP, Brazil).

Figure 9 - Animal model: 25 Nelore cows were synchronized and randomly divided into two groups: fixed-time artificial insemination (FTAI, n = 14) or sham-inseminated (Control, n = 11).

Source: own authorship.

5.2. Sample collection and groups formation

Reproductive tracts were processed within 30 minutes after slaughter. Oviducts ipsilateral to the corpus luteum (CL) were dissected and separated from the utero-tubal junction. Next, oviducts were divided in ampulla and isthmus through the ampullary–isthmic junction, identified as the place where the oviductal diameter starts to decrease, as described by MAILLO et al. (50) and GONELLA-DIAZA et al. (51). The isthmus was individually flushed with 1 ml of calcium and magnesium-free PBS with 0.1% of polyvinylpyrrolidone (PVP). The presence or absence of the embryo within the isthmus portion was used for group formation and confirmed in the oviductal flushing using a stereo microscope. After washing, epithelial cells of each isthmus were obtained by squeezing the tissue using a sterile glass slide and frozen in liquid nitrogen until further use (Figure 10).

Figure 10 - Sample collection: the reproductive tracts were dissected, and the ipsilateral oviduct was divided into portions (ampulla and isthmus) and the isthmus was washed individually and later OECs were collected.

Source: own authorship.

In addition to the criterion of the presence or absence of the embryo, we also analyzed pre-ovulatory follicle (POF) diameter, weight, and diameter of the CL and serum concentration of progesterone (P4) and estrogen (E2). The POF diameter was measured by ultrasound (MyLab Delta, Esaote, Italy) on day 9 of the estrous synchronization protocol. For CL weight and size, CLs were dissected from the ovary, weighed, and their diameter was calculated as the average between two perpendicular axes measured. For serum concentration of P4 and E2, blood samples were collected from the jugular vein in serum blood collection tubes (BD, São Paulo/SP, Brazil) at the time of slaughter. The serum was separated by centrifugation at 1,500 xg for 30 min, stored at -20°C, and the measurement of E2 and P4 was performed using chemiluminescence immunoassay (ADVIA Centaur®-Siemens) at Pasin Laboratory (Santa Maria/RS, Brazil).

5.3. Isolation of OF-sEVs

OF-sEVs were obtained from the isthmus of ipsilateral oviducts from pregnant and non-pregnant groups according to the isolation protocol previously used by da Silveira et al., (DA SILVEIRA et al., 2017); Alminaña et al. (ALMIÑANA et al., 2018), and Ávila et al. (DE ÁVILA et al., 2020). Briefly, the oviductal flushing was centrifuged at 300 xg for 10 min to remove live cells, 2,000 xg for 10 min to remove cellular debris,

16,500 xg for 30 min to remove large vesicles, and the remaining supernatant was placed at -80°C until further use. The supernatant was filtered through a 0.20 µm sterile syringe filter (PES membrane; Corning) in order to remove any remaining large EVs. For sEVs isolation, filtered oviductal flushing was centrifuged twice at 119,700 xg for 70 min at 4 °C (Optima XE-90 Ultracentrifuge; rotor 70 Ti; Beckman Coulter). After this procedure, the supernatant was discarded, and the sEVs pellets were resuspended in 20 µL of phosphate-buffered saline (1X Ca²⁺/Mg²⁺ free PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) and used for further analysis (Figure 11). Due to the small volume of oviductal flushing obtained from a single animal, the protocol to isolate OF-sEVs was validated using female reproductive tracts obtained from slaughterhouses, as described in APPENDIX B.

Figure 11 - Isolation protocol of OF-sEVs. The oviductal flushing was centrifuged at 300 xg for 10 min to remove live cells, 2,000 xg for 10 min to remove cellular debris, 16,500 xg for 30 min to remove large vesicles. The supernatant was filtered and centrifuged twice at 119,700 xg for 70 min. The isolated sEVs from pregnant and non-pregnant cows were characterized regarding size, concentration, and miRNA profile.

Source: own authorship.

5.4. Nanoparticle tracking analysis

The OF-sEVs isolated from pregnant and non-pregnant groups were analyzed based on particle mode size and concentration by nanoparticle tracking analysis (NTA). OF-sEVs isolated from 200 µL of each oviductal flushing were individually centrifuged, and their pellets were resuspended in 20 µL of 1X Ca²⁺/Mg²⁺ free PBS. As described by Ávila et al. (2020), with minimal modifications, particle size, and

concentration were measured by nanoparticle tracking analysis using Nanosight (NS300; Malvern). Briefly, for this analysis, the dilution factor used was 1: 100 in 1X $\text{Ca}^{2+}/\text{Mg}^{2+}$ free PBS. Five videos of 30 sec were taken for each sample, captured by a scientific complementary metal–oxide–semiconductor (sCMOS) camera at camera level 13 and under a controlled temperature of 38.5 °C. Considering the threshold level of 5, these captured images were tracked by the NanoSight NTA 3.4 Analytical Software (NTA 3.4 Build 3.4.003; Malvern) that measures and provides information about particle size and concentrations of each sample.

5.5. Total RNA extraction, reverse transcription, and real-time PCR

Total RNA, including miRNAs, from OF-sEVs and OECs was extracted according to the TRIzol reagent (Thermo Fisher Scientific) manufacturer's instruction and with the addition of 1.33 μL the coprecipitator GlycoBlue (Thermo Fisher Scientific) to the aqueous phase before RNA precipitation, as previously described by Da Silveira et al. (54) with minimal modifications. Total RNA concentrations were analyzed using spectrometry (NanoDrop 2000, Thermo Fisher Scientific). The RNA was treated with DNaseI (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions.

MiRNA analysis of OF-sEVs and OECs were performed as described in Da Silveira et al. (54). Briefly, for cDNA synthesis, reverse transcription was performed with the commercial miSCRIPT II RT kit (QIAGEN), using the MiScript HiFlex Buffer and 200 ng of total RNA. For quantitative RT-PCR, the miScript SYBR® Green PCR Kit (QIAGEN) was used according to the manufacturer's instructions. The relative levels of 383 miRNAs were evaluated for each sample. The 384-well plates were assembled using a multichannel electronic pipetting system and analyzed by RT-PCR with QuantStudio 6 Flex (Applied Biosystems). MiRNAs were considered present when cycle threshold (CT) was less than 37 in at least three out of six biological repetitions with an adequate melting curve. For both OF-sEVs and OECs, the CT values were normalized using the geometric mean CT of the endogenous genes Hm/Ms/Rt U1 snRNA and bta-miR-99b (55). The relative expression values were calculated using the ΔCt method, and the normalized data were transformed by $2^{-\Delta\text{Ct}}$ for graphical representation of the relative transcript levels (56).

Regarding mRNAs analysis of OECs, we evaluated the relative expression of 14 mRNAs selected from key pathways known to influence oviductal function and two endogenous controls (Table 1) for each sample. Briefly, reverse transcription reaction was performed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), according to the manufacturer's protocol. For quantitative RT-PCR, the GoTaq® qPCR Master Mix (Promega) was used according to the manufacturer's instructions and using the QuantStudio 6 Flex (Applied Biosystems) with the following PCR cycle conditions: 95°C for 10 min, 45 cycles of 95°C for 15 s and 60°C for 60 s. The amplification of single cDNA products was confirmed by melt curve analysis. CT values were normalized using the geometric mean CT of the endogenous genes PPIA and GAPDH, as previously described for similar tissues (49). The relative expression values were calculated using the ΔC_t method, and the normalized data were transformed by $2^{-\Delta C_t}$ for graphical representation of the relative transcript levels (56).

Table 1 - Primer sequences of genes analyzed by real-time PCR. Gene selected from key pathways known to influence oviductal function and endogenous controls. F: Forward and R: Reverse.

Key function	Gene symbol	Gene name	Primer sequences (5'-3')	GenBank number	Reference
Inflammatory and immune response	<i>IL1B</i>	Interleukin 1 Beta	F: TCCAGCCAACCTTCATTGC R: ACAGCTCATTCTCGTCACTGTAGTAAG	NM_174093.1	LFEM*
	<i>IL2RA</i>	Interleukin 2 Receptor Subunit Alpha	F: CTGCTCATATCCCCAAAGG R: CATGGGTTTGCTTGTCTT	NM_174358.2	LFEM*
	<i>IL6R</i>	Interleukin 6 Receptor	F: ACCACCAAGGCCGTGTACT R: GGCACACACAGGACAATA	NM_001110785.1	(LOPES, 2016)
	<i>TAB1</i>	TGF-Beta-Activated Kinase 1-Binding Protein 1	F: GACGCGCTGGCTGAGAAG R: GCCTTGAGTCTTCGAGGATCTT	NM_001102057.1	(LOPES, 2016)
	<i>TGFB1</i>	Transforming Growth Factor Beta 1	F: CCTGCTGAGGCTCAAGTAAAAAG R: GCCACTGCCGACAACCTC	NM_001166068.1	(SCOLARI et al., 2017)
	<i>TNFR1</i>	Tumor Necrosis Factor Receptor Type 1	F: AGGACCCAGGCACTACAGTACTATTAC R: CACCGCTGGTAGCGACATG	NM_174674.1	(LOPES, 2016)
	<i>RGS2</i>	Regulator of G Protein Signaling 2	F: CCTGAACACCCAGGCCTGAAT R: GAACCCCATGCCACATGAGA	NM_001075596.1	LFEM*
	<i>PTGES2</i>	Prostaglandin E synthase 2	F: GTGGGCGGACGACTGGTTGG R: CGGAGGTGGTGCTGCGTTT	NM_001166554.1	(OLIVEIRA et al., 2017)
	<i>PTGES3</i>	Prostaglandin E synthase 3	F: CAGTCATGGCCAAGTTAACAAA R: ATCACCACCCATGTTGTTTCATC	NM_001007806.2	(OLIVEIRA et al., 2017)
Pregnancy recognition	<i>IFNAR2</i>	Interferon (alpha, beta and omega) receptor 2	F: CTGGTCATTTGTATGGGCTCTTT R: GTATCCCGGGACTGTGCAATT	NM_174553.2	(SPONCH IADO et al., 2017)
	<i>MX1</i>	MX dynamin-like GTPase 1	F: GTACGAGCCGAGTTCTCCAA R: ATGTCCACAGCAGGCTCTTC	NM_173940.2	(GIFFORD et al., 2007)
	<i>MX2</i>	MX dynamin-like GTPase 2	F: CTTAGAGAGCGCCTCAGTCG R: TGAAGCAGCCAGGAATAGT	NM_173941	(GIFFORD et al., 2007)
	<i>OAS1Y</i>	2',5'-oligoadenylate synthetase 1	F: GCGGACCCTACAGGAAATGT R: TGTTCCTGGGGCGACACATC	NM_001040606.1	LFEM*
	<i>ISG15</i>	ISG15 ubiquitin-like modifier	F: AGAGAGCCTGGCACCAGAAC R: TTCTGGGCGATGAACTGCTT	NM_174366.1	(SPONCH IADO et al., 2017)
Endogenous control	<i>PPIA</i>	Peptidylprolyl isomerase A	F: GCCATGGAGCGCTTTGG R: CCACAGTCAGCAATGGTGATCT	NM_178320.2	(ARAÚJO et al., 2016)
	<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	F: GCCATCAATGACCCCTTCAT R: TGCCGTGGGTGGAATCA	NM_001034034.2	(ARAÚJO et al., 2016)

* Provided by Molecular Endocrinology Physiology Laboratory (LFEM-FMVZ-USP)

Source: MAZZARELLA, R. et al. Changes in Oviductal Cells and Small Extracellular Vesicles miRNAs in Pregnant Cows. **Frontiers in Veterinary Science**, v. 8, n. March, p. 1–14, 4 mar. 2021.

5.6. Statistical and bioinformatics analysis

The data obtained were tested for outliers, normality by Shapiro–Wilk test and the normalized RT-PCR data were analyzed by the Student's T-test. For all analyzes,

$p < 0.05$ was considered significant unless otherwise stated. Statistical analyzes were performed with JMP 14 (SAS Institute Inc.). For bioinformatics analysis and identification of biological pathways, the miRNAs differently detected within the groups were evaluated by miRWalk 2.0 bovine database (STICHT et al., 2018). MiRWalk is an online database that, through their TarPmiR algorithm, allowed the searching of validated and predicted interactions between miRNAs and gene sequences. Then, the biological pathways predicted as modulated by these miRNAs of interest were identified through the gene set enrichment analysis (GSEA) function within the MiRWalk platform, assessing the geneset "KEGG Pathways". Only pathways with a predicted probability lower than 0.05 that a candidate target site is a true target site were considered as significant.

6. RESULTS

6.1. Sample collection and groups formation

Two out of 14 cows from the FTAI group had not an ovulated follicle between 24 and 36 hours after GnRH analog application and were not kept in this study. Embryos between 4 and 16 cell stage were found in six out of the 12 cows slaughtered from the FTAI group, and these animals formed the Pregnant group (n= 6, Figure 12). Six out of the 11 sham-inseminated cows that followed the analyzed variables described in Table 2 formed the Non-Pregnant group (n=6). No differences were found on diameter POF size, weight, and diameter of the CL as well as serum concentration of P4 and E2 between the two groups, ensuring that these variables did not interfere with our results.

Figure 12 - Recovered embryos. Recovery rate and embryo stages recovered in six out of the 12 cows slaughtered from the FTAI group.

Source: own authorship.

Table 2 - Variables analyzed for group formation. Values are expressed as means $\pm$ SEM.

Variables	Group		P-value (T test)
	Non-pregnant (n=6)	Pregnant (n=6)	
Pre-ovulatory follicle (mm)	13.30 $\pm$ 1.19	13.08 $\pm$ 2.53	0.8546
Corpus luteum weight (g)	1.40 $\pm$ 0.27	1.36 $\pm$ 0.50	0.8492
Corpus luteum diameter (cm)	1.57 $\pm$ 0.08	1.45 $\pm$ 0.26	0.3330
Serum progesterone concentration (ng/mL)	3.96 $\pm$ 1.17	3.82 $\pm$ 1.90	0.8757
Serum estrogen concentration (pg/mL)	25.07 $\pm$ 2.32	26.52 $\pm$ 7.92	0.6824

Source: MAZZARELLA, R. et al. Changes in Oviductal Cells and Small Extracellular Vesicles miRNAs in Pregnant Cows. **Frontiers in Veterinary Science**, v. 8, n. March, p. 1–14, 4 mar. 2021.

6.2. Isolation of OF-sEVs

Initially, the efficiency of the sEVs isolation protocol was confirmed using OF-sEVs obtained as described in the Appendix B. TEM was used to analyze the morphology and size of the isolated OF-sEVs. Throughout the images, we were able to identify cup-shaped particles with characteristic sizes resembling sEVs (FIGURE APPENDIX B). Therefore, the isolation protocol was adequate for the isolation of OF-sEVs.

6.3. Nanoparticle tracking analysis

In order to analyze particle concentration and mode size of sEVs, we performed NTA using OF-sEVs isolated from non-pregnant (n=6) and pregnant cows (n=6). No differences were identified in mean particle concentration (OF-sEV/Non-pregnant: $4.3 \times 10^9 \pm 2.3 \times 10^9$ particles/mL; OF-sEV/Pregnant: $3.5 \times 10^9 \pm 1.2 \times 10^9$ particles/mL; Figure 13A) or mean mode size (OF-sEV/Non-pregnant: 140.7 ± 10.1 nm; OF-sEV/Pregnant: 135.4 ± 11.6 nm; Figure 13B) between the groups. From this result, we concluded that the pregnancy does not affect concentration and size of sEVs in the oviductal flushing.

Figure 13 - Characterization of OF-sEVs by nanoparticle analysis. Nanoparticle tracking analysis showing no difference in particles concentration (A) and mode size (B) between the groups OF-sEV/Non-pregnant (n=6) and OF-sEV/Pregnant (n=6). Error bars represents SEM.

Source: MAZZARELLA, R. et al. Changes in Oviductal Cells and Small Extracellular Vesicles miRNAs in Pregnant Cows. **Frontiers in Veterinary Science**, v. 8, n. March, p. 1–14, 4 mar. 2021.

6.4. MiRNA profile of OF-sEVs and bioinformatics analyses

To analyze the miRNAs profile, the relative expression levels of 383 miRNAs were investigated in OF-sEVs obtained from six oviducts from pregnant and six oviducts non-pregnant cows. The presence of 248 and 205 miRNAs were identified in OF-sEVs isolated from the non-pregnant and pregnant group, respectively. Of these, 192 were commonly detected between the two groups, 56 were exclusive to OF-sEV/Non-pregnant, and 13 were exclusive to OF-sEV/Pregnant (for CT levels, see APPENDIX 3). Among the 192 miRNAs detected in both groups, 8 miRNAs (bta-miR-126-5p, bta-miR-129, bta-miR-140, bta-miR-188, bta-miR-219, bta-miR-345-3p, bta-miR-4523 and bta-miR-760-3p) were up-regulated in OF-sEV/Pregnant and one miRNA (bta-miR-331-5p) was up-regulated in OF-sEV/Non-pregnant ($p < 0.05$; Figure 14).

Figure 14 - MiRNAs profile of OF-sEVs isolated from non-pregnant and pregnant cows. Venn diagram representing the 261 miRNAs detected: 192 in common between the two groups, 56 exclusives to OF-sEV/Non-pregnant and 13 exclusive to OF-sEV/Pregnant. Among the 192 miRNAs detected in both groups, 8 miRNAs (bta-miR-126-5p, bta-miR-129, bta-miR-140, bta-miR-188, bta-miR-219, bta-miR-345-3p, bta-miR-4523 and bta-miR-760-3p) were up-regulated in OF-sEV/Pregnant and one miRNA (bta-miR-331-5p) was up-regulated in OF-sEV/Non-pregnant. The asterisk (*) indicates miRNAs with relative expression significantly different ($p < 0.05$) within the groups. Samples size were $n=6$ for both groups. Error bars represent SEM.

Source: MAZZARELLA, R. et al. Changes in Oviductal Cells and Small Extracellular Vesicles miRNAs in Pregnant Cows. **Frontiers in Veterinary Science**, v. 8, n. March, p. 1–14, 4 mar. 2021.

To investigate the biological functions of miRNAs differently expressed in OF-sEVs, we performed bioinformatics analysis. All the analyses were performed with up-regulated miRNAs. Based on their potential relevance for the oviductal milieu, 21 pathways were predicted to be modulated by the miRNA up-regulated in OF-sEVs from the non-pregnant group (Figure 15A), and 41 pathways predicted to be modulated by the miRNAs up-regulated in OF-sEVs from the pregnant group (Figure 15B) were

selected. Our results demonstrated that differentially expressed OF-sEVs miRNAs modulate different pathways, such as PI3K-Akt signaling pathway, MAPK signaling pathway, and mTOR signaling pathway. For all identified pathways ($p < 0.05$), see APPENDIX D e E.

Figure 15 - Selected biological pathways predicted to be regulated by miRNAs with increased expression in OF-sEVs. (A) Pathways predicted as regulated by the miRNA (bta-miR-331-5p) up-regulated in OF-sEV/Non-pregnant. (B) Pathways predicted as regulated by the 8 miRNAs (bta-miR-126-5p, bta-miR-129, bta-miR-140, bta-miR-188, bta-miR-219, bta-miR-345-3p, bta-miR-4523 and bta-miR-760-3p) up-regulated in OF-sEV/Pregnant. Pathways were selected based on the potential relevance for oviductal functions and $p < 0.05$. Underlined pathways are modulated only in the group that they are present.

Source: MAZZARELLA, R. et al. Changes in Oviductal Cells and Small Extracellular Vesicles miRNAs in Pregnant Cows. **Frontiers in Veterinary Science**, v. 8, n. March, p. 1–14, 4 mar. 2021.

6.5. MiRNA contents of OECs and bioinformatics analyses

Next, we evaluated the levels of 383 miRNAs in OECs from the non-pregnant group (OEC/Non-pregnant) and the pregnant group (OEC/Pregnant). We identified a total of 355 and 353 miRNAs in OEC/Non-pregnant and OEC/Pregnant, respectively (for CT levels, see APPENDIX F). Of these, 345 miRNAs were commonly detected between the two groups, 10 were exclusive to OEC/Non-pregnant, and 8 were exclusive to OEC/Pregnant. Among the 345 miRNAs detected in both groups, 6 miRNAs (bta-miR-133b, bta-miR-205, bta-miR-584, bta-miR-551a, bta-miR-1193, and bta-miR-1225-3p) were up-regulated in OEC/Non-pregnant, and none was up-regulated in OEC/Pregnant group and ($p < 0.05$; Figure 16). The prediction of biological functions modulated by miRNAs differently detected in OECs demonstrated 37 selected pathways predicted as regulated by 6 miRNAs (bta-miR-133b, bta-miR-205, bta-miR-584, bta-miR-551a, bta-miR-1193, and bta-miR-1225-3p) up-regulated in OECs from non-pregnant compared with pregnant cows (Figure 17). Our results suggest that differentially expressed OECs miRNAs modulate pathways such as chemokine, oxytocin, and TNF signaling pathways. For all identified pathways ($p < 0.05$), see APPENDIX G.

Figure 16 - MiRNAs profile of OECs from non-pregnant and pregnant cows. Venn diagram representing the detection of 363 miRNAs: 345 common between the two groups, 10 exclusives to OEC/Non-pregnant and 8 exclusive to OEC/Pregnant. Among the 345 miRNAs detected in both groups, 6 miRNAs (bta-miR-133b, bta-miR-205, bta-miR-584, bta-miR-551a, bta-miR-1193 and bta-miR-1225-3p) were up-regulated in OEC/Non-pregnant. The asterisk (*) indicates miRNAs with relative expression significantly different ($p < 0.05$) within the groups. Samples size were $n=6$ for both groups. Error bars represent SEM.

Source: MAZZARELLA, R. et al. Changes in Oviductal Cells and Small Extracellular Vesicles miRNAs in Pregnant Cows. **Frontiers in Veterinary Science**, v. 8, n. March, p. 1–14, 4 mar. 2021.

Figure 17 - Biological pathways predicted using up-regulated miRNAs in OECs. Pathways predicted as regulated by 6 miRNAs (bta-miR-133b, bta-miR-205, bta-miR-584, bta-miR-551a, bta-miR-1193 and bta-miR-1225-3p) up-regulated in OECs from non-pregnant compared with OECs from pregnant cows. The left Y-axis values represent the number of genes predicted as modulated by the miRNAs for the respective pathways. The right Y-axis represents the enrichment score ($-\log_{10}$ of the p value) for each pathway.

Source: MAZZARELLA, R. et al. Changes in Oviductal Cells and Small Extracellular Vesicles miRNAs in Pregnant Cows. **Frontiers in Veterinary Science**, v. 8, n. March, p. 1–14, 4 mar. 2021.

6.6. Relative transcripts levels for selected genes

To evaluate genes present in predicted pathways regulated by miRNAs differently expressed in OECs, we analyzed the relative levels of 14 mRNAs selected from inflammatory, immune response, and pregnancy recognition pathways, known to influence oviductal function during early pregnancy. Considering a significant difference of $p < 0.1$, the genes TGFB1 ($p = 0.029$), TNFR1 ($p = 0.096$) and PTGES2 ($p = 0.068$) were up-regulated in OECs from the pregnant group (Figure 18). Thus, our

results demonstrate that the presence of one single embryo can affect the gene expression of those pathways in OECs.

Figure 18 - Relative mRNA expression of selected genes in OECs from non-pregnant compared with pregnant cows. Relative mRNA expression of selected genes in OECs from non-pregnant compared with pregnant cows. Relative expression of up and downregulated mRNAs are shown as mean of $2^{-\Delta Ct} \pm$ SEM. One asterisk (*) indicates genes with $p < 0.05$ and two asterisks (**) indicates genes with $p < 0.1$ within the groups. Samples size were $n=6$ for both groups.

Source: MAZZARELLA, R. et al. Changes in Oviductal Cells and Small Extracellular Vesicles miRNAs in Pregnant Cows. **Frontiers in Veterinary Science**, v. 8, n. March, p. 1–14, 4 mar. 2021.

7. DISCUSSION

Here we identified the miRNA contents of OF-sEVs and OECs as well as selected genes in OECs from pregnant and non-pregnant cows. This study indicates that communication within the pregnant animals, mediated by sEVs, is potentially initiated in the oviduct. Although the passage of spermatozoa and the presence of a single embryo does not interfere with the OF-sEVs concentration, it modulates miRNAs' contents in both OF-sEVs and OECs, as well as the transcript levels of the selected genes *TGFB1*, *TNFR1*, and *PTGES2* in OECs.

Oviductal flushing samples from the isthmus of ipsilateral oviducts were collected and used for sEVs isolation. The validation of the OF-sEVs isolation was based on the Minimal information for studies of extracellular vesicles 2018 (MISEV 2018; 28). No differences were identified in mode size or mean particle concentration between the groups; additionally, our results showed a population of vesicles with the expected size for sEVs, <200nm (THÉRY et al., 2018). For instance, the passage of the spermatozoa and embryo presence do not induce changes in the oviductal secretion of sEVs, and the amount of sEVs secreted by the embryo were not enough to cause a detectable increase in the total amount of sEVs. However, we were able to identify miRNAs with distinct levels between the pregnant and non-pregnant groups in both OF-sEVs and OECs.

Pregnancy stimulated changes in the miRNA profile of oviductal sEVs. We identified eight miRNAs up-regulated in pregnant and one miRNA up-regulated in non-pregnant cows. The presence of sEVs in the bovine oviductal fluid and their functional effects on the embryo are already recognized (ALMIÑANA et al., 2017; LOPERA-VASQUEZ et al., 2016, 2017c). MiRNAs from oviductal EVs have been studied in bovines and murine across the estrous cycle, and in both models, it was showed a hormonal effect on the miRNAs in cyclic animals (ALMIÑANA et al., 2018; FERESHTEH et al., 2018). We showed that the passage of the spermatozoa and embryo presence could induce differential secretion of miRNAs in OF-sEVs, although the FTAI occurred 108 hours before the sample collection. Among the up-regulated miRNAs in the pregnant group, the bta-miR-140 was also detected across the bovine estrous cycle, without differences among the stages (ALMIÑANA et al., 2018). In the same study, the miRNAs bta-miR-129, bta-miR-188, bta-miR-219, bta-miR-345-3p,

bta-miR-4523, bta-miR-760-3p identified in our study as up-regulated in pregnant cows were not detected across the estrous cycle. Similarly, the bta-miR-331-5p, up-regulated in our study in the non-pregnant group, was not detected across the estrus cycle (ALMIÑANA et al., 2018). Thus, these differences in miRNA contents could be due to differences in the isolation protocol and mainly in the technique used for miRNAs analysis and not necessarily that these miRNAs originated from the embryo itself since they are also present in low levels in the non-pregnant cows.

Differentially expressed miRNAs in OF-sEVs modulate pathways such as PI3K/AKT, mTOR, and MAPK, which are predicted to be modulated by up-regulated miRNAs from OF-sEVs from the pregnant group. These pathways are related to transcription, translation, proliferation, growth, control of the cytoskeletal organization, metabolism, and survival (ENGELMAN; LUO; CANTLEY, 2006; ERSAHIN; TUNCBAG; CETIN-ATALAY, 2015; HANSEN; DENICOL; DOBBS, 2014; JOUSAN; HANSEN, 2007; YU; CUI, 2016). Previous studies have demonstrated that PI3K/mTOR signaling pathways are related to angiogenesis, an essential process in the female's reproductive system and essential for pregnancy establishment (KARAR; MAITY, 2011; RIZOV; ANDREEVA; DIMOVA, 2017). Additionally, PI3K/AKT pathway can mediate cell growth and survival in pre-implantation embryos. In early mouse embryos, PI3K-Akt subunits are expressed from zygote to blastocyst stage and are involved with blastomere proliferation (FIORENZA et al., 2020; LU et al., 2004). In bovine pre-implantation embryos, the activation PI3K signaling is also required for apoptosis control (JOUSAN; HANSEN, 2007). Together, these studies demonstrate that the presence and function of the PI3K/AKT/mTOR pathway are essential for embryo development. We suggest that the oviduct recognizes the embryo presence and through the miRNAs within sEVs from the oviductal fluid could influence embryo development in the oviduct, mediating maternal-embryonic communication during early embryo development. In order to test that, in future studies we aim to utilize oviductal sEVs collected from oviductal fluid from the pregnant group as a tool to modulate the embryo environment *in vitro*, as well as to study the effects of those sEVs on embryo quality and development.

Pregnancy also stimulated changes in the miRNA profile of OECs. Six miRNAs were up-regulated in OECs from the non-pregnant group (bta-miR-133b, bta-miR-205, bta-miR-584, bta-miR-551a, bta-miR-1193 and bta-miR-1225-3p) and none in the

pregnant group. It is well known that sex-steroid hormones, E2 and P4, coordinate changes in the oviductal transcriptome and secretory function across the estrous cycle (BAUERSACHS et al., 2003, 2004; BUHI; ALVAREZ; KOUBA, 2000; LAMY et al., 2016). Also, considering day 4 of the estrous cycle, the POF and CL size potentially affect the oviductal environment (GONELLA-DIAZA et al., 2015). In our results, no differences were found in POF and CL size as well as no differences in E2 and P4 between our groups, ensuring that they did not interfere with our results. The miRNAs bta-miR-133b, bta-miR-205, bta-miR-584, bta-miR-551a, identified in our study as up-regulated in non-pregnant cows, were already detected on bovine OECs exposed to low and high E2/P4 levels without the presence of the embryo, but with no differences in transcript levels (GONELLA-DIAZA et al., 2021). Thus, these miRNAs are not modulated by steroid hormones, supporting our data indicating that the passage of the spermatozoa and embryo presence can induce changes in miRNAs within oviductal cells in the same stage of the estrous cycle. Taken together, our results suggest that the passage of the spermatozoa and presence of an embryo within the oviduct can modulate the oviductal microenvironment, specifically the contents of miRNAs in OF-sEVs and OECs.

Based on bioinformatics analysis, we decided to evaluate the modulation of key-genes transcripts involved in the immune system, inflammation, and pregnancy recognition on OECs. Our study suggests that pregnancy stimulates changes in *TGFB1*, *PTGES2*, and *TNFR1* levels. *TGFB1* is an anti-inflammatory cytokine present in OECs throughout the estrous cycle and crucial for tolerance to non-self-cells (YOUSEF et al., 2016). Increased expression level of *TGFB1*, as found in our pregnant group, is also present in bovine OECs cultured with embryos *in vitro* (TALUKDER et al., 2018). However, it cannot be disregarded that the increase in *TGFB1* levels is also related to sperm binding and induction of sperm-tolerance (MORILLO et al., 2020; YOUSEF et al., 2016).

Regarding the sperm effect on the oviductal milieu it is worth mentioning that there is no consensus on the exact period of sperm permanence in the female reproductive tract. Competent sperm arrives in the oviducts 6 to 8 h after insemination in sheep and cattle (HUNTER, 1988). By 12 to 24 h after artificial insemination, most of the spermatozoa is lost, and only a few percentages persist in the reproductive tract (HAWK, 1987). In cattle, the sperm may spend up to 22 h in the isthmus reservoir

(HUNTER; WILMUT, 1984) and remains in the cow's reproductive tract for at least 48 to 72 h after insemination (HAWK, 1987). Importantly, our study used frozen-thawed semen, which has a detrimental effect on the number of sperm that reaches oviducts and also on sperm viability (HAWK, 1983; SHANNON; VISHWANATH, 1995). Considering that our animal was slaughtered 108h after insemination, at the time of our sample collection, the presence of sperm was probably none or extremely low. Additionally, the gene expression from mRNA transcription to protein production occurs within a few hours (BUCCITELLI; SELBACH, 2020). Thus, the effect of the sperm at the time of slaughter may be only residual, while the presence of the embryo had enough time to generate changes in the transitional profile of the oviduct. Nevertheless, YOUSEF et al. (2016) showed that the sperm-bovine OEC binding *in vitro* modulates the *TGFB1* and also the *IL1B* expression. In our results, even with the sperm presence, we did not detect a difference in *IL1B* expression, supporting that the sperm effect was minimal at the time of sampling collection.

Synergistically with *TGFB1*, the *PTGES2*, up-regulated in our pregnant group, participates in the eicosanoid metabolic process that will drive the production of PGE2 (WANG; DEY, 2005), promoting immune tolerance in the bovine oviduct (BARATELLI et al., 2005; TALUKDER et al., 2018). Talukder et al. (2018) and Maillo et al. (2015) suggested that the embryo generates an anti-inflammatory response by suppressing key factors, such as NFkB2 and NFkBIA. The activation of NFkB transcriptional responses can be triggered by TNFR1 (TING; BERTRAND, 2016). In our results, *TNFR1* was increased in OECs from the pregnant cows. The TNFR1 is the receptor for the pro-inflammatory cytokines TNF- α . However, this cytokine also stimulates the productions of contractive substances, such as prostaglandins, regulating gametes transport, and ensuring the embryo migration into the uterus (WIJAYAGUNAWARDANE; MIYAMOTO, 2004). Thus, it is imperative the adequate modulation of *TNFR1*, and among the modulators of the inflammatory responses of mammals are miRNAs (IBRAHIM et al., 2015). We identified a classic relationship between miR-584 and its predicted target TNFR1 in OECs. The bta-miR-584 was down-regulated in OECs from the pregnant group, while TNFR1 is increased in this group, while the opposite was observed in the non-pregnant group. Our results suggest that miRNAs may be decisive for the modulation of pro- and anti-inflammatory cytokines, leading to a proper immune response. Regarding genes involved in

pregnancy recognition, no differences were identified between OECs groups. These results can be explained as the OECs from the pregnant group were exposed to embryos between 4 and 16 cells stage, and not morulas or blastocysts. Thereby, the interferon tau mechanism might not be initiated during this stage or it causes a local effect that was diluted when the OECs were collected from the entire isthmus portion. Similar results were also found *in vitro*, where the presence of day-04 embryo did not influence the expression of interferon-stimulated genes (such as *ISG15*, *OAS1*, *MX2*) and *IFNAR2* on bovine OECs (TALUKDER et al., 2018). Taken together, pregnancy can dynamically modulate the immune system and inflammation response as early as day 4, affecting the oviductal environment and possibly embryo survival and development.

In this study, we identify the modulation of miRNAs and mRNAs in the oviduct. However, some limitations should be noticed since our experimental model cannot determine if differently expressed miRNAs are a direct or indirect effect of the spermatozoa or the embryo. Nevertheless, previous studies have shown that the embryo has a local effect on both, the oviduct and the endometrium (MAILLO et al., 2015; SPONCHIADO et al., 2017, 2020). Additionally, the miRNAs up-regulated in OF-sEVs from the pregnant group are potentially secreted by the embryo, by the oviduct as a result of the embryonic stimulus, or from both. Although the ideal model to isolate the embryo effect is to transfer embryos directly into the oviduct (MAILLO et al., 2015), we must not disregard that the physiological environment is dynamic and depends on a set of synchronous factors and signals that together will guarantee the success of the pregnancy (LI; WINUTHAYANON, 2017). Therefore, we chose not to remove these factors or to amplify the embryo's signal by transferring a large number of embryos as it would not represent the physiological response of the oviductal milieu. Additionally, future approaches should consider a larger number of animals and also small RNA sequencing analysis for a more comprehensive understanding of the expression and regulation of miRNAs associated with pregnancy in OECs and sEVs.

8. CONCLUSION

In conclusion, our results demonstrate that pregnancy can modulate the miRNA contents of sEVs as well as the miRNAs and mRNAs levels in OECs in cows. The functional enrichment of miRNAs highlights pathways involved in physiological processes such as inflammation, cell proliferation, and immune response, crucial for both the reproductive tract and embryo development. Furthermore, our results confirm *in vivo* that the embryo-maternal crosstalk starts within the oviduct, enhancing oviductal receptivity, allowing proper fertilization and early embryo development, thus leading to a successful pregnancy. Importantly, unveiling the sEVs contents and their effects is crucial for developing biomarkers, new strategies for *in vitro* embryo production, and therapies that could assist human and animal reproduction.

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APPENDIX A – Published research: MAZZARELLA, R. et al. Changes in Oviductal Cells and Small Extracellular Vesicles miRNAs in Pregnant Cows. *Frontiers in Veterinary Science*, v. 8, n. March, p. 1–14, 4 mar. 2021.

Changes in Oviductal Cells and Small Extracellular Vesicles miRNAs in Pregnant Cows

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Early embryonic development occurs in the oviduct, where an ideal microenvironment is provided by the epithelial cells and by the oviductal fluid produced by these cells. The oviductal fluid contains small extracellular vesicles (sEVs), which through their contents, including microRNAs (miRNAs), can ensure proper cell communication between the mother and the embryo. However, little is known about the modulation of miRNAs within oviductal epithelial cells (OECs) and sEVs from the oviductal fluid in pregnant cows. In this study, we evaluate the miRNAs profile in sEVs from the oviductal flushing (OF-sEVs) and OECs from pregnant cows compared to non-pregnant, at 120 h after ovulation induction. In OF-sEVs, eight miRNAs (bta-miR-126-5p, bta-miR-129, bta-miR-140, bta-miR-188, bta-miR-219, bta-miR-345-3p, bta-miR-4523, and bta-miR-760-3p) were up-regulated in pregnant and one miRNA (bta-miR-331-5p) was up-regulated in non-pregnant cows. In OECs, six miRNAs (bta-miR-133b, bta-miR-205, bta-miR-584, bta-miR-551a, bta-miR-1193, and bta-miR-1225-3p) were up-regulated in non-pregnant and none was up-regulated in pregnant cows. Our results suggest that embryonic maternal communication mediated by sEVs initiates in the oviduct, and the passage of gametes and the embryo presence modulate miRNAs contents of sEVs and OECs. Furthermore, we demonstrated the transcriptional levels modulation of selected genes in OECs in pregnant cows. Therefore, the embryonic-maternal crosstalk potentially begins during early embryonic development in the oviduct through the modulation of miRNAs in OECs and sEVs in pregnant cows.

Keywords: small extracellular vesicles, miRNAs, oviductal cells, oviductal fluid, embryo maternal-communication, reproduction, bovine

INTRODUCTION

The oviduct provides the ideal microenvironment for early embryonic development (1, 2). The bovine embryo develops in the oviduct for 4 days, staying in contact with oviductal epithelial cells (OECs) as well as its secretions, the oviductal fluid (3). The oviductal epithelium is composed of ciliated cells and secretory cells that produce the oviductal fluid (4). The oviductal

fluid is composed of simple and complex carbohydrates, proteins, ions, inorganic salts, lipids, phospholipids, small metabolites, and vesicles (4, 5). Changes in this fluid and the oviductal cells are regulated by different factors, such as the oviduct region (ampulla or isthmus), the ipsilateral or contralateral side to the corpus luteum (6–8), and the concentration of steroid hormones (9, 10).

The oviductal fluid and OECs are also affected by the presence of gametes and embryos. Different studies have demonstrated changes in gene expression patterns of bovine OECs co-cultured with embryos (11, 12). *In vivo*, despite no differences in OECs gene expression were found using microarray analysis in the presence of a single embryo in artificially inseminated heifers compared to cyclic heifers by Maillou et al. (13) or using target genes analysis by Rodríguez-Alonso et al. (14), the same group identified differentially expressed genes by RNA-seq when multiples embryos were transferred (13). In the oviductal fluid, different proteins were described indicating that the fluid composition could be influenced by the presence of gametes (15). Recently, Rodríguez-Alonso et al. (16) demonstrated that the oviductal fluid presents a different composition of carbohydrates, proteins, and amino acids in pregnant cows (artificially inseminated) when compared to cyclic cows (non-breed). Thus, it is possible that other components within the oviductal environment, such as extracellular vesicles present in the oviductal fluid, can also be modulated in pregnant animals.

EVs are nanoparticles secreted by cells in the extracellular environment (17) and contain bioactive materials, such as proteins (18), lipids (19), mRNAs, and non-coding RNAs, including microRNAs (miRNAs) (20, 21). EVs are classified as exosomes, microvesicles, and apoptotic bodies according to their size, biogenesis, and secretion (22). However, due to the difficulty to establish the specific origin of these EVs, they can be classified according to their size as small EVs (sEVs; <200 nm) or large EVs (lEVs; >200 nm) (23). In the oviduct, EVs have been identified in the oviductal fluid and are potentially related to the maternal crosstalk with gametes and embryos (24–28). Functionally, both sEVs originating from the oviductal fluid and bovine OECs conditioned culture medium are internalized by bovine embryos and improve *in vitro* embryo development and quality (29–31). However, the protein profile of sEVs that originated from OECs *in vivo* is different from the sEVs produced by OECs *in vitro* (31). Lopera-Vasquez et al. (29) demonstrated that sEVs from the isthmus have a better effect on *in vitro* embryo quality than sEVs from the ampulla. Although they have not studied the content of these sEVs, it can be inferred that the contents from the isthmus are somehow different and better for the embryo. The molecular contents of oviductal sEVs (RNA, protein, and small ncRNA, including miRNAs) are also regulated by steroid hormones across the bovine estrous cycle (32). To our knowledge, there are no studies on how the sEVs miRNA contents are modulated in pregnant cows.

miRNAs are small non-coding molecules (~22 nucleotides) that act in post-transcriptional regulation (33, 34). miRNAs are transcribed as primary miRNAs (pri-miRNAs), which are processed in precursor miRNA (pre-miRNA) and then transferred to the cytoplasm for further processing into mature miRNAs sequences (35). In mammals, the partial complementary

base pairing miRNA-mRNA can induce transcription inhibition, thus regulating protein levels (36). miRNAs are involved in different reproductive events, such as spermatogenesis (37), follicular development (38, 39), oocyte maturation (40, 41), and embryo development (42). To our knowledge, there are no studies on miRNAs regulation and function in OECs from pregnant cows. miRNAs are also involved in cell-to-cell communication through sEVs (43). miRNAs within sEVs may play a role in modulating embryo-maternal communication during early pregnancy (44, 45). EVs can mediate the delivery of its miRNAs contents into embryos, a phenomenon already implicated in embryo development and maternal-embryonic communication (45–48). However, there is no evidence of the effects on miRNA contents of the oviductal sEVs from pregnant cows during the first embryo cleavages at the oviduct.

Therefore, the modulation and function of miRNAs within oviductal epithelial cells and EVs from the oviductal fluid in early pregnant cows is still elusive. Thus, we hypothesize that the miRNAs contents in small extracellular vesicles from the oviductal flushing (OF-sEVs) and in oviductal epithelial cells (OECs) undergo differential modulations in pregnant cows. The present study evaluated the profile of 383 miRNAs in OF-sEVs and OECs in artificially inseminated (pregnant group) and in sham-inseminated (non-pregnant group) cows. Additionally, we access in OECs genes known to influence the immune system, inflammation, and early pregnancy development. To our knowledge, this is the first study to characterize the changes in miRNA profile from both OF-sEVs and OECs obtained from pregnant and non-pregnant cows. Additionally, functional enrichment analysis was performed to evaluate the predicted pathways related to differentially expressed miRNAs between pregnant and non-pregnant cows.

MATERIALS AND METHODS

All the reagents were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO) unless otherwise stated. The present study was approved by the University of São Paulo Research Ethics Committee (protocol number: 4909010817).

Animal Model

This study was conducted at the University of São Paulo (campus of Pirassununga, SP, Brazil) with Nelore (*Bos indicus*) multiparous cows with a body condition score 4 (0-emaciated; 5-obese) according to Ayres et al. (49). Cows were fed with corn silage, supplemented with mineral salt, and free access to potable water. In order to obtain the oviducts exposed or not exposed to embryos, 25 cows were submitted to estrous synchronization protocol. As described by Gonella-Díaz (50) with minor modifications: on the first day, all the cows received 2 mg of estradiol benzoate (2 ml, i.m., Sincrodiol, Ourofino Saúde Animal, Brazil), 0.53 mg of sodium cloprostenol (2 ml, i.m., Sincrocio, Ourofino Saúde Animal, Brazil) and an intravaginal P4-releasing device (1 g; Sincrogest®, Ourofino Saúde Animal, Brazil). On day 7, the P4 device was removed, and cows received 0.53 mg of sodium cloprostenol (2 ml, i.m., Sincrocio, Ourofino Saúde Animal, Brazil). On day 9, cows received 0.01 mg of

gonadotrophin releasing hormone (GnRH) analog (2.5 ml, i.m., Sincroforte, Ourofino Saúde Animal, Brazil). Twelve hours after GnRH analog application, cows were randomly assigned into two groups: fixed-time artificial inseminated (FTAI, $n = 14$) or sham-inseminated (control, $n = 11$). In the group FTAI, cows were artificially inseminated with frozen-thawed semen from the same bull of proved fertility. In the control group, cows were sham-inseminated with deposition of depleted semen extender in the uterine tract. For sperm depletion, straws from the same bull used in the FTAI group were thawed, centrifuged at $4,000 \times g$ for 15 min, and the supernatant was filled into a new straw. The ovulation time was controlled by ultrasound (MyLab Delta, Esaote, Italy) and only animals that had an ovulated follicle between 24 and 36 h after GnRH analog application were kept in this study. All cows were slaughtered about 120 h after ovulation induction with GnRH at the slaughterhouse at University of São Paulo (campus of Pirassununga/SP, Brazil).

Sample Collection and Groups Formation

Reproductive tracts were processed within 30 min after slaughter. Oviducts ipsilateral to the corpus luteum (CL) were dissected and separated from the utero-tubal junction. Next, oviducts were divided in ampulla and isthmus through the ampullary-isthmic junction, identified as the place where the oviductal diameter starts to decrease, as described by Maillo et al. (51) and Gonella-Díaz et al. (52). The isthmus was individually flushed with 1 ml of calcium and magnesium-free PBS with 0.1% of polyvinylpyrrolidone (PVP). The presence or absence of the embryo within the isthmus portion was used for group formation and confirmed in the oviductal flushing using a stereo microscope. After washing, epithelial cells of each isthmus were obtained by squeezing the tissue using a sterile glass slide and frozen in liquid nitrogen until further use.

In addition to the criterion of the presence or absence of the embryo, we also analyzed pre-ovulatory follicle (POF) diameter, weight, and diameter of the CL and serum concentration of progesterone (P4) and estrogen (E2). The POF diameter was measured by ultrasound (MyLab Delta, Esaote, Italy) on day 9 of the estrous synchronization protocol. For CL weight and size, CLs were dissected from the ovary, weighed, and their diameter was calculated as the average between two perpendicular axes measured. For serum concentration of P4 and E2, blood samples were collected from the jugular vein in serum blood collection tubes (BD, São Paulo/SP, Brazil) at the time of slaughter. The serum was separated by centrifugation at $1,500 \times g$ for 30 min, stored at -20°C , and the measurement of E2 and P4 was performed using chemiluminescence immunoassay (ADVIA Centaur®-Siemens) at Pasin Laboratory (Santa Maria/RS, Brazil).

Isolation of OF-sEVs

OF-sEVs were obtained from the isthmus of ipsilateral oviducts from pregnant and non-pregnant groups according to the isolation protocol previously used by da Silveira et al. (53), Almiñana et al. (32), and Ávila et al. (54). Briefly, the oviductal flushing was centrifuged at $300 \times g$ for 10 min to remove live cells, $2,000 \times g$ for 10 min to remove cellular debris, $16,500$

$\times g$ for 30 min to remove large vesicles, and the remaining supernatant was placed at -80°C until further use. The supernatant was filtered through a $0.20 \mu\text{m}$ sterile syringe filter (PES membrane; Corning) in order to remove any remaining large EVs. For sEVs isolation, filtered oviductal flushing was centrifuged twice at $119,700 \times g$ for 70 min at 4°C (Optima XE-90 Ultracentrifuge; rotor 70 Ti; Beckman Coulter). After this procedure, the supernatant was discarded, and the sEVs pellets were resuspended in $20 \mu\text{L}$ of phosphate-buffered saline ($1 \times \text{Ca}^{2+}/\text{Mg}^{2+}$ free PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 2 mM KH_2PO_4) and used for further analysis. Due to the small volume of oviductal flushing obtained from a single animal, the protocol to isolate OF-sEVs was validated using female reproductive tracts obtained from slaughterhouses, as described in **Supplementary Data 1**.

Nanoparticle Tracking Analysis

The OF-sEVs isolated from pregnant and non-pregnant groups were analyzed based on particle mode size and concentration by nanoparticle tracking analysis (NTA). OF-sEVs isolated from $200 \mu\text{L}$ of each oviductal flushing were individually centrifuged, and their pellets were resuspended in $20 \mu\text{L}$ of $1 \times \text{Ca}^{2+}/\text{Mg}^{2+}$ free PBS. As described by Ávila et al. (54), with minimal modifications, particle size, and concentration were measured by nanoparticle tracking analysis using Nanosight (NS300; Malvern). Briefly, for this analysis, the dilution factor used was 1:100 in $1 \times \text{Ca}^{2+}/\text{Mg}^{2+}$ free PBS. Five videos of 30 s were taken for each sample, captured by a scientific complementary metal-oxide-semiconductor (sCMOS) camera at camera level 13 and under a controlled temperature of 38.5°C . Considering the threshold level of 5, these captured images were tracked by the NanoSight NTA 3.4 Analytical Software (NTA 3.4 Build 3.4.003; Malvern) that measures and provides information about particle size and concentrations of each sample.

Total RNA Extraction, Reverse Transcription, and Real-Time PCR

Total RNA, including miRNAs, from OF-sEVs and OECs was extracted according to the TRIzol reagent (Thermo Fisher Scientific) manufacturer's instruction and with the addition of $1.33 \mu\text{L}$ the coprecipitator GlycoBlue (Thermo Fisher Scientific) to the aqueous phase before RNA precipitation, as previously described by Da Silveira et al. (55) and Gonella-Díaz et al. (56) with minimal modifications. Total RNA concentrations were analyzed using spectrometry (NanoDrop 2000, Thermo Fisher Scientific). The RNA was treated with DNaseI (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions.

miRNA analysis of OF-sEVs and OECs were performed as described in Da Silveira et al. (55). Briefly, for cDNA synthesis, reverse transcription was performed with the commercial miScript II RT kit (QIAGEN), using the MiScript HiFlex Buffer and 200 ng of total RNA. For quantitative RT-PCR, the miScript SYBR® Green PCR Kit (QIAGEN) was used according to the manufacturer's instructions. The relative levels of 383 miRNAs were evaluated for each sample. Three hundred eighty four-well plates were assembled using a multichannel electronic pipetting system and analyzed by RT-PCR with QuantStudio

6 Flex (Applied Biosystems). miRNAs were considered present when cycle threshold (CT) was <37 in at least three out of six biological repetitions with an adequate melting curve. For both OF-sEVs and OECs, the CT values were normalized using the geometric mean CT of the endogenous genes Hm/Ms/Rt U1 snRNA and bta-miR-99b (57). The relative expression values were calculated using the ΔCt method, and the normalized data were transformed by $2^{-\Delta\text{Ct}}$ for graphical representation of the relative transcript levels (58).

Regarding mRNAs analysis of OECs, we evaluated the relative expression of 14 mRNAs selected from key pathways known to influence oviductal function and two endogenous controls (Table 1) for each sample. Briefly, reverse transcription reaction was performed with 30 ng of total RNA per gene using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), according to the manufacturer's protocol. For

quantitative RT-PCR, the GoTaq[®] qPCR Master Mix (Promega) was used according to the manufacturer's instructions and using the QuantStudio 6 Flex (Applied Biosystems) with the following PCR cycle conditions: 95°C for 10 min, 45 cycles of 95°C for 15 s, and 60°C for 60 s. The amplification of single cDNA products was confirmed by melt curve analysis. CT values were normalized using the geometric mean CT of the endogenous genes PPIA and GAPDH, as previously described for similar tissues (50). The relative expression values were calculated using the ΔCt method, and the normalized data were transformed by $2^{-\Delta\text{Ct}}$ for graphical representation of the relative transcript levels (58).

Statistical and Bioinformatics Analysis

The data obtained were tested for outliers' presence, normality by Shapiro-Wilk test, and the normalized RT-PCR data were analyzed by the Student's *t*-test. For all analyzes, $p < 0.05$

TABLE 1 | Primer sequences of genes analyzed by real-time PCR.

Key function	Gene symbol	Gene name	Primer sequences (5'-3')	GenBank number	References
Inflammatory and immune response	<i>IL1B</i>	Interleukin 1 Beta	F: TCCAGCCAACCTTCATTGG R: ACAGCTCATTCTCGTCACTGTAGTAAG	NM_174093.1	LFEM*
	<i>IL2RA</i>	Interleukin 2 Receptor Subunit Alpha	F: CTGCTCATATCCCCAAAGG R: CATGGGTTTGCTTGCTGCTT	NM_174358.2	LFEM*
	<i>IL6R</i>	Interleukin 6 Receptor	F: ACCACCAAGGCCGTGTTACT R: GCGACACACAGGACAATA	NM_001110785.1	(53)
	<i>TAB1</i>	TGF- β -Activated Kinase 1-Binding Protein 1	F: GACGCGCTGGCTGAGAAG R: GCCTTGAGTCTTTCGAGGATCTT	NM_001102057.1	(59)
	<i>TGFB1</i>	Transforming Growth Factor Beta 1	F: CCGTCTGAGGCTCAAGTTAAAG R: GCGACTGCGGCACAACTC	NM_001168068.1	(60)
	<i>TNFR1</i>	Tumor Necrosis Factor Receptor Type 1	F: AGGACCCAGGCACTACAGTACTATTAC R: CACCGCTGGTAGCGACATG	NM_174674.1	(59)
	<i>RGS2</i>	Regulator Of G Protein Signaling 2	F: CCTGAACACCGCAGGCTGAAT R: GAACCCCATGCCACATGAGA	NM_001075596.1	LFEM*
	<i>PTGES2</i>	Prostaglandin E synthase 2	F: GTGGGCGGACGACTGGTTGG R: CGGAGGTGGTCCCTGCCGTTT	NM_001168554.1	(61)
	<i>PTGES3</i>	Prostaglandin E synthase 3	F: CAGTCATGGCCAAGGTTAACAAA R: ATCACCACCCATGTTGTTATC	NM_001007806.2	(61)
Pregnancy recognition	<i>IFNAR2</i>	Interferon (alpha, beta and omega) receptor 2	F: CTGGTCATTGTATGGGCTCTT R: GTATCCCGGACTGTGCAATT	NM_174553.2	(62)
	<i>MX1</i>	MX dynamin-like GTPase 1	F: GTACGAGCCGAGTTCCTCAA R: ATGTCCACAGCAGGCTCTTC	NM_173940.2	(63)
	<i>MX2</i>	MX dynamin-like GTPase 2	F: GTTCAGAGACGCTCAGTGG R: TGAAGCAGCCAGGAATAGT	NM_173941	(63)
	<i>OAS1Y</i>	2',5'-oligoadenylate synthetase 1	F: GCGGACCCTACAGGAATGT R: TGTCTTGGGGCGACACATC	NM_001040606.1	LFEM*
	<i>ISG15</i>	ISG15 ubiquitin-like modifier	F: AGAGAGCCTGGCACCAGAAC R: TTCTGGCGATGAAGTGGT	NM_174366.1	(62)
Endogenous control	<i>PPIA</i>	Peptidylprolyl isomerase A	F: GCGATGGAGCGCTTTGG R: CCACAGTCAGCAATGGTGATCT	NM_178320.2	(64)
	<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	F: GCGATCAATGACCCCTTCAT R: TGCCGTGGGTGGAATCA	NM_001034034.2	(64)

Gene selected from key pathways known to influence oviductal function and endogenous controls. F, Forward and R, Reverse.

*Provided by Molecular Endocrinology Physiology Laboratory (LFEM-FMVZ-USP).

was considered significant unless otherwise stated. Statistical analyses were performed with JMP 14 (SAS Institute Inc.). For bioinformatics analysis and identification of biological pathways, the miRNAs differently detected within the groups were evaluated by miRWalk 2.0 bovine database (65). MiRWalk is an online database that, through their TarPmiR algorithm, allowed the searching of validated and predicted interactions between miRNAs and gene sequences. Then, the biological pathways predicted as modulated by these miRNAs of interest were identified through the gene set enrichment analysis (GSEA) function within the MiRWalk platform, assessing the geneset “KEGG Pathways.” Only pathways with a *p*-value lower than 0.05 were considered as significant.

RESULTS

Sample Collection and Groups Formation

Embryos were found in six out of the 14 cows in FTAI group, and these samples formed the Pregnant group (*n* = 6). Six out of the 11 sham-inseminated cows that followed the analyzed variables described in Table 2 formed the Non-Pregnant group (*n* = 6). No differences were found on diameter POF size, weight, and diameter of the CL as well as serum concentration of P4 and E2 between the two groups, ensuring that these variables did not interfere with our results.

Isolation of OF-sEVs

Initially, the efficiency of the sEVs isolation protocol was confirmed using OF-sEVs obtained as described in the Supplementary Data 1. TEM was used to analyze the morphology and size of the isolated OF-sEVs. Throughout the images, we were able to identify cup-shaped particles with characteristic sizes resembling sEVs (Supplementary Figure 1). Therefore, the isolation protocol was adequate for the isolation of OF-sEVs.

Nanoparticle Tracking Analysis

In order to analyze particle concentration and mode size of sEVs, we performed NTA using OF-sEVs isolated from non-pregnant and pregnant cows. No differences were identified in mean particle concentration (OF-sEV/Non-pregnant: $4.3 \times 10^9 \pm 2.3 \times 10^9$ particles/mL; OF-sEV/Pregnant: $3.5 \times 10^9 \pm 1.2 \times$

10^9 particles/mL; Figure 1A) or mean mode size (OF-sEV/Non-pregnant: 140.7 ± 10.1 nm; OF-sEV/Pregnant: 135.4 ± 11.6 nm; Figure 1B) between the groups. From this result, we concluded that the pregnancy does not affect concentration and size of sEVs in the oviductal flushing.

miRNA Profile of OF-sEVs and Bioinformatics Analyses

To analyze the miRNAs profile, the relative expression levels of 383 miRNAs were investigated in OF-sEVs from six oviducts from pregnant and six oviducts non-pregnant cows. The presence of 248 and 205 miRNAs were identified in OF-sEVs isolated from the non-pregnant and pregnant group, respectively. Of these, 192 were commonly detected between the two groups, 56 were exclusive to OF-sEV/Non-pregnant, and 13 were exclusive to OF-sEV/Pregnant (for CT levels, see Supplementary Table 1). Among the 192 miRNAs detected in both groups, eight miRNAs (bta-miR-126-5p, bta-miR-129, bta-miR-140, bta-miR-188, bta-miR-219, bta-miR-345-3p, bta-miR-4523, and bta-miR-760-3p) were up-regulated in OF-sEV/Pregnant and one miRNA (bta-miR-331-5p) was up-regulated in OF-sEV/Non-pregnant (*p* < 0.05; Figure 2).

To investigate the biological functions of miRNAs differently expressed in OF-sEVs, we performed bioinformatics analysis. All the analyses were performed with up-regulated miRNAs. Based on their potential relevance for the oviductal milieu, 21 pathways were predicted to be modulated by the miRNA up-regulated in OF-sEVs from the non-pregnant group (Figure 3A), and 41 pathways predicted to be modulated by the miRNAs up-regulated in OF-sEVs from the pregnant group (Figure 3B) were selected. Our results demonstrated that differentially expressed OF-sEVs miRNAs modulate different pathways, such as PI3K-Akt signaling pathway, MAPK signaling pathway, and mTOR signaling pathway. For all identified pathways (*p* < 0.05), see Supplementary Tables 2, 3.

miRNA Contents of OECs and Bioinformatics Analyses

Next, we evaluated the levels of 383 miRNAs in OECs from the non-pregnant group (OEC/Non-pregnant) and the pregnant group (OEC/Pregnant). We identified a total of 355 and 353 miRNAs in OEC/Non-pregnant and OEC/Pregnant, respectively

TABLE 2 | Variables analyzed for group formation.

Variables	Group		P-value (t-test)
	Non-pregnant (<i>n</i> = 6)	Pregnant (<i>n</i> = 6)	
Pre-ovulatory follicle (mm)	13.30 ± 1.19	13.08 ± 2.53	0.8546
Corpus luteum weight (g)	1.40 ± 0.27	1.36 ± 0.50	0.8492
Corpus luteum diameter (cm)	1.57 ± 0.08	1.45 ± 0.26	0.3330
Serum progesterone concentration (ng/mL)	3.96 ± 1.17	3.82 ± 1.90	0.8757
Serum estrogen concentration (pg/mL)	25.07 ± 2.32	26.52 ± 7.92	0.6824

Values are expressed as means ± SEM.

(for CT levels, see **Supplementary Table 4**). Of these, 345 miRNAs were commonly detected between the two groups, 10 were exclusive to OEC/Non-pregnant, and eight were exclusive to OEC/Pregnant. Among the 345 miRNAs detected in both groups, six miRNAs (bta-miR-133b, bta-miR-205, bta-miR-584, bta-miR-551a, bta-miR-1193, and bta-miR-1225-3p) were up-regulated in OEC/Non-pregnant, and none was up-regulated in OEC/Pregnant group and ($p < 0.05$; **Figure 4**).

The prediction of biological functions modulated by miRNAs differently detected in OECs demonstrated 37 selected pathways predicted as regulated by six miRNAs (bta-miR-133b, bta-miR-205, bta-miR-584, bta-miR-551a, bta-miR-1193, and bta-miR-1225-3p) up-regulated in OECs from non-pregnant compared with pregnant cows (**Figure 5**). Our results suggest that differentially expressed OECs miRNAs modulate pathways, such as chemokine, oxytocin, and TNF signaling pathways. For all identified pathways ($p < 0.05$), see **Supplementary Table 5**.

Relative Transcripts Levels for Selected Genes

To evaluate genes present in predicted pathways regulated by miRNAs differently expressed in OECs, we analyzed the relative levels of 14 mRNAs selected from inflammatory, immune response, and pregnancy recognition pathways,

known to influence oviductal function during early pregnancy. Considering a significant difference of $p < 0.1$, the genes *TGFB1* ($p = 0.029$), *TNFR1* ($p = 0.096$), and *PTGES2* ($p = 0.068$) were up-regulated in OECs from the pregnant group (**Figure 6**). Thus, our results demonstrate that the presence of one single embryo can affect the gene expression of those pathways in OECs.

DISCUSSION

Here we identified the miRNA contents of OF-sEVs and OECs as well as selected genes in OECs from pregnant and non-pregnant cows. This study indicates that communication within the pregnant animals, mediated by sEVs, is potentially initiated in the oviduct and, although the passage of spermatozoa and presence of a single embryo does not interfere with the OF-sEVs concentration, it modulates the miRNAs' contents in both OF-sEVs and OECs, as well as the transcript levels of the selected genes *TGFB1*, *TNFR1*, and *PTGES2* in OECs.

Oviductal flushing samples from the isthmus of ipsilateral oviducts were collected and used for sEVs isolation. The validation of the OF-sEVs isolation was based on the Minimal information for studies of extracellular vesicles 2018 [MISEV 2018; (28)]. No differences were identified in mode size or

mean particle concentration between the groups; additionally, our results showed a population of vesicles with the expected size for sEVs, <200 nm (23). For instance, the passage of the spermatozoa and embryo presence do not induce changes in the oviductal secretion of sEVs, and the amount of sEVs secreted

by the embryo were not enough to cause a detectable increase in the total amount of sEVs. Although there is no influence on the concentration of sEVs in pregnant cows, we were able to identify miRNAs with distinct levels between the pregnant and non-pregnant groups in both OF-sEVs and OECs.

In OF-sEVs, we identified eight miRNAs up-regulated in pregnant and one miRNA up-regulated in non-pregnant cows. The presence of EVs in the bovine oviductal fluid and their functional effects on the embryo are already recognized (30, 31, 66). miRNAs from oviductal EVs have been studied in bovines and murine across the estrous cycle, and in both models, it was showed a hormonal effect on the miRNAs in cyclic animals (32, 67). We showed that the passage of the spermatozoa and embryo presence could induce differential secretion of miRNAs in OF-sEVs. Among the up-regulated miRNAs in the pregnant group, the bta-miR-140 was also detected across the bovine estrous cycle, without differences among the stages (32). In the same study, the miRNAs bta-miR-129, bta-miR-188, bta-miR-219, bta-miR-345-3p, bta-miR-4523, bta-miR-760-3p identified in our study as up-regulated in pregnant cows were not detected across the estrous cycle. Similarly, the bta-miR-331-5p, up-regulated in our study in the non-pregnant group, was not detected across the estrus cycle (32). Thus, these differences in miRNA contents could be due to differences in the isolation protocol and mainly in the technique used for miRNAs analysis and not necessarily that these miRNAs originated from the embryo itself since they are also present in low levels in the non-pregnant cows.

Differentially expressed miRNAs in OF-sEVs modulate pathways, such as PI3K/AKT, mTOR, and MAPK, which are predicted to be modulated by up-regulated miRNAs from OF-sEVs from the pregnant group. These pathways are related to transcription, translation, proliferation, growth, control of the cytoskeletal organization, metabolism, and survival (68–72). Previous studies have demonstrated that PI3K/mTOR signaling pathways are related to angiogenesis, a crucial process in the female's reproductive system and essential for pregnancy establishment (73, 74). Additionally, PI3K/AKT pathway can mediate cell growth and survival in pre-implantation embryos. In early mouse embryos, PI3K-Akt subunits are expressed from zygote to blastocyst stage and are involved with blastomere proliferation (75, 76). In bovine pre-implantation embryos, the activation PI3K signaling is also required for apoptosis control (71). Together, these studies demonstrate that the presence and function of the PI3K/AKT/mTOR pathway are essential for embryo development. We suggest that these miRNAs within sEVs could influence embryo development in the oviduct, mediating maternal-embryonic communication during early embryo development. In order to test that, in future studies we aim to utilize oviductal sEVs collected from oviductal fluid from the pregnant group as a tool to modulate the embryo environment *in vitro*, as well as to study the effects of those sEVs on embryo quality and development.

In OECs, six miRNAs were up-regulated in the non-pregnant group (bta-miR-133b, bta-miR-205, bta-miR-584, bta-miR-551a, bta-miR-1193, and bta-miR-1225-3p) and none in the pregnant group. It is well-known that sex-steroid hormones, E2 and P4, coordinate changes in the oviductal transcriptome and secretory function across the estrous cycle (6, 77–79). Also, considering day 4 of the estrous cycle, the POF and CL size potentially affect the oviductal environment (50). In our results, no differences were found in POF and CL size as well as no differences in E2 and P4 between our groups, ensuring that they did not interfere with our

results. The miRNAs bta-miR-133b, bta-miR-205, bta-miR-584, bta-miR-551a, identified in our study as up-regulated in non-pregnant cows, were already detected on bovine OECs exposed to low and high E2/P4 levels without the presence of the embryo, but with no differences in transcript levels (56). Thus, these miRNAs are not modulated by steroid hormones, supporting our data indicating that the passage of the spermatozoa and embryo presence can induce changes in miRNAs within oviductal cells in the same stage of the estrous cycle. Taken together, our results suggest that the passage of the spermatozoa and presence of an embryo within the oviduct can modulate the oviductal microenvironment, specifically the contents of miRNAs in OF-sEVs and OECs.

Based on bioinformatics analysis, we decided to evaluate the modulation of key-genes transcripts involved in the immune system, inflammation, and pregnancy recognition on OECs. Our study suggests that pregnancy stimulates changes in *TGFB1*, *PTGES2*, and *TNFR1* levels. *TGFB1* is an anti-inflammatory cytokine present in OECs throughout the estrous cycle and crucial for tolerance to non-self-cells (80). Increased expression level of *TGFB1*, as found in our pregnant group, is also present in bovine OECs cultured with embryos *in vitro* (81). However, it cannot be disregarded that the increase in *TGFB1* levels is also related to sperm binding and induction of sperm-tolerance (80, 82). Synergistically with *TGFB1*, the *PTGES2*, up-regulated in our pregnant group, participates in the eicosanoid metabolic process that will drive the production of PGE2 (83), promoting immune tolerance in the bovine oviduct (81, 84). Talukder et al. (81) and Maillo et al. (13) suggested that the embryo generates an anti-inflammatory response by suppressing key factors, such as NFkB2 and NFkBIA. The activation of NFkB transcriptional responses can be triggered by *TNFR1* (85). In our results, *TNFR1* was increased in OECs from the pregnant cows. The *TNFR1* is the receptor for the pro-inflammatory cytokines TNF- α . However, this cytokine also stimulates the productions of contractive substances, such as prostaglandins, regulating gametes transport, and ensuring the embryo migration into the uterus (86). Thus, it is imperative the adequate modulation of *TNFR1*, and among the modulators of the inflammatory responses of mammals are miRNAs (87). We identified a classic relationship between miR-584 and its predicted target *TNFR1* in OECs. The bta-miR-584 was down-regulated in OECs from the pregnant group, while *TNFR1* is increased in this group, while the opposite was observed in the non-pregnant group. Our results suggest that miRNAs may be decisive for the modulation of pro- and anti-inflammatory cytokines, leading to a proper immune response. Regarding genes involved in pregnancy recognition, no differences were identified between OECs groups. These results can be explained as the OECs from the pregnant group were exposed to embryos between 4 and 16 cells stage, and not morulas or blastocysts. Thereby, the interferon tau mechanism might not be initiated during this stage. Similar results were also found *in vitro*, where the presence of day-04 embryo did not influence the expression of interferon-stimulated genes (such as *ISG15*, *OAS1*, *MX2*) and *IFNAR2* on bovine OECs (81). Taken together, pregnancy can dynamically modulate the immune system and inflammation response as

early as day 4, affecting the oviductal environment and possibly embryo survival and development.

In this study, we identify the modulation of miRNAs and mRNAs in the oviduct. However, some limitations should be noticed since our experimental model cannot determine if differently expressed miRNAs are a direct or indirect effect of the spermatozoa or the embryo. Nevertheless, previous studies have shown that the embryo has a local effect on both, the oviduct and the endometrium (13, 62, 88). Additionally, the miRNAs up-regulated in OF-sEVs from the pregnant group are potentially secreted by the embryo, by the oviduct as a result of the embryonic stimulus, or from both. Although the ideal model to isolate the embryo effect is to transfer embryos directly into the oviduct (13), we must not disregard that the physiological environment is dynamic and depends on a set of synchronous factors and signals that together will guarantee the success of the pregnancy (89). Therefore, we chose not to remove these factors or to amplify the embryo's signal by transferring a large number of embryos as it would not represent the physiological response of the oviductal milieu. Additionally, future approaches should consider increase the number of animals and also small RNA sequencing analysis for a more comprehensive understanding of the expression and regulation of miRNAs associated with pregnancy in OECs and sEVs.

In conclusion, our results demonstrate that pregnancy can modulate the miRNA contents of sEVs as well as the miRNAs and mRNAs levels in OECs in cows. The functional enrichment of miRNAs highlights pathways involved in physiological processes, such as inflammation, cell proliferation, and immune response, crucial for both the reproductive tract and embryo development. Furthermore, our results confirm *in vivo* that the embryo-maternal crosstalk starts within the oviduct, enhancing oviductal receptivity, allowing proper fertilization and early embryo development, thus leading to a successful pregnancy.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

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ETHICS STATEMENT

The animal study was reviewed and approved by University of São Paulo Research Ethics Committee (Protocol Number 4909010817).

AUTHOR CONTRIBUTIONS

RM and JS designed the study. RM, NB, AB, MC, GA, and CP collected the samples and performed the experiments. RM, FP, and JS analyzed the data and wrote the manuscript. JP and GP performed the ultrasonography and artificial insemination. LS provided the conditions for cattle housing and management. FM and GP contributed the expertise and reagents. All authors discussed, reviewed, and agreed to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2021.639752/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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APPENDIX B – Confirmation of the isolation of OF-sEVs.

The isolation of OF-sEVs was confirmed using transmission electron microscopy (TEM). Due to the small volume of oviductal flush obtained from a single animal, the TEM was performed with oviducts obtained from slaughterhouses. In order to be as close as possible to our animal model, only reproductive tracts with a CL classified as stage 1 (early corpus luteum, red and recently ovulated), according to Ireland et al. (1980), were used. The isthmus of the ipsilateral oviducts was flushed, and the OF-sEVs were obtained as previously described.

SEVs were isolated from 3 mL of oviductal flush and according to the methods described by DE ÁVILA et al. (2020), with minimal modifications. The obtained pellet was diluted in 50 µL of fixation solution (1% sodium cacodylate; 2% glutaraldehyde; 2% paraformaldehyde; pH 7.2) for 2h at room temperature. After that, the OF-sEVs were diluted in 2 mL of PBS and centrifuged (119700 xg, 70 min, 4 °C) in order to remaining fixative. The pellet obtained was diluted in 20 µL of buffer solution (1% sodium cacodylate; pH 7.2) and kept refrigerated until analysis. The contents were placed in a copper grid for 20 min at room temperature to air dry. Subsequently, a drop of 2% uranyl acetate, which enhances the contrast, was added to the grid and examined in a FEI Tecnai 20 (LAB6 emission) at 200 KV. Through the images, we were able to identify cup-shaped particles with characteristic size resemble sEVs.

FIGURE APPENDIX B - Transmission electron microscopy analysis of small extracellular vesicles showing OF-sEVs isolated from the oviduct. White arrows indicate sEVs.

Source: MAZZARELLA, R. et al. Changes in Oviductal Cells and Small Extracellular Vesicles miRNAs in Pregnant Cows. *Frontiers in Veterinary Science*, v. 8, n. March, p. 1–14, 4 mar. 2021

APPENDIX C – Cycle threshold levels of the 261 miRNAs detected in OF-sEVs isolated from oviductal flushes from pregnant (OF-sEV/Pregnant) and non-pregnant (OF-sEV/Non-pregnant) cows.

MicroRNAs	Group											
	OF-sEV/Non-pregnant						Of-sEV/Pregnant					
	1	2	3	4	5	6	1	2	3	4	5	6
(n) Detected in sEV/Non-pregnant												
1 bta-let-7a-3p	35.94	.	.	.	34.89	31.82	.	34.30	.	.	.	.
2 bta-miR-107	34.43	36.91	.	.	.	34.60	33.46	.	34.58	.	.	.
3 bta-miR-10b	33.84	34.90	33.93	35.80	.	33.47	32.08	.	34.50	.	.	.
4 bta-let-7i	33.54	36.11	33.42	.	34.53	34.51	30.16	34.52	.	.	.	.
5 bta-miR-124a	34.29	35.33	.	.	32.97	36.64	32.37	34.31	.	.	.	.
6 bta-miR-101	31.55	.	33.99	34.88	.	31.74	30.73	34.82	.	.	.	.
7 bta-miR-128	34.76	.	36.98	.	.	34.17	32.41	.	.	.	.	.
8 bta-miR-139	33.43	.	.	34.22	34.64	.	31.59	.	34.46	.	.	.
9 bta-miR-133a	32.81	35.15	34.64	.	34.73	.	30.76	.	.	.	.	34.95
10 bta-miR-145	29.72	31.24	31.12	30.98	31.93	33.89	.	.	30.05	.	.	.
11 bta-miR-15a	30.73	.	34.64	.	36.12	.	30.06	.	33.95	.	.	.
12 bta-miR-149-5p	33.46	32.46	35.67	33.48	35.02	34.91	29.83	35.03	.	.	.	.
13 bta-miR-181a	34.64	.	34.02	.	34.50	34.34	30.46	.	34.00	.	.	.
14 bta-miR-192	34.43	.	34.38	36.35	33.29	.	31.92	.	34.27	.	.	.
15 bta-miR-185	32.56	34.58	33.93	33.84	33.94	35.33	29.78	.	35.96	.	.	.
16 bta-miR-193b	32.94	34.90	35.29	35.84	33.68	.	32.83	34.92	.	.	.	.
17 bta-miR-196b	35.41	.	33.52	36.79	32.91	35.89	35.18	.	.	.	33.20	.
18 bta-miR-19b	33.52	.	32.90	.	33.43	31.77	33.28	.	34.13	.	.	.
19 bta-miR-21-5p	34.89	.	34.12	34.98	.	.	.	.	.	36.69	.	.
20 bta-miR-212	.	.	35.26	35.18	.	33.96	.	.	.	.	33.68	.
21 bta-miR-216a	37.00	35.41	.	.	36.48	.	31.73	.	.	.	.	35.91
22 bta-miR-218	35.28	36.12	.	.	.	35.46	.	.	.	.	.	.
23 bta-miR-27a-5p	32.94	.	33.72	36.30	34.00	.	32.60	.	35.63	.	.	.
24 bta-miR-301b	35.55	35.94	35.94	.	35.89	.	31.83	.	35.93	.	.	.
25 bta-miR-330	33.53	34.15	36.77	33.64	.	35.32	30.49	.	.	.	.	.
26 bta-miR-331-3p	34.80	.	.	34.59	.	33.92	31.76	.	.	.	.	.
27 bta-miR-338	34.86	35.90	.	35.97	.	.	32.84	.	.	.	.	.
28 bta-miR-324	34.91	33.33	34.06	.	.	.	31.11	.	.	.	.	.
29 bta-miR-365-3p	32.88	.	33.34	34.39	34.32	34.09	32.76	.	33.10	.	.	.
30 bta-miR-342	33.51	34.89	.	34.23	.	.	32.88	.	34.31	.	.	.
31 bta-miR-369-5p	34.50	33.86	35.03	36.03	33.78	.	.	.	33.93	.	.	.
32 bta-miR-374b	33.24	.	34.14	35.97	.	33.03	31.87	.	.	.	.	.
33 bta-miR-362-3p	.	.	34.48	35.89	34.06	34.78	33.77	.	36.93	.	.	.
34 bta-miR-376a	36.79	33.63	35.19	.	.	.	34.04	.	.	.	.	.
35 bta-miR-376d	32.51	33.35	32.26	.	.	.	36.00	.	.	.	.	.
36 bta-miR-376e	32.13	33.51	32.83	.	.	.	.	.	33.95	.	.	.
37 bta-miR-380-5p	.	36.34	34.95	33.93	32.82	33.02	.	.	35.73	.	.	.
38 bta-miR-449d	32.44	33.11	32.81	32.84	32.56	33.97	.	.	32.50	.	.	34.97

39	bta-miR-424-5p	31.42	36.62	32.87	.	36.07	32.81	29.70	33.24	.	.	.	.
40	bta-miR-452	.	.	.	34.90	34.81	33.28	33.76	.	.	.	.	.
41	bta-miR-431	32.63	34.92	33.27	35.32	33.78	35.30	.	35.67	32.88	.	.	.
42	bta-miR-432	35.57	34.10	.	35.56	35.85	.	31.82	.	33.10	.	.	.
43	bta-miR-497	32.76	34.85	34.78	33.84	33.85	.	28.73	.	33.24	.	.	.
44	bta-miR-488	33.95	34.98	.	35.55	.	.	30.87	.	32.86	.	.	.
45	bta-miR-490	.	35.64	.	34.39	31.78	.	30.81	.	.	.	.	.
46	bta-miR-491	33.01	.	.	34.66	32.37	33.14	30.43	.	34.69	.	.	.
47	bta-miR-532	32.85	33.15	31.61	36.49	33.95	33.97	30.73	.	31.80	.	.	.
48	bta-miR-652	32.76	34.26	35.45	.	.	33.38	.	.	35.41	.	.	.
49	bta-miR-658	33.18	34.62	34.59	34.31	.	.	31.70	.	33.98	.	.	.
50	bta-miR-660	32.52	36.37	.	.	34.03	.	31.76	.	.	.	.	35.76
51	bta-miR-665	29.73	29.69	31.78	30.80	31.66	34.09	.	.	.	.	32.89	.
52	bta-miR-671	34.14	36.22	.	35.25	35.06	.	.	.	.	.	.	35.61
53	bta-miR-769	33.33	35.88	.	.	.	33.55	.	.	.	.	.	.
54	bta-miR-708	33.61	35.59	33.94	34.32	34.27	.	31.83	.	34.42	.	.	.
55	bta-miR-744	33.32	.	.	.	32.97	35.03	.	35.21	.	.	.	35.16
56	bta-miR-1306	36.84	33.31	.	33.10	33.40	34.33	30.77	.	.	36.85	.	.

(n) Detected only in OF-sEV/Pregnant

1	bta-miR-133b	.	.	.	.	.	.	36.91	.	36.25	.	33.48	.
2	bta-miR-147	.	.	.	.	.	32.10	32.01	35.68	35.47	.	.	33.41
3	bta-miR-196a	.	.	.	.	33.24	33.98	.	.	.	36.67	31.41	33.96
4	bta-miR-215	34.68	.	.	36.01	.	.	32.94	.	35.05	.	35.87	34.80
5	bta-miR-23b-5p	.	.	.	.	34.33	32.63	33.65	.	.	.	32.00	33.34
6	bta-miR-29d-5p	34.92	.	.	.	.	34.04	32.00	35.80	35.90	.	.	36.61
7	bta-miR-29e	33.94	.	34.39	.	.	.	32.57	34.63	34.12	.	.	.
8	bta-miR-371	34.23	35.64	.	.	.	.	32.57	.	33.99	.	36.49	.
9	bta-miR-409a	33.94	.	.	34.92	.	.	35.97	.	35.97	.	36.14	35.36
10	bta-miR-409b	.	.	.	.	.	.	36.65	.	35.86	.	36.80	.
11	bta-miR-379	.	.	.	.	.	.	34.00	34.45	37.00	.	.	.
12	bta-miR-483	.	.	34.91	.	.	.	33.88	36.64	35.49	.	.	.
13	bta-miR-670	34.35	.	.	.	.	34.14	35.41	.	34.54	.	32.98	.

(n) Detected in OF-sEV/Pregnant and OF-sEV/Non-pregnant

1	bta-miR-103	30.75	35.84	33.33	33.40	31.71	32.84	28.28	36.54	31.85	.	.	34.39
2	bta-let-7a-5p	27.51	31.69	30.18	.	29.59	28.72	26.55	30.67	30.34	35.03	33.48	33.95
3	bta-let-7b	26.74	28.91	28.76	28.75	27.75	28.35	23.65	29.17	27.60	33.81	33.83	31.69
4	bta-miR-105b	35.11	34.12	35.00	33.99	33.62	.	30.99	33.95	34.10	.	.	.
5	bta-let-7c	26.74	29.50	29.43	29.01	28.02	27.75	24.31	29.15	27.69	34.40	33.85	31.78
6	bta-miR-106a	29.69	34.77	31.82	33.65	30.64	30.84	29.09	33.26	33.50	.	33.15	32.66
7	bta-let-7d	28.73	33.96	32.06	31.89	29.96	30.29	27.70	30.79	31.11	.	.	.
8	bta-miR-106b	30.60	32.18	33.41	32.99	32.81	32.85	28.16	32.92	.	.	31.97	32.64
9	bta-let-7e	26.96	30.39	29.83	29.75	28.09	27.83	25.81	29.61	29.23	34.15	.	33.08
10	bta-let-7f	29.56	35.05	31.89	33.48	31.71	30.70	28.80	31.74	32.16	.	34.76	35.47
11	bta-miR-10a	31.71	33.97	34.38	.	34.68	34.15	30.81	36.78	35.63	.	.	32.88
12	bta-let-7g	30.77	.	34.26	.	33.36	32.40	30.82	34.07	32.79	.	34.93	.
13	bta-miR-122	.	.	33.45	35.97	33.03	35.94	36.15	35.11	35.20	34.47	.	.

14	bta-miR-100	30.51	32.59	31.86	33.99	32.87	33.06	29.05	.	34.16	.	34.94	35.60
15	bta-miR-124b	33.92	.	.	35.56	32.87	.	32.18	.	.	.	33.93	33.64
16	bta-miR-125a	30.12	34.81	32.03	32.88	31.92	31.79	27.69	34.78	33.36	36.51	35.68	.
17	bta-miR-125b	27.75	32.09	29.64	30.22	29.27	28.78	25.73	30.65	29.97	34.45	34.81	33.48
18	bta-miR-126-3p	31.64	31.88	31.38	31.73	32.16	31.68	31.56	32.62	31.63	32.35	31.90	32.86
19	bta-miR-126-5p	33.05	35.67	35.42	35.72	33.97	34.04	31.43	32.91	35.68	36.03	33.41	32.86
20	bta-miR-134	36.13	.	.	35.18	35.44	.	31.34	35.17	33.14	.	.	.
21	bta-miR-127	27.92	28.82	29.81	28.86	29.64	31.90	25.10	29.40	28.21	33.44	31.81	31.32
22	bta-miR-135a	30.30	34.74	32.80	32.71	32.35	32.51	28.75	34.26	32.75	.	.	.
23	bta-miR-135b	30.71	.	33.98	32.90	33.94	32.74	29.79	34.62	33.27	.	.	.
24	bta-miR-129	33.60	.	35.96	34.70	.	33.95	29.72	33.05	33.94	.	.	35.54
25	bta-miR-129-3p	34.45	.	.	36.72	33.89	.	32.86	.	34.97	.	33.97	35.26
26	bta-miR-129-5p	32.02	34.88	34.62	.	32.31	.	29.42	.	32.29	.	.	36.94
27	bta-miR-138	34.86	34.45	35.19	33.93	33.56	34.12	29.68	.	.	.	34.25	33.63
28	bta-miR-130a	31.33	31.52	31.79	31.55	31.64	32.98	30.74	31.40	31.73	32.19	32.23	32.61
29	bta-miR-130b	30.12	30.75	31.44	.	30.49	31.59	28.34	30.74	30.03	32.65	31.56	32.19
30	bta-miR-140	33.31	.	33.85	35.06	33.04	.	31.35	32.89	32.40	.	32.62	.
31	bta-miR-132	33.41	.	34.03	34.72	34.29	32.94	33.78	35.04	35.01	.	34.99	35.52
32	bta-miR-141	28.90	31.85	30.48	.	30.74	30.84	27.49	32.61	.	.	33.82	32.37
33	bta-miR-151-3p	31.49	33.94	35.00	33.93	32.84	.	29.66	34.36	32.75	.	26.48	33.87
34	bta-miR-143	32.68	34.26	32.91	32.66	30.70	30.62	31.60	32.90	31.84	35.14	32.31	31.68
35	bta-miR-151-5p	31.11	.	33.67	34.36	32.35	.	29.67	36.17	32.28	.	33.69	34.94
36	bta-miR-152	32.93	.	35.45	.	36.14	.	32.59	35.99	35.01	.	.	.
37	bta-miR-154a	.	34.07	33.08	35.96	35.71	.	31.73	35.52	35.95	36.89	.	.
38	bta-miR-154b	31.39	30.82	30.35	29.84	29.82	.	30.70	31.88	30.56	30.05	29.46	30.66
39	bta-miR-154c	34.38	.	36.01	34.80	36.42	.	34.06	34.08	35.01	36.80	35.48	34.53
40	bta-miR-148a	27.96	31.07	30.50	30.33	29.31	28.79	27.20	32.82	30.91	33.91	.	34.92
41	bta-miR-148b	28.45	33.97	30.74	32.15	30.30	29.44	27.74	31.90	31.79	35.96	35.11	35.21
42	bta-miR-149-3p	25.77	26.24	27.73	26.78	28.58	31.09	.	27.47	25.55	33.32	32.27	29.82
43	bta-miR-15b	31.87	34.74	34.29	33.31	32.89	.	30.45	33.87	32.91	36.37	34.73	.
44	bta-miR-16a	30.43	32.98	32.68	33.00	31.59	.	29.50	33.96	33.32	.	33.87	33.25
45	bta-miR-16b	29.64	33.66	31.40	33.73	31.37	.	28.76	32.86	32.61	.	33.98	32.94
46	bta-miR-188	33.80	34.05	36.90	36.50	34.79	.	30.71	36.28	33.96	35.82	32.26	32.39
47	bta-miR-17-5p	33.86	33.18	33.93	33.21	31.97	36.07	31.79	35.34	33.85	34.95	30.77	31.71
48	bta-miR-181b	31.96	34.43	.	.	33.08	34.82	30.56	.	35.26	.	32.93	.
49	bta-miR-190b	34.34	.	36.37	.	34.69	34.47	33.24	.	34.99	.	35.59	.
50	bta-miR-181d	32.50	33.54	35.10	.	32.56	35.23	30.09	.	32.51	.	.	35.71
51	bta-miR-191	30.75	33.70	33.85	34.04	32.13	31.64	.	32.29	32.99	17.17	33.15	32.91
52	bta-miR-184	35.04	34.51	35.42	.	.	..	33.41	34.14	33.88	.	.	.
53	bta-miR-193a-5p	31.31	33.84	32.35	34.17	33.41	33.06	29.50	33.91	32.72	.	.	36.03
54	bta-miR-186	33.11	34.61	35.07	33.62	36.68	34.39	31.49	.	34.54	.	.	35.32
55	bta-miR-187	30.60	32.70	31.24	31.88	30.52	31.85	28.76	32.94	32.08	.	37.00	32.86
56	bta-miR-195	30.46	33.93	31.18	32.78	31.81	33.52	29.26	33.44	33.16	32.85	33.00	.
57	bta-miR-200c	27.83	35.09	29.83	30.99	29.64	28.68	26.63	31.69	30.71	.	31.82	32.87
58	bta-miR-204	31.45	33.92	33.34	34.92	33.20	33.91	28.78	33.97	32.50	.	36.65	.
59	bta-miR-197	31.55	32.97	32.27	34.02	36.77	32.27	28.73	34.43	31.83	.	.	36.68
60	bta-miR-205	30.93	33.60	34.22	32.90	32.64	.	29.57	36.69	33.69	.	.	.
61	bta-miR-206	34.93	.	35.90	34.43	.	.	32.93	36.96	35.33	.	.	.

62	bta-miR-199c	33.25	34.68	33.29	32.13	36.93	33.55	29.67	32.91	34.01	.	35.12	35.10
63	bta-miR-20a	30.63	33.42	32.84	32.98	31.84	30.98	28.98	33.85	33.85	.	33.11	34.61
64	bta-miR-19a	31.84	.	.	34.70	32.13	31.81	33.71	33.86	34.03	.	.	.
65	bta-miR-20b	30.81	.	33.14	34.79	32.40	32.55	29.80	33.12	33.87	.	32.44	34.61
66	bta-miR-200a	31.73	.	31.67	33.68	33.84	32.68	29.80	32.29	33.93	.	.	35.72
67	bta-miR-200b	26.54	31.10	29.05	29.81	28.74	26.88	25.50	29.74	29.63	34.90	33.93	31.67
68	bta-miR-210	30.37	33.25	32.54	32.90	31.71	31.72	28.31	32.87	31.38	.	.	33.94
69	bta-miR-211	31.95	34.97	33.92	34.11	35.05	.	29.37	35.66	33.54	.	.	34.99
70	bta-miR-22-5p	32.77	34.27	33.09	.	36.03	36.10	32.14	33.68	.	.	.	34.04
71	bta-miR-221	31.90	30.74	32.67	32.89	32.11	.	28.48	34.50	30.53	.	36.50	34.93
72	bta-miR-214	29.47	31.91	31.59	.	31.54	34.16	26.05	31.54	29.32	.	.	33.94
73	bta-miR-222	30.70	30.57	34.55	31.58	31.36	33.06	26.63	33.33	30.28	.	.	35.18
74	bta-miR-223	33.42	34.15	.	.	33.99	.	32.00	35.64	32.90	.	.	.
75	bta-miR-23a	28.38	33.11	29.84	30.85	29.57	29.29	26.42	31.97	30.60	.	33.92	31.73
76	bta-miR-23b-3p	31.80	32.85	31.75	32.38	30.53	31.78	28.64	32.76	32.90	.	.	33.67
77	bta-miR-219	33.15	33.68	33.95	34.60	32.26	34.19	29.85	.	33.14	.	.	33.87
78	bta-miR-219-3p	33.96	36.09	35.77	36.33	.	36.82	32.01	36.49	35.05	.	.	32.87
79	bta-miR-24-3p	28.38	30.87	30.75	30.57	29.39	29.45	26.62	30.76	30.70	34.93	.	.
80	bta-miR-25	29.82	32.22	32.39	32.48	31.83	31.86	27.69	34.60	31.71	.	34.81	.
81	bta-miR-22-3p	3.45	2.72	2.93	.	2.66	2.68	3.40	2.92	3.08	2.93	2.54	2.69
82	bta-miR-26a	27.78	32.90	29.46	30.73	28.84	28.68	26.38	31.43	30.60	.	34.38	32.78
83	bta-miR-26b	31.60	.	31.26	33.90	34.88	30.72	29.26	34.27	34.29	.	34.96	34.91
84	bta-miR-29d-3p	30.74	35.06	31.89	34.00	32.64	32.86	28.61	35.59	31.57	.	.	33.88
85	bta-miR-27a-3p	30.03	35.29	32.23	31.53	31.58	30.61	29.17	33.00	32.53	.	35.05	35.21
86	bta-miR-301a	34.64	34.57	.	.	35.05	.	32.39	.	35.82	34.91	.	.
87	bta-miR-27b	30.78	.	31.61	32.57	32.85	30.81	29.23	.	33.34	.	.	33.50
88	bta-miR-296-3p	31.81	32.45	32.44	33.02	32.10	31.90	29.20	36.04	32.94	35.02	32.75	32.63
89	bta-miR-296-5p	32.21	27.80	32.69	32.34	32.94	.	30.71	29.01	27.63	.	35.38	32.85
90	bta-miR-29a	28.25	31.63	30.59	30.65	29.49	29.74	26.19	31.69	29.78	34.93	.	34.05
91	bta-miR-29b	30.04	33.90	32.42	34.16	33.27	32.59	29.40	34.45	33.89	.	.	.
92	bta-miR-30a-5p	31.54	34.57	32.87	34.54	32.21	32.24	29.77	35.23	35.18	.	34.82	32.88
93	bta-miR-29c	28.03	31.90	29.76	.	29.49	29.72	26.22	31.17	29.96	.	35.57	33.98
94	bta-miR-30b-5p	32.74	32.95	.	35.02	31.68	33.65	29.97	.	32.91	.	.	36.81
95	bta-miR-328	30.74	34.36	32.18	31.68	29.73	28.45	29.08	33.02	32.32	33.95	35.67	35.49
96	bta-miR-30c	30.51	.	31.87	33.06	31.83	31.90	28.70	33.05	32.48	.	34.90	34.17
97	bta-miR-30d	32.68	.	32.89	32.74	32.76	31.72	30.02	35.35	33.68	.	35.80	34.54
98	bta-miR-30e-5p	32.65	.	33.16	32.36	32.83	32.01	29.83	34.58	.	.	32.72	33.52
99	bta-miR-30f	31.68	.	35.85	34.96	32.87	32.89	29.77	33.19	33.45	.	.	.
100	bta-miR-31	29.41	32.89	31.60	31.54	29.52	30.49	27.33	32.65	31.38	.	32.21	.
101	bta-miR-331-5p	31.72	31.96	30.83	31.40	31.81	33.16	31.69	33.80	32.60	35.64	35.17	36.50
102	bta-miR-335	33.72	.	36.26	34.86	34.81	36.98	35.93	.	35.06	.	33.05	34.93
103	bta-miR-320a	25.80	27.33	27.60	26.80	27.67	30.18	23.15	27.90	26.59	33.88	31.98	29.81
104	bta-miR-320b	31.38	31.51	31.57	32.08	33.21	.	27.75	33.96	30.36	.	34.21	.
105	bta-miR-339a	31.81	33.96	36.25	32.86	.	35.00	28.82	35.03	32.23	.	.	.
106	bta-miR-323	17.41	17.64	17.83	16.71	16.76	17.46	17.28	17.41	17.36	18.73	17.05	16.76
107	bta-miR-339b	31.29	34.82	33.38	31.62	31.89	32.88	27.96	34.22	.	35.03	.	.
108	bta-miR-326	31.81	33.13	34.79	36.04	35.75	36.55	29.93	36.76	33.40	.	.	.
109	bta-miR-33b	34.00	33.89	33.79	34.89	36.48	.	34.33	33.66	34.32	35.90	35.51	.

110	bta-miR-365-5p	33.38	33.66	.	33.00	.	.	30.75	33.99	33.56	.	.	34.77
111	bta-miR-345-3p	33.98	36.08	35.88	34.15	.	.	.	33.88	34.00	.	.	35.40
112	bta-miR-345-5p	28.67	30.65	31.02	29.70	30.77	33.49	26.69	31.40	29.06	36.09	35.39	33.91
113	bta-miR-346	31.30	30.90	31.87	32.55	33.68	34.64	26.45	34.14	30.82	.	36.80	36.63
114	bta-miR-34a	33.42	.	33.93	33.98	.	34.13	31.80	36.89	33.47	.	.	.
115	bta-miR-370	32.86	33.87	34.15	33.86	33.98	.	30.77	36.58	34.99	34.81	36.54	33.43
116	bta-miR-34b	29.10	33.30	31.47	31.74	31.92	29.54	28.80	31.70	31.67	.	.	35.21
117	bta-miR-34c	29.63	34.23	31.78	31.62	30.84	30.27	28.60	32.24	31.72	.	.	.
118	bta-miR-361	32.31	34.45	33.83	34.34	34.84	33.57	31.03	36.38	34.87	.	.	.
119	bta-miR-375	30.18	31.41	31.50	31.06	31.51	32.93	28.48	31.66	31.14	31.87	31.60	33.71
120	bta-miR-382	29.05	28.83	29.43	29.83	30.07	29.84	29.66	29.79	28.73	31.54	30.51	31.41
121	bta-miR-383	27.56	30.09	30.75	36.00	36.48	.	32.11	33.24	31.71	.	.	.
122	bta-miR-378	32.86	33.19	33.66	34.82	33.10	31.65	29.80	.	33.84	.	34.05	.
123	bta-miR-378b	32.28	34.32	35.71	34.81	32.92	32.91	31.10	35.22	35.53	.	.	36.48
124	bta-miR-411a	32.52	31.88	32.74	32.79	32.62	31.66	.	32.90	32.49	34.15	32.80	31.66
125	bta-miR-378c	34.96	36.68	34.35	32.82	33.90	34.55	36.82	.	34.90	33.91	33.16	32.77
126	bta-miR-411b	36.43	.	34.90	33.85	33.84	34.36	31.24	.	35.71	.	32.96	35.25
127	bta-miR-378d	32.70	35.13	33.31	32.17	32.89	33.56	.	33.42	31.90	33.96	33.85	34.24
128	bta-miR-421	31.93	32.54	32.35	30.39	32.91	31.67	.	33.16	31.18	29.68	30.37	31.11
129	bta-miR-423-3p	30.17	31.77	34.74	31.88	31.57	31.78	28.06	32.33	31.53	.	.	.
130	bta-miR-423-5p	29.51	32.85	31.99	32.77	31.43	32.47	28.05	31.76	31.73	.	.	.
131	bta-miR-449c	33.81	34.91	33.74	34.05	.	35.62	30.53	36.31	34.42	.	.	.
132	bta-miR-425-3p	25.38	26.35	27.39	26.00	26.94	29.99	21.68	27.81	24.70	34.36	31.82	29.53
133	bta-miR-425-5p	31.33	36.68	33.98	34.36	33.02	32.28	29.26	34.05	33.30	34.92	.	.
134	bta-miR-429	29.48	30.19	29.70	29.48	30.55	30.83	28.81	29.50	29.61	32.26	31.01	30.66
135	bta-miR-4523	33.33	35.98	34.19	34.65	34.11	34.44	31.76	34.67	34.82	35.06	33.05	33.92
136	bta-miR-453	33.11	35.81	32.87	33.71	35.16	.	32.60	32.64	33.55	34.16	33.88	35.03
137	bta-miR-433	30.15	30.38	30.02	28.69	29.31	29.83	29.60	30.53	30.65	28.42	28.34	28.78
138	bta-miR-449a	31.16	33.54	32.61	32.39	32.56	30.72	28.57	33.34	31.93	36.04	.	.
139	bta-miR-449b	31.90	34.35	33.82	.	32.85	32.45	29.35	33.90	33.32	.	.	.
140	bta-miR-484	32.22	33.92	.	32.98	.	33.13	29.69	34.85	34.23	.	.	.
141	bta-miR-485	33.74	35.24	35.99	35.14	36.10	33.98	32.06	35.78	34.43	.	35.88	.
142	bta-miR-486	27.18	27.53	28.80	27.29	29.00	33.40	.	29.06	25.74	34.97	35.37	30.91
143	bta-miR-500	.	.	.	36.04	34.79	33.90	31.24	34.79	33.29	.	.	.
144	bta-miR-502b	33.97	36.76	36.47	34.83	.	33.38	33.49	.	.	.	31.77	32.34
145	bta-miR-489	31.89	31.82	30.98	29.99	31.20	32.31	30.54	32.08	31.16	30.55	29.84	31.90
146	bta-miR-503-3p	30.49	30.84	32.76	30.85	31.63	32.38	28.13	31.52	30.63	34.94	33.46	33.88
147	bta-miR-493	27.40	28.74	29.40	28.39	30.21	33.87	25.43	29.80	28.24	.	33.97	31.82
148	bta-miR-505	28.81	29.81	29.87	29.23	29.81	31.72	25.80	30.84	29.32	.	34.33	32.66
149	bta-miR-494	17.75	19.24	20.42	18.33	18.96	21.71	15.30	19.62	18.11	.	24.98	22.64
150	bta-miR-541	30.80	30.73	30.61	30.13	30.82	31.97	29.66	30.82	30.69	31.71	31.39	31.60
151	bta-miR-584	31.86	31.79	32.89	31.94	33.10	34.60	28.17	33.91	.	.	.	35.05
152	bta-miR-592	33.43	33.90	35.16	36.31	.	34.52	33.53	36.97	36.58	.	.	.
153	bta-miR-615	9.78	9.69	9.52	9.72	10.76	11.84	9.66	9.79	9.71	9.34	9.68	10.79
154	bta-miR-631	19.50	19.58	19.75	18.91	18.74	18.48	19.23	19.38	19.54	20.16	18.80	18.79
155	bta-miR-574	27.85	29.37	29.20	30.11	29.65	29.65	25.82	30.35	28.71	32.94	33.92	31.85
156	bta-miR-656	35.98	30.47	31.23	30.80	32.09	33.19	.	.	.	33.45	32.02	32.84
157	bta-miR-664a	32.00	32.83	32.99	33.76	34.04	35.87	29.75	.	32.99	.	.	36.43

158	bta-miR-760-3p	33.93	32.70	33.89	34.40	33.32	.	29.84	.	32.78	.	.	33.94
159	bta-miR-664b	33.96	35.77	35.20	35.84	34.37	.	30.79	34.18	32.85	.	34.78	.
160	bta-miR-760-5p	30.66	31.66	32.00	31.47	31.78	33.64	27.70	32.54	30.46	.	35.33	33.38
161	bta-miR-669	30.96	31.51	32.46	33.08	31.86	33.46	27.85	33.00	30.54	.	34.22	34.57
162	bta-miR-763	32.78	32.64	32.96	32.71	32.62	36.98	29.37	33.87	32.98	.	32.90	33.37
163	bta-miR-764	33.88	35.06	35.82	34.22	.	36.31	30.37	36.06	34.90	.	.	.
164	bta-miR-767	31.44	31.57	31.65	31.67	31.87	33.06	30.64	31.51	31.00	33.37	33.05	33.64
165	bta-miR-677	32.61	32.63	35.00	34.74	34.80	35.90	28.70	34.98	32.92	.	.	.
166	bta-miR-874	30.77	32.29	32.84	32.83	32.06	32.96	28.05	32.84	30.64	35.69	.	33.10
167	bta-miR-877	29.76	31.05	31.62	30.92	31.72	32.83	27.66	31.34	29.67	.	35.09	32.71
168	bta-miR-885	31.87	34.58	32.85	32.88	.	33.98	28.82	33.98	.	.	.	35.39
169	bta-miR-99a-5p	29.59	34.15	31.55	31.45	31.68	30.82	27.99	32.09	30.88	.	33.95	.
170	bta-miR-9-5p	31.87	.	33.95	.	33.69	33.11	32.93	36.66	33.22	35.76	.	33.12
171	bta-miR-92a	28.29	30.66	30.76	31.12	30.61	30.58	26.60	32.16	30.70	.	34.01	32.84
172	bta-miR-92b	26.80	27.76	28.68	28.07	27.78	28.33	24.77	28.56	26.99	32.43	31.80	30.50
173	bta-miR-93	30.68	34.94	33.20	33.89	31.73	30.92	29.32	33.15	33.87	35.10	31.73	30.71
174	bta-miR-935	32.86	32.35	32.58	33.27	32.76	32.04	29.50	.	32.55	.	36.86	35.38
175	bta-miR-940	25.36	23.46	26.83	26.52	27.82	29.49	22.83	25.68	23.70	31.65	30.70	29.73
176	bta-miR-1224	23.68	24.33	24.73	24.78	25.50	27.81	20.38	26.13	23.74	30.24	28.71	26.75
177	bta-miR-1225-3p	26.71	25.25	28.41	28.20	29.05	30.18	24.46	27.41	25.57	34.23	32.53	31.56
178	bta-miR-1246	21.75	24.94	22.63	.	24.87	24.53	20.44	.	22.42	.	28.64	28.01
179	bta-miR-1247-3p	29.76	31.60	32.19	30.85	31.79	31.87	27.57	31.89	30.46	35.04	33.32	33.99
180	bta-miR-1296	32.02	32.17	36.57	33.70	34.38	36.03	28.67	33.92	31.35	.	.	.
181	bta-miR-1247-5p	26.73	27.30	29.07	28.18	28.74	30.64	23.81	28.81	26.73	.	32.93	30.88
182	bta-miR-1248	34.26	32.88	34.73	33.96	35.66	.	29.41	.	33.86	.	33.98	.
183	bta-miR-1301	.	36.10	.	33.51	33.55	32.20	33.87	.	.	36.00	35.03	.
184	bta-miR-1249	32.88	32.85	33.98	34.45	31.76	31.18	29.42	36.12	32.14	.	32.50	32.86
185	bta-miR-1260b	22.75	25.71	24.16	24.77	24.41	26.83	20.84	25.64	23.93	30.83	28.62	26.83
186	bta-miR-1307	23.79	24.22	26.04	24.41	25.41	27.70	20.82	25.54	23.60	32.83	25.60	27.75
187	bta-miR-1343-3p	29.69	31.45	31.84	31.97	30.84	31.42	27.66	31.72	30.68	.	26.34	32.77
188	bta-miR-1343-5p	25.09	24.71	27.04	26.36	27.71	30.23	22.45	26.82	24.32	34.38	.	29.43
189	bta-miR-1281	30.82	29.70	31.03	31.45	30.78	30.82	27.27	31.21	29.36	.	22.37	31.76
190	bta-miR-1388-3p	32.26	31.69	31.80	31.81	32.22	34.14	28.10	32.81	31.27	.	28.51	36.39
191	bta-miR-1287	33.20	34.87	34.55	35.44	.	36.06	32.88	.	33.07	.	35.04	35.11
192	bta-miR-1291	33.97	32.88	.	.	32.93	.	32.67	.	34.15	.	30.92	.

Source: MAZZARELLA, R. et al. Changes in Oviductal Cells and Small Extracellular Vesicles miRNAs in Pregnant Cows. *Frontiers in Veterinary Science*, v. 8, n. March, p. 1–14, 4 mar. 2021

APPENDIX D – Full list of biological pathways with significant p-value ($p < 0.05$) predicted as modulated by the 8 miRNAs (bta-miR-126-5p, bta-miR-129, bta-miR-140, bta-miR-188, bta-miR-219, bta-miR-345-3p, bta-miR-4523 and bta-miR-760-3p) up-regulated in OF-sEVs from pregnant compared to non-pregnant cows.

Pathways	Number of genes	P-value
bta00564 Glycerophospholipid metabolism	57	0.0003
bta01100 Metabolic pathways	552	0.0005
bta04070 Phosphatidylinositol signaling system	53	0.0008
bta04514 Cell adhesion molecules (CAMs)	75	0.0012
bta04012 ErbB signaling pathway	46	0.0012
bta04144 Endocytosis	106	0.0017
bta04152 AMPK signaling pathway	60	0.0021
bta04014 Ras signaling pathway	104	0.0022
bta05200 Pathways in cancer	209	0.0024
bta04928 Parathyroid hormone synthesis, secretion and action	52	0.0027
bta04071 Sphingolipid signaling pathway	58	0.0028
bta04921 Oxytocin signaling pathway	70	0.0029
bta04931 Insulin resistance	54	0.003
bta04659 Th17 cell differentiation	55	0.0032
bta04910 Insulin signaling pathway	65	0.0035
bta00562 Inositol phosphate metabolism	39	0.0036
bta04150 mTOR signaling pathway	71	0.0039
bta04072 Phospholipase D signaling pathway	69	0.0041
bta04810 Regulation of actin cytoskeleton	90	0.0048
bta04360 Axon guidance	78	0.0048
bta01521 EGFR tyrosine kinase inhibitor resistance	41	0.0051
bta05017 Spinocerebellar ataxia	47	0.0055
bta04010 MAPK signaling pathway	118	0.0058
bta05205 Proteoglycans in cancer	87	0.006
bta05214 Glioma	39	0.0071
bta04722 Neurotrophin signaling pathway	56	0.0074
bta04270 Vascular smooth muscle contraction	60	0.0076
bta04961 Endocrine and other factor-regulated calcium reabsorption	28	0.0078
bta04261 Adrenergic signaling in cardiomyocytes	66	0.0082
bta04066 HIF-1 signaling pathway	51	0.0088
bta04310 Wnt signaling pathway	70	0.0091
bta05211 Renal cell carcinoma	36	0.0092
bta04142 Lysosome	59	0.0093
bta04530 Tight junction	75	0.0102
bta05231 Choline metabolism in cancer	46	0.012
bta04130 SNARE interactions in vesicular transport	20	0.0124
bta05100 Bacterial invasion of epithelial cells	36	0.0126
bta04020 Calcium signaling pathway	83	0.0129
bta04062 Chemokine signaling pathway	78	0.013

bta05220 Chronic myeloid leukemia	37	0.0154
bta00760 Nicotinate and nicotinamide metabolism	22	0.0161
bta04670 Leukocyte transendothelial migration	50	0.0176
bta04721 Synaptic vesicle cycle	37	0.0178
bta04022 cGMP-PKG signaling pathway	70	0.0181
bta04510 Focal adhesion	80	0.0191
bta04750 Inflammatory mediator regulation of TRP channels	46	0.0196
bta04216 Ferroptosis	24	0.0196
bta04658 Th1 and Th2 cell differentiation	44	0.0207
bta04666 Fc gamma R-mediated phagocytosis	42	0.0218
bta04919 Thyroid hormone signaling pathway	51	0.0226
bta05167 Kaposi sarcoma-associated herpesvirus infection	82	0.023
bta01522 Endocrine resistance	42	0.0246
bta05135 Yersinia infection	55	0.0247
bta05321 Inflammatory bowel disease (IBD)	33	0.0255
bta01230 Biosynthesis of amino acids	34	0.0267
bta04922 Glucagon signaling pathway	45	0.0269
bta04151 PI3K-Akt signaling pathway	138	0.027
bta05202 Transcriptional misregulation in cancer	76	0.0276
bta05212 Pancreatic cancer	35	0.0278
bta04120 Ubiquitin mediated proteolysis	58	0.0287
bta04371 Apelin signaling pathway	58	0.0287
bta04211 Longevity regulating pathway	40	0.0293
bta04015 Rap1 signaling pathway	84	0.0318
bta05170 Human immunodeficiency virus 1 infection	90	0.0328
bta04024 cAMP signaling pathway	88	0.0349
bta04728 Dopaminergic synapse	55	0.0364
bta00230 Purine metabolism	55	0.0364
bta04664 Fc epsilon RI signaling pathway	32	0.0365
bta05163 Human cytomegalovirus infection	93	0.0382
bta05215 Prostate cancer	42	0.0387
bta04668 TNF signaling pathway	49	0.0401
bta04730 Long-term depression	28	0.0405
bta04611 Platelet activation	50	0.0407
bta04660 T cell receptor signaling pathway	45	0.0412
bta05235 PD-L1 expression and PD-1 checkpoint pathway in cancer	40	0.0412
bta04925 Aldosterone synthesis and secretion	41	0.0422
bta05230 Central carbon metabolism in cancer	30	0.0439
bta04370 VEGF signaling pathway	27	0.0445
bta04924 Renin secretion	32	0.0469
bta04725 Cholinergic synapse	47	0.0471

Source: MAZZARELLA, R. et al. Changes in Oviductal Cells and Small Extracellular Vesicles miRNAs in Pregnant Cows. **Frontiers in Veterinary Science**, v. 8, n. March, p. 1–14, 4 mar. 2021

APPENDIX E – Full list of biological pathways with significant p-value ($p < 0.05$) predicted as modulated by the miRNAs (bta-miR-331-5p) up-regulated in OF-sEVs from non-pregnant compared to pregnant cows.

Pathways	Number of genes	P-value
bta05211 Renal cell carcinoma	12	0
bta04919 Thyroid hormone signaling pathway	13	0.0003
bta04910 Insulin signaling pathway	13	0.0012
bta04931 Insulin resistance	11	0.0016
bta04012 ErbB signaling pathway	9	0.0027
bta04360 Axon guidance	14	0.0034
bta04662 B cell receptor signaling pathway	9	0.0034
bta05210 Colorectal cancer	9	0.0039
bta04370 VEGF signaling pathway	7	0.0043
bta05163 Human cytomegalovirus infection	17	0.0046
bta05212 Pancreatic cancer	8	0.005
bta04022 cGMP-PKG signaling pathway	13	0.0055
bta05200 Pathways in cancer	30	0.0061
bta04922 Glucagon signaling pathway	9	0.0091
bta05221 Acute myeloid leukemia	7	0.0093
bta04140 Autophagy	11	0.0095
bta00510 N-Glycan biosynthesis	6	0.0096
bta00564 Glycerophospholipid metabolism	9	0.0096
bta04310 Wnt signaling pathway	12	0.0096
bta04660 T cell receptor signaling pathway	9	0.0113
bta04024 cAMP signaling pathway	15	0.0115
bta04512 ECM-receptor interaction	8	0.0116
bta04066 HIF-1 signaling pathway	9	0.0132
bta05170 Human immunodeficiency virus 1 infection	15	0.0136
bta04072 Phospholipase D signaling pathway	11	0.0148
bta05225 Hepatocellular carcinoma	12	0.0156
bta04728 Dopaminergic synapse	10	0.0162
bta05213 Endometrial cancer	6	0.0163
bta05205 Proteoglycans in cancer	13	0.0218
bta04550 Signaling pathways regulating pluripotency of stem cells	10	0.0226
bta04152 AMPK signaling pathway	9	0.0241
bta04810 Regulation of actin cytoskeleton	13	0.0264
bta04921 Oxytocin signaling pathway	10	0.033
bta05226 Gastric cancer	10	0.0342
bta04510 Focal adhesion	12	0.0352
bta04380 Osteoclast differentiation	9	0.0375
bta04670 Leukocyte transendothelial migration	8	0.0376
bta04390 Hippo signaling pathway	10	0.038
bta05235 PD-L1 expression and PD-1 checkpoint pathway in cancer	7	0.0384
bta04514 Cell adhesion molecules (CAMs)	10	0.0406

bta05165 Human papillomavirus infection	18	0.0416
bta05412 Arrhythmogenic right ventricular cardiomyopathy (ARVC)	6	0.0443
bta05220 Chronic myeloid leukemia	6	0.0465
bta05231 Choline metabolism in cancer	7	0.0499

Source: MAZZARELLA, R. et al. Changes in Oviductal Cells and Small Extracellular Vesicles miRNAs in Pregnant Cows. **Frontiers in Veterinary Science**, v. 8, n. March, p. 1–14, 4 mar. 2021

APPENDIX F – Cycle threshold levels of the 363 miRNAs detected in oviductal epithelial cells from non-pregnant (OEC/Non-pregnant) or pregnant (OEC/Pregnant) cows.

MicroRNAs	Group											
	OEC/Non-pregnant						OEC/Pregnant					
	1	2	3	4	5	6	1	2	3	4	5	6
(n) Detected in OEC/Non-pregnant												
1 bta-miR-124b	29.83	.	29.83	31.70	.	29.65	29.83	.	.	.	.	29.72
2 bta-miR-217	34.98	.	.	36.33	33.78	33.89	.	34.43	.	.	.	33.71
3 bta-miR-329a	36.04	34.90	.	.	.	35.89	33.97	34.39	.	.	.	.
4 bta-miR-377	36.04	.	.	35.73	35.91	36.76	.	.	.	.	.	35.39
5 bta-miR-411c-5p	33.56	.	.	34.59	.	34.39	35.70	.	.	36.75	.	.
6 bta-miR-487a	29.73	32.35	.	31.61	.	.	.	30.81	.	.	.	.
7 bta-miR-539	32.60	35.01	33.00	33.94	31.23	34.16	.	.	.	.	35.57	33.98
8 bta-miR-655	32.90	.	34.42	34.34	.	36.25	33.17	.	.	.	.	36.02
9 bta-miR-875	36.95	.	35.13	.	33.02	33.91	35.00	35.71	.	.	.	.
10 bta-miR-9-3p	34.57	36.41	.	.	34.75	36.85	.	.	.	.	.	35.04
(n) Detected only in OEC/Pregnant												
1 bta-miR-127	.	.	.	.	.	26.58	25,79	25,63	.	.	.	25,83
2 bta-miR-302b	35.99	35.21	.	.	.	.	.	34,49	.	.	35,80	35,37
3 bta-miR-331-5p	.	.	27.92	.	.	.	.	.	.	29,12	29,45	27,92
4 bta-miR-4523	.	.	28.66	.	.	.	.	.	.	30,03	30,57	28,60
5 bta-miR-504	.	.	27.65	27.28	.	.	.	29,77	27,79	27,59	.	26,77
6 bta-miR-542-5p	.	.	33.71	.	.	.	.	.	32,96	33,81	36,61	32,43
7 bta-miR-544a	31.55	.	32.82	.	.	.	32,39	32,51	.	34,10	35,73	33,14
8 bta-miR-1197	32.95	.	33.70	.	.	.	33,91	35,38	35,05	33,54	34,97	33,18
(n) Detected in OEC/Non-pregnant and OEC/Pregnant												
1 bta-let-7a-3p	26.37	27.86	27.32	25.75	23.80	25.67	25.75	25.73	26.06	26.03	26.88	25.60
2 bta-miR-103	24.76	26.75	25.47	24.08	22.66	24.14	25.09	24.81	25.09	24.81	26.12	23.75
3 bta-let-7a-5p	18.15	20.05	18.24	18.73	17.66	18.66	18.50	18.33	19.42	18.77	20.41	18.20
4 bta-miR-105a	30.11	31.48	30.78	31.77	28.45	31.44	.	.	32.94	31.29	.	30.47
5 bta-let-7b	16.75	18.20	16.84	17.43	16.55	17.66	16.93	16.74	18.00	17.27	18.56	17.30
6 bta-miR-105b	28.74	30.34	29.76	28.79	25.68	28.62	29.05	28.53	29.26	29.35	29.54	28.70
7 bta-let-7c	16.96	18.67	16.89	17.63	16.49	17.77	17.27	17.01	18.00	17.69	18.83	17.35
8 bta-miR-106a	25.09	27.34	25.88	23.80	22.56	23.41	24.98	25.40	24.67	25.20	25.33	23.00
9 bta-let-7d	19.42	21.14	19.49	19.84	18.80	20.04	19.58	19.74	21.03	20.13	21.74	19.55
10 bta-miR-106b	.	28.36	26.83	25.45	23.81	25.79	26.40	26.76	26.57	26.88	27.14	25.29
11 bta-let-7e	17.57	19.43	17.51	18.00	16.76	18.67	17.79	17.83	18.89	18.12	19.74	17.57
12 bta-miR-107	28.33	29.84	29.55	29.01	26.80	28.80	29.00	28.43	29.74	29.80	30.48	28.78
13 bta-let-7f	20.73	22.85	21.08	21.41	19.99	21.13	20.79	21.22	22.10	21.63	22.92	20.64
14 bta-miR-10a	24.08	26.01	24.77	23.45	22.78	22.71	24.74	24.61	24.44	24.35	25.84	22.58
15 bta-let-7g	22.90	24.70	23.21	22.92	21.60	22.79	23.01	23.07	24.42	23.34	24.75	22.24
16 bta-miR-10b	24.75	26.69	25.50	23.87	23.10	23.46	25.34	25.48	24.85	24.88	26.35	22.83
17 bta-let-7i	23.78	25.69	24.40	24.54	22.10	24.21	23.83	24.27	24.75	24.33	25.66	24.29

18	bta-miR-1	31.20	33.36	31.74	32.02	29.88	30.92	32.28	.	31.84	32.15	32.67	30.51
19	bta-miR-124a	.	31.00	30.74	33.87	.	30.47	.	.	31.80	30.66	.	30.25
20	bta-miR-100	23.11	24.69	23.78	21.46	20.83	21.46	22.50	22.35	22.69	22.33	22.99	21.57
21	bta-miR-101	27.51	29.98	27.89	25.40	23.33	25.82	27.61	28.09	27.07	27.89	27.76	24.90
22	bta-miR-125a	22.01	22.84	22.12	21.65	20.59	21.40	21.82	22.05	22.78	21.70	23.84	21.64
23	bta-miR-125b	20.30	21.35	20.51	19.73	18.79	20.12	19.83	19.85	21.53	20.31	21.81	20.06
24	bta-miR-133b	32.31	32.91	.	32.92	.	31.87	32.82	32.48	35.24	34.53	34.14	32.99
25	bta-miR-126-3p	28.80	31.43	30.49	27.45	27.13	28.75	28.82	31.83	28.96	28.75	30.75	27.72
26	bta-miR-133c	31.91	35.81	32.88	.	32.66	33.32	31.78	32.77	35.95	.	.	34.82
27	bta-miR-126-5p	27.68	29.95	28.84	27.49	27.37	29.05	27.76	29.83	29.31	28.60	30.32	28.07
28	bta-miR-134	26.94	28.78	26.90	28.07	26.19	27.05	27.48	27.74	28.74	28.81	.	25.80
29	bta-miR-135a	22.83	24.10	23.77	21.67	20.02	21.54	21.81	22.08	22.00	21.75	22.26	21.89
30	bta-miR-128	25.93	27.55	26.06	25.82	24.56	25.81	25.78	25.82	26.62	25.78	27.81	25.57
31	bta-miR-135b	24.19	25.70	25.52	22.96	21.68	22.94	23.03	23.62	23.58	23.62	23.72	23.57
32	bta-miR-129	26.75	27.93	26.87	27.71	25.68	27.16	26.74	27.33	27.89	28.00	28.73	26.80
33	bta-miR-136	31.28	32.64	32.78	33.23	31.22	32.80	31.81	30.88	33.82	32.36	34.30	31.94
34	bta-miR-129-3p	29.02	30.81	30.57	29.74	28.68	29.77	29.77	30.33	30.44	29.63	31.87	29.39
35	bta-miR-137	34.78	.	36.83	36.34	30.72	35.21	36.43	35.00	33.49	32.90	34.02	.
36	bta-miR-129-5p	26.68	27.99	27.16	27.78	25.73	26.93	26.94	.	27.81	27.85	28.68	27.02
37	bta-miR-138	25.34	27.84	25.89	.	23.71	26.68	25.89	25.68	26.61	.	27.43	26.22
38	bta-miR-130a	29.32	30.40	29.68	29.21	26.77	28.70	29.17	29.57	28.98	28.83	30.72	29.47
39	bta-miR-139	27.96	28.93	27.78	27.80	26.18	27.49	26.62	27.45	28.02	27.95	28.79	27.75
40	bta-miR-130b	20.25	20.81	20.73	20.72	19.85	20.69	20.49	20.11	21.46	20.78	21.75	20.46
41	bta-miR-140	27.46	28.96	27.78	27.18	25.63	26.87	27.54	27.68	28.25	27.34	29.22	26.64
42	bta-miR-132	27.55	28.19	27.14	28.49	27.03	27.79	27.78	27.81	28.68	27.59	28.24	27.36
43	bta-miR-141	24.23	26.79	25.65	21.81	19.48	22.05	24.33	24.57	22.74	23.83	23.33	21.15
44	bta-miR-133a	27.75	.	27.80	.	25.72	27.99	28.23	27.84	.	28.78	.	28.08
45	bta-miR-142-3p	30.75	34.72	35.57	29.38	27.01	27.88	30.80	34.04	29.40	30.40	30.79	27.90
46	bta-miR-142-5p	30.36	.	31.86	31.70	28.80	31.02	30.60	31.82	31.77	31.43	34.20	30.28
47	bta-miR-151-3p	23.83	25.27	24.49	23.71	22.79	23.46	24.16	.	24.36	23.95	25.32	23.55
48	bta-miR-143	24.36	26.14	25.06	24.69	22.89	24.71	24.69	24.53	25.76	25.05	26.85	24.66
49	bta-miR-151-5p	21.81	23.52	22.45	21.97	20.76	21.86	22.10	21.93	22.73	22.46	23.78	21.86
50	bta-miR-152	.	31.88	29.08	30.52	27.45	28.93	.	29.70	.	31.53	31.81	29.39
51	bta-miR-145	25.80	.	26.63	25.84	23.32	26.61	26.59	26.01	25.84	25.62	.	26.47
52	bta-miR-153	29.70	31.72	31.37	27.11	24.82	26.70	28.84	29.26	26.21	27.30	26.67	26.84
53	bta-miR-146a	29.38	33.49	29.75	31.79	28.43	31.43	31.56	30.28	28.94	29.59	33.95	29.40
54	bta-miR-154a	30.10	30.47	31.06	30.68	.	30.58	30.66	29.80	31.36	30.90	31.88	30.70
55	bta-miR-146b	29.39	31.82	29.52	31.73	27.81	30.82	30.32	29.59	28.80	29.10	32.82	.
56	bta-miR-154b	31.58	30.29	29.74	31.13	30.13	30.54	31.68	30.77	31.83	30.90	30.77	30.08
57	bta-miR-147	27.47	29.71	28.75	29.46	27.38	29.22	27.80	28.52	28.72	30.49	31.49	28.69
58	bta-miR-154c	33.53	32.80	33.52	33.06	31.78	32.85	33.10	32.95	34.01	34.96	33.43	33.09
59	bta-miR-148a	21.98	23.87	22.35	21.04	19.27	21.52	21.74	22.18	22.58	22.34	22.74	21.67
60	bta-miR-155	26.77	28.20	27.30	27.02	27.52	27.81	27.22	27.30	27.76	27.72	28.76	26.56
61	bta-miR-148b	22.65	24.62	22.77	21.64	19.52	22.00	22.42	22.55	22.92	22.80	23.17	21.77
62	bta-miR-15a	25.15	27.14	25.69	24.51	22.77	24.12	25.35	24.88	25.18	24.98	25.68	23.80
63	bta-miR-15b	23.02	24.40	23.47	23.61	23.07	23.51	23.27	22.97	23.89	23.24	24.74	23.18
64	bta-miR-149-5p	26.71	28.42	.	27.92	25.54	27.46	26.81	27.22	28.10	27.74	28.73	26.79
65	bta-miR-16a	22.73	24.69	23.68	22.25	21.17	22.06	22.76	22.72	23.20	22.73	23.81	21.87

66	bta-miR-150	27.06	28.44	28.43	27.75	26.60	26.79	27.65	27.98	28.24	27.37	30.27	26.82
67	bta-miR-16b	21.70	23.73	22.76	21.74	20.65	21.50	21.84	21.83	22.50	22.01	22.99	21.47
68	bta-miR-17-3p	28.75	30.74	.	.	27.17	28.97	28.68	28.74	30.02	30.08	29.94	28.58
69	bta-miR-188	27.71	29.23	28.56	29.63	25.77	28.38	28.88	28.55	.	.	29.52	28.74
70	bta-miR-17-5p	27.67	29.65	28.78	26.86	25.07	26.24	27.73	27.95	27.43	27.99	28.25	26.21
71	bta-miR-18a	30.28	31.83	31.70	29.66	27.71	28.74	29.94	.	29.85	29.70	30.49	28.05
72	bta-miR-181a	28.48	29.65	29.33	27.06	25.31	26.77	28.30	28.38	27.77	27.83	28.73	27.43
73	bta-miR-18b	.	34.32	31.88	30.91	29.08	30.60	31.88	31.68	30.78	32.49	32.11	29.78
74	bta-miR-181b	26.29	27.97	26.77	26.46	24.75	26.46	26.72	26.54	26.78	26.28	27.80	26.76
75	bta-miR-190a	34.14	.	34.28	29.38	28.69	29.19	31.62	32.72	31.65	31.16	31.81	29.37
76	bta-miR-181c	29.55	30.80	30.00	28.75	26.55	28.29	29.77	29.54	.	29.34	29.76	28.50
77	bta-miR-190b	25.18	26.83	25.90	25.29	23.84	24.59	25.70	25.46	25.94	25.20	25.74	24.51
78	bta-miR-181d	24.96	26.92	25.59	25.57	24.42	25.72	25.52	25.44	25.96	25.14	26.46	25.80
79	bta-miR-191	22.67	23.53	22.81	22.70	21.66	22.68	22.60	22.60	23.80	22.80	24.47	22.38
80	bta-miR-182	27.47	29.14	27.91	27.23	25.84	27.00	27.19	27.76	28.01	27.64	28.62	27.26
81	bta-miR-192	28.61	29.75	.	28.69	26.69	28.73	28.78	.	30.01	29.43	30.66	28.32
82	bta-miR-183	27.13	29.18	27.81	27.73	26.83	27.76	27.39	27.81	28.44	27.77	29.17	27.79
83	bta-miR-193a	32.36	33.45	32.48	.	.	32.35	32.69	32.67	.	33.98	.	31.43
84	bta-miR-184	32.97	34.50	31.66	33.90	.	.	31.09	32.70	33.02	33.89	34.12	32.32
85	bta-miR-193a-3p	.	31.53	.	30.01	.	29.06	29.87	.	.	29.80	32.04	29.58
86	bta-miR-185	26.63	29.11	26.84	26.64	25.49	26.73	26.99	26.70	27.80	27.49	28.90	26.22
87	bta-miR-193a-5p	25.77	26.14	25.73	25.82	24.64	25.78	25.81	25.77	26.49	25.82	26.71	25.70
88	bta-miR-186	24.93	.	25.44	24.49	22.42	24.53	25.28	25.02	25.70	25.11	26.70	23.99
89	bta-miR-187	26.64	.	27.10	28.97	24.46	27.28	26.81	27.41	27.82	27.95	28.17	27.25
90	bta-miR-194	27.69	29.54	28.68	26.23	25.42	26.24	27.86	28.50	27.86	27.81	28.13	25.99
91	bta-miR-195	24.19	26.23	24.80	24.31	22.88	24.32	24.25	24.05	24.91	24.64	25.74	24.03
92	bta-miR-200c	18.73	19.84	18.82	18.62	18.64	18.76	18.80	18.80	19.41	18.72	20.12	18.44
93	bta-miR-196a	30.19	32.63	30.54	30.81	29.80	30.69	32.05	31.35	29.52	31.03	31.89	30.68
94	bta-miR-202	.	.	33.86	34.80	30.34	.	34.93	.	33.88	.	34.51	33.39
95	bta-miR-196b	.	33.79	31.87	30.08	29.74	30.70	32.83	.	29.60	31.88	32.94	31.42
96	bta-miR-204	.	26.66	25.88	26.49	23.79	27.01	24.79	25.05	26.72	25.73	26.64	23.64
97	bta-miR-197	23.71	24.59	23.97	24.75	23.49	24.60	24.53	24.05	25.06	24.49	25.77	23.84
98	bta-miR-205	23.82	25.60	23.84	24.40	23.28	23.91	25.21	25.74	24.76	24.72	27.04	24.52
99	bta-miR-199a-3p	28.51	33.10	28.78	27.96	26.72	28.68	29.79	30.61	29.14	28.25	31.03	28.26
100	bta-miR-206	27.83	29.77	.	29.37	26.68	28.66	28.50	28.12	29.37	.	30.31	28.58
101	bta-miR-199a-5p	29.35	33.82	.	30.30	27.78	30.71	30.84	31.37	30.38	29.50	32.59	30.71
102	bta-miR-208a	31.58	33.04	31.89	33.34	30.80	32.58	.	31.92	34.12	33.65	33.88	32.31
103	bta-miR-199b	29.78	34.45	30.65	29.91	28.15	30.71	31.06	32.07	31.56	29.53	32.19	30.30
104	bta-miR-208b	32.92	32.88	33.28	32.67	31.78	33.00	32.87	32.88	32.67	33.13	34.46	32.34
105	bta-miR-199c	27.76	30.51	28.36	26.94	25.83	27.75	28.69	29.52	28.03	26.98	30.36	27.06
106	bta-miR-20a	25.08	27.14	25.99	23.70	22.67	23.18	25.10	25.46	24.58	24.90	25.03	23.10
107	bta-miR-19a	27.67	30.72	28.61	27.55	25.70	27.36	28.25	28.97	.	28.99	29.83	27.09
108	bta-miR-20b	26.44	28.79	27.28	25.13	23.70	24.67	26.65	26.72	25.95	26.67	26.74	24.71
109	bta-miR-19b	27.82	30.59	28.63	27.35	25.49	27.07	28.51	28.79	.	28.50	29.43	26.74
110	bta-miR-21-3p	.	30.65	29.48	30.19	.	30.22	30.22	29.79	30.76	30.41	.	.
111	bta-miR-200a	24.99	27.28	25.78	23.13	21.06	23.71	25.29	25.08	24.68	24.84	25.76	22.89
112	bta-miR-21-5p	25.81	28.17	26.39	25.78	24.81	25.70	26.53	26.52	26.67	26.21	27.82	25.25
113	bta-miR-200b	17.52	19.07	17.81	17.16	16.73	17.22	17.70	17.53	18.31	17.47	19.03	16.72

114 bta-miR-210	25.04	27.29	25.69	26.44	24.56	25.97	26.15	25.33	27.59	26.38	27.88	25.85
115 bta-miR-211	25.92	27.50	25.80	26.81	24.40	27.36	25.51	25.77	27.76	26.50	27.57	24.62
116 bta-miR-22-5p	27.72	29.76	28.49	27.03	25.54	27.63	27.76	27.62	28.07	27.55	28.63	27.55
117 bta-miR-212	30.80	31.86	.	32.85	.	.	30.81	.	33.01	32.96	33.52	31.44
118 bta-miR-221	25.11	26.83	26.79	27.17	21.54	26.08	25.78	25.59	26.45	26.41	26.57	26.53
119 bta-miR-214	26.57	28.54	27.19	28.77	25.01	.	26.95	27.32	28.62	.	28.81	27.37
120 bta-miR-222	23.46	24.40	23.89	24.46	22.81	24.14	23.62	23.72	24.46	23.97	24.82	23.72
121 bta-miR-215	29.24	31.51	30.36	28.56	27.75	28.73	29.45	30.44	29.87	29.27	29.96	28.46
122 bta-miR-223	32.51	31.84	30.03	30.78	29.88	31.88	31.01	30.73	33.27	32.15	31.81	30.81
123 bta-miR-216a	28.14	29.99	28.69	29.77	26.86	29.59	28.16	28.31	29.70	29.52	29.15	29.15
124 bta-miR-224	26.79	27.93	27.30	26.91	25.15	26.72	26.80	26.76	27.74	26.68	28.55	27.32
125 bta-miR-216b	28.82	30.74	29.42	30.39	28.48	29.83	29.41	.	30.81	30.83	31.67	28.89
126 bta-miR-23a	19.40	20.82	19.72	19.12	18.81	19.49	19.80	19.56	20.21	19.49	20.85	18.93
127 bta-miR-23b-3p	22.19	23.81	22.58	22.27	21.67	22.23	22.60	22.63	22.99	22.63	23.79	21.80
128 bta-miR-218	30.28	32.63	30.75	29.92	27.85	30.09	30.71	30.18	30.17	29.64	31.62	29.87
129 bta-miR-23b-5p	.	31.09	29.43	30.08	27.72	30.09	.	29.67	29.76	31.82	30.64	29.59
130 bta-miR-24	31.71	35.64	33.16	30.08	28.16	30.38	32.32	32.05	29.96	32.84	31.62	.
131 bta-miR-219-3p	.	.	27.69	28.41	26.11	28.04	27.68	.	.	28.41	28.70	27.71
132 bta-miR-24-3p	22.81	24.61	22.87	21.67	20.52	21.49	22.71	23.30	22.76	22.55	24.03	21.25
133 bta-miR-219-5p	35.41	.	.	35.74	31.82	.	34.07	.	.	34.47	33.94	34.19
134 bta-miR-25	21.66	23.12	22.16	21.70	21.35	21.42	22.09	21.85	22.33	21.69	23.26	21.32
135 bta-miR-22-3p	2.92	3.05	3.07	2.82	2.49	3.17	2.84	2.92	3.03	2.89	2.93	2.84
136 bta-miR-26a	19.82	21.41	19.81	19.07	17.74	18.77	19.81	19.77	20.25	19.72	20.85	18.79
137 bta-miR-26b	22.65	24.54	22.77	21.70	20.36	21.54	22.46	22.81	22.88	22.46	23.66	21.29
138 bta-miR-29d-3p	24.23	25.82	24.75	22.76	20.81	22.73	23.58	24.13	23.92	24.00	24.06	22.48
139 bta-miR-26c	32.86	.	33.82	34.92	31.88	.	34.63	34.98	.	.	36.63	33.96
140 bta-miR-29d-5p	26.41	28.13	27.32	26.73	24.76	26.48	26.78	26.85	27.72	26.79	28.06	26.78
141 bta-miR-27a-3p	23.55	25.53	23.72	22.82	20.72	22.81	23.81	23.49	24.01	23.57	24.89	22.79
142 bta-miR-29e	29.66	35.74	30.51	26.80	24.55	27.41	29.18	30.59	28.82	29.62	28.63	26.58
143 bta-miR-27a-5p	29.62	.	29.73	29.52	27.64	29.35	29.64	.	31.05	29.72	30.28	29.10
144 bta-miR-301a	33.34	33.86	.	32.81	30.04	33.63	33.56	31.84	32.91	33.34	.	32.16
145 bta-miR-27b	23.24	24.98	23.34	22.66	21.25	22.48	23.70	23.42	23.83	23.53	24.71	21.81
146 bta-miR-301b	32.34	33.14	33.80	32.64	.	33.04	31.88	33.37	33.50	32.32	33.83	31.80
147 bta-miR-28	27.17	28.80	27.56	25.88	25.46	25.62	27.48	27.43	27.24	27.30	28.18	25.73
148 bta-miR-302a	.	33.51	.	35.81	.	34.49	.	36.21	36.38	.	35.88	33.97
149 bta-miR-296-3p	25.96	27.62	26.62	27.31	24.72	26.74	.	26.61	27.32	27.25	27.70	26.45
150 bta-miR-296-5p	26.43	28.23	.	.	25.28	26.92	26.94	.	27.83	27.77	28.76	.
151 bta-miR-302c	31.87	34.99	31.90	32.84	30.82	32.06	32.13	32.76	35.29	33.98	32.88	32.27
152 bta-miR-299	34.65	34.89	32.92	.	32.21	.	35.84	35.25	35.49	33.37	.	36.23
153 bta-miR-302d	33.89	33.90	31.42	.	.	32.86	34.89	31.82	.	34.01	.	31.68
154 bta-miR-29a	20.53	22.00	20.41	19.22	18.24	19.32	19.94	20.57	20.84	20.22	21.39	19.32
155 bta-miR-3064	28.10	32.52	28.00	32.49	27.73	28.30	27.93	30.39	30.50	30.20	32.70	28.73
156 bta-miR-29b	26.15	29.79	27.27	24.80	21.93	25.04	26.55	26.99	26.19	26.47	26.71	24.58
157 bta-miR-30a-5p	24.65	26.23	25.39	23.80	21.86	23.39	24.69	24.76	25.18	24.77	25.82	23.43
158 bta-miR-29c	20.54	22.07	20.50	19.31	18.20	19.36	19.97	20.62	20.73	20.26	21.28	19.31
159 bta-miR-30b-3p	28.28	29.51	27.84	28.91	27.71	28.81	29.06	28.21	29.16	28.50	29.22	28.73
160 bta-miR-30b-5p	23.78	25.61	24.15	23.27	21.53	23.17	24.08	23.80	25.33	24.35	25.80	22.74
161 bta-miR-328	24.73	25.49	24.68	25.48	23.84	24.64	25.40	24.77	25.82	25.22	25.80	24.35

162	bta-miR-30c	22.70	23.69	23.01	22.78	20.94	22.19	23.20	22.76	23.97	23.24	24.83	21.76
163	bta-miR-30d	24.74	25.97	25.27	23.69	22.00	23.54	24.79	24.82	25.24	24.93	25.76	23.54
164	bta-miR-329b	31.29	35.72	.	34.77	30.61	31.59	32.10	32.93	33.50	36.72	35.99	31.84
165	bta-miR-30e-5p	24.63	26.22	25.29	23.83	21.89	23.54	24.84	24.86	25.23	24.90	26.10	23.55
166	bta-miR-330	.	29.62	27.33	.	25.81	27.73	27.76	.	.	28.93	29.66	27.79
167	bta-miR-30f	24.17	24.85	24.51	24.02	22.25	23.49	24.65	24.26	25.34	24.72	.	23.46
168	bta-miR-331-3p	26.59	26.73	25.79	24.73	24.23	24.66	25.80	26.80	25.36	24.84	27.34	25.16
169	bta-miR-31	22.91	25.46	23.26	22.69	21.11	22.22	23.58	23.32	24.09	23.36	25.13	21.63
170	bta-miR-32	31.86	32.82	33.81	30.69	28.48	31.02	32.26	31.77	29.89	.	31.68	30.90
171	bta-miR-335	30.78	35.29	32.95	32.62	29.46	32.62	31.88	33.51	31.90	33.70	30.87	31.55
172	bta-miR-320a	21.57	22.80	22.19	22.54	20.59	22.28	22.20	21.88	22.67	22.35	23.12	22.24
173	bta-miR-338	30.23	31.98	30.81	27.89	25.52	27.93	30.43	31.52	28.98	29.52	29.57	28.07
174	bta-miR-320b	26.80	28.70	.	28.60	24.35	27.87	27.39	27.04	27.84	28.31	27.84	27.76
175	bta-miR-339a	24.74	25.77	24.76	24.93	22.88	24.58	25.08	24.83	25.42	25.25	26.84	24.27
176	bta-miR-323	17.49	17.82	17.51	17.65	17.50	17.72	17.27	17.88	17.36	17.69	17.71	17.67
177	bta-miR-339b	23.77	24.81	23.59	23.87	21.78	23.47	24.04	23.79	24.67	24.13	25.83	23.48
178	bta-miR-324	28.50	29.12	28.79	27.49	.	26.89	28.17	28.82	28.19	27.81	.	26.67
179	bta-miR-33a	30.19	32.34	31.14	32.27	28.58	31.71	31.20	31.63	30.69	32.28	32.13	31.53
180	bta-miR-326	.	27.82	25.97	27.27	.	26.44	25.99	26.25	.	.	.	26.41
181	bta-miR-33b	30.80	32.91	31.81	32.45	29.97	32.03	.	31.08	32.29	33.30	34.65	30.73
182	bta-miR-340	32.54	33.16	31.13	30.62	29.64	29.72	30.73	30.99	31.54	30.73	31.92	30.86
183	bta-miR-365-3p	26.31	27.75	25.97	25.98	25.29	25.75	26.84	25.96	27.52	26.55	28.16	25.27
184	bta-miR-342	27.73	29.42	28.60	27.78	26.55	27.37	27.92	28.79	28.76	28.00	30.36	26.81
185	bta-miR-365-5p	26.60	.	25.99	28.92	25.06	26.06	26.92	28.09	29.71	.	29.68	26.69
186	bta-miR-345-3p	27.53	29.64	27.73	28.79	26.16	27.71	28.06	27.78	28.80	28.97	.	28.00
187	bta-miR-345-5p	26.88	28.56	27.68	28.02	.	.	27.57	.	27.99	27.86	.	.
188	bta-miR-369-3p	33.99	.	.	35.46	31.83	.	35.44	.	35.79	33.69	35.21	34.27
189	bta-miR-346	23.23	25.57	23.78	25.34	21.78	23.79	23.98	23.28	24.79	24.82	25.15	24.65
190	bta-miR-369-5p	34.30	34.49	33.74	.	32.57	33.90	.	33.91	34.48	34.09	32.95	34.27
191	bta-miR-34a	25.49	27.77	25.75	25.68	23.04	25.80	26.19	25.78	26.45	25.91	27.69	25.23
192	bta-miR-370	26.11	.	27.00	27.62	25.48	27.24	.	26.70	.	27.83	28.03	26.67
193	bta-miR-34b	22.16	24.38	21.71	21.80	20.41	21.37	22.86	22.27	22.85	22.23	24.53	20.84
194	bta-miR-371	.	33.27	30.78	31.05	30.62	31.90	30.82	31.09	31.87	32.89	33.03	31.37
195	bta-miR-34c	22.15	24.19	21.72	21.69	20.39	21.16	22.98	22.17	22.82	22.12	24.48	20.80
196	bta-miR-374a	26.61	28.11	27.15	24.66	24.50	25.42	.	26.60	25.85	25.81	26.32	25.03
197	bta-miR-361	26.07	28.01	26.72	25.71	24.62	25.76	26.72	26.20	26.58	26.46	26.86	25.68
198	bta-miR-374b	23.74	25.33	24.22	23.74	22.92	23.65	23.59	23.79	24.57	23.72	25.18	23.19
199	bta-miR-362-3p	28.96	31.50	30.69	27.79	26.15	28.06	.	29.26	29.29	28.72	29.72	27.74
200	bta-miR-375	21.76	22.63	21.64	21.84	21.61	21.83	22.63	21.83	22.79	22.10	23.79	21.68
201	bta-miR-362-5p	28.92	31.41	30.27	29.30	27.19	29.10	29.77	29.82	29.88	29.25	30.77	29.29
202	bta-miR-376a	33.78	.	34.69	34.96	32.14	.	36.41	.	35.24	34.56	.	34.73
203	bta-miR-363	30.30	33.46	31.56	30.32	28.68	29.88	31.50	31.86	30.97	30.30	32.78	29.70
204	bta-miR-376b	34.40	34.90	32.94	34.50	33.71	36.08	34.77	35.50	.	36.88	.	33.82
205	bta-miR-382	30.33	29.92	29.15	30.81	28.34	28.80	30.60	29.19	30.67	29.73	29.31	28.79
206	bta-miR-376d	31.83	34.58	32.69	.	31.84	32.39	32.86	34.17	.	.	.	33.86
207	bta-miR-383	.	29.34	28.70	30.13	26.64	29.53	29.18	28.68	29.64	29.75	30.09	.
208	bta-miR-376e	33.68	.	33.86	34.13	32.61	35.54	31.32	36.24	.	.	.	32.86
209	bta-miR-409a	32.47	34.73	32.97	34.70	.	32.19	33.72	32.08	33.98	.	33.86	32.88

210	bta-miR-409b	31.46	34.24	32.81	33.93	29.70	33.98	.	.	32.18	35.27	33.80	32.86
211	bta-miR-378	26.59	27.48	26.79	26.46	25.82	25.83	26.70	27.29	27.15	27.40	29.41	25.79
212	bta-miR-410	30.75	33.03	.	32.86	.	30.84	30.58	31.61	31.60	32.02	35.36	32.13
213	bta-miR-378b	26.81	27.62	27.32	26.45	26.34	25.82	27.52	27.40	27.53	27.42	29.52	25.89
214	bta-miR-378c	29.72	31.76	29.68	30.69	.	30.02	30.34	30.58	30.93	30.72	32.91	29.80
215	bta-miR-411b	28.86	29.72	29.32	29.61	28.54	29.70	28.95	29.02	29.71	29.55	30.54	29.74
216	bta-miR-378d	28.69	29.69	28.79	29.89	26.82	28.71	28.70	29.14	29.73	30.09	.	.
217	bta-miR-411c-3p	31.78	35.40	.	33.92	30.35	34.89	.	32.91	33.80	33.89	33.32	32.69
218	bta-miR-379	28.26	29.53	28.70	29.67	26.49	29.34	28.02	28.64	28.38	28.73	29.01	29.03
219	bta-miR-380-3p	30.74	33.11	31.77	33.14	28.78	31.64	31.76	31.85	33.36	33.26	36.72	.
220	bta-miR-412	.	34.11	31.76	35.75	29.71	31.99	32.90	31.84	34.99	.	35.16	32.43
221	bta-miR-380-5p	30.00	31.34	30.32	.	28.94	30.30	30.40	30.72	32.67	31.81	30.59	30.51
222	bta-miR-421	26.76	28.30	27.10	26.52	25.75	26.59	26.11	26.73	27.31	27.27	28.14	26.58
223	bta-miR-381	.	30.86	.	.	28.69	29.75	29.80	31.30	31.31	32.57	31.29	29.92
224	bta-miR-423-3p	24.76	25.61	24.83	24.65	23.21	.	24.82	24.72	25.01	24.76	26.05	24.05
225	bta-miR-423-5p	23.42	24.48	23.82	24.16	22.82	23.72	23.83	23.73	24.08	23.14	24.83	23.36
226	bta-miR-449c	28.11	28.83	28.38	28.29	26.12	27.77	27.79	27.75	28.90	27.92	28.83	26.77
227	bta-miR-424-3p	28.90	.	30.75	31.01	27.72	31.94	29.42	30.86	29.71	31.01	32.59	30.79
228	bta-miR-449d	25.81	27.73	26.38	27.46	24.39	26.43	26.59	26.63	26.97	27.52	27.59	25.93
229	bta-miR-424-5p	24.52	30.14	27.83	26.47	23.40	27.78	25.56	28.50	24.67	26.82	26.73	27.60
230	bta-miR-450a	30.71	.	34.50	33.35	30.25	33.98	31.43	35.67	31.78	32.80	32.93	34.79
231	bta-miR-425-3p	21.26	22.46	22.10	22.51	20.67	22.78	21.92	21.61	22.32	22.68	22.82	22.21
232	bta-miR-450b	32.35	.	.	33.85	31.24	34.55	32.82	34.49	33.65	33.50	33.81	33.57
233	bta-miR-425-5p	23.78	24.67	24.57	23.74	22.47	23.56	23.70	23.56	23.81	23.47	24.71	23.49
234	bta-miR-451	28.45	30.69	27.91	29.74	24.00	30.07	27.79	28.44	27.57	25.73	29.75	29.68
235	bta-miR-429	22.91	24.70	23.89	22.30	21.54	22.57	23.31	22.91	23.34	22.71	23.90	22.14
236	bta-miR-452	29.65	31.75	29.59	29.99	27.80	29.71	29.59	29.75	30.00	.	31.56	28.86
237	bta-miR-431	28.76	30.34	28.65	.	26.71	28.23	28.69	28.83	30.23	.	30.62	28.71
238	bta-miR-432	.	31.05	29.89	.	28.06	30.03	.	.	31.94	30.81	31.59	30.02
239	bta-miR-433	27.17	29.75	.	29.71	26.58	28.81	28.22	28.04	.	29.71	.	28.58
240	bta-miR-454	32.06	33.41	32.74	33.02	30.53	31.80	31.66	32.82	32.65	31.91	32.83	31.79
241	bta-miR-448	32.61	35.73	33.48	34.27	.	33.92	33.74	33.91	33.73	33.96	33.88	32.32
242	bta-miR-455-3p	27.40	28.80	27.15	26.83	27.69	27.59	28.50	28.69	28.25	27.55	30.16	27.67
243	bta-miR-449a	23.91	25.13	24.31	23.79	21.40	23.62	23.79	22.88	24.76	23.50	24.79	21.94
244	bta-miR-455-5p	30.80	33.91	31.83	29.61	28.70	30.25	31.68	31.63	30.20	31.55	32.82	29.60
245	bta-miR-449b	26.84	27.63	26.77	26.48	24.31	26.57	26.82	25.80	27.71	26.24	27.42	25.11
246	bta-miR-483	29.78	32.25	29.69	32.93	28.45	29.89	30.22	30.50	31.85	32.30	33.67	29.75
247	bta-miR-484	26.47	27.51	26.73	27.11	25.13	26.62	.	26.76	27.76	27.15	28.72	26.32
248	bta-miR-496	.	33.97	32.14	34.28	30.43	32.92	32.86	34.40	.	.	35.53	32.11
249	bta-miR-485	.	36.32	.	36.34	32.05	32.81	33.40	33.92	34.31	35.16	36.30	.
250	bta-miR-497	26.52	27.17	26.82	26.79	25.85	26.75	26.70	26.52	27.27	26.71	27.82	26.77
251	bta-miR-486	24.09	25.19	24.82	25.14	22.06	24.79	24.73	24.39	24.79	24.95	25.29	24.70
252	bta-miR-499	31.81	35.97	33.30	31.90	29.04	30.21	31.74	32.25	32.90	.	32.27	31.52
253	bta-miR-500	27.71	29.61	28.25	27.34	25.32	26.93	.	27.57	27.70	27.96	.	27.57
254	bta-miR-487b	32.20	36.66	31.89	.	30.78	32.81	32.55	32.54	33.64	33.63	34.13	.
255	bta-miR-502a	.	32.02	31.89	32.87	29.59	30.79	32.56	.	31.54	32.01	33.42	32.37
256	bta-miR-488	27.79	28.76	28.58	29.03	27.16	28.77	28.39	27.82	28.46	28.69	28.96	28.48
257	bta-miR-502b	28.43	.	.	28.16	25.97	27.85	28.74	28.47	28.65	28.18	.	28.06

258	bta-miR-489	.	32.80	33.15	32.34	.	33.32	32.70	.	.	33.88	33.99	33.39
259	bta-miR-503-3p	25.37	27.25	26.16	26.84	23.32	26.03	26.18	25.72	26.41	26.58	27.26	25.93
260	bta-miR-490	27.80	.	28.40	29.10	25.83	28.22	28.62	28.26	29.81	29.22	29.94	28.05
261	bta-miR-503-5p	28.79	31.03	29.63	30.41	27.84	30.39	29.81	29.65	30.69	31.33	30.83	30.49
262	bta-miR-491	27.47	29.69	27.83	26.37	26.25	26.08	27.64	27.62	27.74	27.64	27.76	25.86
263	bta-miR-493	25.21	.	25.52	27.43	22.53	25.65	25.86	25.71	26.80	26.91	26.83	25.23
264	bta-miR-505	24.57	25.28	24.99	24.73	23.65	24.52	25.03	24.67	25.84	25.02	25.93	24.66
265	bta-miR-494	16.80	17.40	17.99	17.45	12.80	16.78	17.39	16.73	16.54	16.84	16.40	17.25
266	bta-miR-532	.	30.04	29.80	29.78	25.95	28.67	29.77	28.81	29.83	29.86	29.26	28.41
267	bta-miR-495	34.56	.	32.87	31.99	.	34.00	35.84	34.57	36.89	33.99	.	32.48
268	bta-miR-541	27.66	29.18	27.71	28.49	24.41	27.96	27.81	27.81	28.66	28.51	28.54	27.21
269	bta-miR-584	.	28.30	27.19	.	25.23	27.37	27.41	.	28.07	28.32	28.49	27.41
270	bta-miR-543	33.06	34.97	31.75	.	.	32.77	33.11	32.68	34.05	33.95	.	33.98
271	bta-miR-592	29.92	33.56	30.77	28.99	27.74	28.06	31.31	30.30	30.18	.	31.00	29.84
272	bta-miR-544b	30.31	34.00	.	35.94	.	30.16	.	31.55	.	35.09	33.88	29.91
273	bta-miR-615	11.70	11.79	11.93	11.99	11.81	12.49	11.80	12.00	12.44	12.42	12.52	12.23
274	bta-miR-545-3p	32.51	.	36.33	32.92	29.51	32.04	.	33.99	33.41	32.92	.	32.49
275	bta-miR-628	29.55	31.75	30.10	29.41	29.21	30.67	29.78	30.40	31.56	30.38	30.71	29.07
276	bta-miR-545-5p	34.49	33.99	33.90	33.19	30.42	31.06	35.79	33.85	32.34	34.14	33.87	32.91
277	bta-miR-631	19.51	19.67	19.23	19.82	19.79	20.12	19.05	19.46	20.49	19.95	20.16	19.85
278	bta-miR-551a	32.93	34.18	31.86	33.94	31.51	32.94	.	33.99	34.18	34.20	36.09	33.06
279	bta-miR-652	26.60	27.71	26.91	25.97	24.63	25.76	26.74	25.97	26.77	26.34	27.28	25.74
280	bta-miR-551b	33.92	35.70	32.94	32.74	29.94	32.88	32.79	33.89	.	32.50	35.06	.
281	bta-miR-653	33.98	36.05	35.49	.	.	.	33.22	36.56	.	.	.	33.87
282	bta-miR-562	33.17	.	33.29	.	.	34.93	32.80	35.52	35.95	35.56	33.88	32.09
283	bta-miR-654	25.73	27.75	26.14	27.47	25.49	26.72	26.43	26.25	26.86	27.51	28.23	26.23
284	bta-miR-574	19.48	20.97	19.76	20.82	18.52	20.12	20.26	19.82	20.76	20.78	21.47	19.79
285	bta-miR-656	32.75	33.99	32.92	33.94	30.72	33.73	33.63	32.50	34.18	32.20	33.89	31.84
286	bta-miR-658	30.16	.	31.29	31.49	.	30.28	31.09	.	29.98	.	30.93	30.80
287	bta-miR-758	.	.	32.76	32.84	31.69	34.43	32.53	.	35.14	33.48	.	32.71
288	bta-miR-660	26.01	28.22	26.96	26.14	24.81	26.18	26.11	26.27	26.75	26.17	27.76	26.42
289	bta-miR-664a	23.64	24.91	24.54	24.55	22.80	24.31	24.40	24.17	24.42	24.28	24.71	24.51
290	bta-miR-760-3p	25.72	27.77	26.66	27.94	24.90	.	26.73	.	27.82	27.77	28.35	26.42
291	bta-miR-664b	20.34	21.92	20.78	21.39	19.50	21.40	21.04	20.37	21.10	21.04	21.82	21.33
292	bta-miR-760-5p	23.06	24.25	22.75	24.16	21.82	23.22	22.92	22.69	.	23.80	24.84	23.53
293	bta-miR-761	29.74	.	30.21	32.00	.	.	30.35	30.18	31.84	31.72	32.11	.
294	bta-miR-669	21.71	23.67	22.50	23.32	19.80	22.42	22.71	22.11	22.34	22.86	23.41	22.28
295	bta-miR-763	27.39	28.87	27.74	28.80	24.66	27.78	28.02	.	28.51	28.78	.	27.78
296	bta-miR-670	.	32.52	34.93	34.81	31.19	31.41	.	32.94	35.53	35.04	32.72	33.85
297	bta-miR-764	29.79	30.51	29.79	31.79	.	30.76	29.57	29.86	31.86	30.79	.	31.48
298	bta-miR-671	29.43	31.09	29.80	29.76	27.68	28.81	.	29.68	30.46	.	.	29.31
299	bta-miR-767	30.44	30.71	30.00	30.47	27.32	30.22	.	30.76	30.08	30.28	30.19	29.84
300	bta-miR-677	22.29	23.76	23.75	22.13	20.34	21.20	22.80	22.28	21.83	22.29	22.73	21.67
301	bta-miR-769	28.73	30.72	28.72	.	26.71	28.70	29.00	.	29.78	29.74	30.48	28.80
302	bta-miR-7	26.88	29.64	27.39	26.52	25.35	25.84	26.86	27.50	27.60	27.42	28.43	25.69
303	bta-miR-873	28.93	.	29.81	30.84	28.40	29.77	.	29.79	.	31.57	30.77	.
304	bta-miR-708	27.47	28.99	28.42	27.83	25.49	27.35	27.68	27.34	27.73	27.55	27.97	27.72
305	bta-miR-874	26.38	27.69	26.84	27.57	24.10	26.60	27.03	26.83	27.72	27.71	28.00	26.76

306 bta-miR-744	25.20	26.82	25.66	25.77	24.99	25.73	26.02	25.79	26.24	25.70	26.47	25.38
307 bta-miR-876	31.09	35.25	33.11	36.34	30.64	32.85	32.85	.	32.90	35.20	34.40	32.67
308 bta-miR-98	26.25	29.10	26.83	26.72	25.00	26.63	26.22	26.68	27.57	26.75	28.50	25.90
309 bta-miR-877	23.80	25.35	24.87	24.54	20.73	23.70	24.75	23.80	23.43	24.40	23.99	23.83
310 bta-miR-99a-3p	28.15	30.75	28.70	28.62	26.72	28.75	28.65	27.81	29.42	28.55	29.65	28.35
311 bta-miR-885	23.64	24.41	23.50	23.95	22.26	24.30	23.70	22.95	24.82	24.09	25.84	24.11
312 bta-miR-99a-5p	22.37	23.82	22.78	20.68	19.79	20.75	21.65	21.51	22.07	21.59	22.35	20.79
313 bta-miR-9-5p	24.67	28.01	26.17	24.74	23.75	24.59	25.40	25.23	24.89	.	25.20	23.67
314 bta-miR-1179	33.43	34.23	32.89	.	.	33.15	33.99	31.84	35.34	.	.	33.63
315 bta-miR-92a	21.26	22.38	21.68	21.76	20.49	20.94	21.73	21.37	21.80	21.68	22.78	20.99
316 bta-miR-92b	19.90	20.64	19.93	20.61	19.55	20.23	20.68	19.78	20.73	20.35	21.31	19.46
317 bta-miR-1193	.	.	34.77	.	31.86	34.24	.	34.98	36.06	35.96	.	.
318 bta-miR-93	23.23	24.51	24.06	23.52	21.48	23.27	23.60	23.51	23.45	23.66	23.94	23.27
319 bta-miR-935	26.71	.	.	27.78	24.18	27.62	27.45	.	.	27.87	.	27.29
320 bta-miR-122	.	35.89	.	34.63	.	33.40	33.84	.	35.64	33.92	.	32.05
321 bta-miR-940	21.96	22.75	22.36	22.83	17.78	22.65	22.30	21.87	22.40	22.83	22.70	22.61
322 bta-miR-1224	19.99	20.94	21.34	21.07	16.47	20.58	20.71	20.29	19.83	20.20	20.21	20.59
323 bta-miR-95	27.20	28.75	26.87	26.20	25.79	25.50	27.57	27.77	28.18	26.63	29.68	25.50
324 bta-miR-1225-3p	22.42	23.67	22.78	.	19.84	22.65	22.81	22.73	23.60	23.35	23.80	22.82
325 bta-miR-96	28.70	30.21	28.35	26.50	24.55	27.12	27.71	28.46	28.80	28.78	28.75	27.90
326 bta-miR-1246	12.64	14.03	13.15	13.49	10.83	13.07	13.25	12.70	12.71	12.91	13.65	13.29
327 bta-miR-1247-3p	24.87	26.51	25.74	26.30	22.15	25.81	25.79	25.56	25.74	25.78	25.89	25.83
328 bta-miR-1296	24.50	26.89	25.60	26.70	.	25.66	25.89	25.39	25.80	26.29	26.65	25.24
329 bta-miR-1247-5p	23.64	24.68	24.69	23.84	19.51	23.78	23.84	23.63	23.65	24.29	23.83	24.17
330 bta-miR-1298	30.80	33.94	.	36.62	.	.	.	.	33.85	36.53	32.90	33.44
331 bta-miR-1248	19.17	20.51	19.57	20.01	17.19	19.78	19.79	19.39	19.66	19.69	19.90	20.10
332 bta-miR-1301	30.85	31.08	31.00	34.03	29.79	30.53	32.18	30.86	32.18	31.85	30.70	29.90
333 bta-miR-1249	24.92	25.87	25.60	26.22	24.60	26.12	24.90	24.76	26.37	26.28	26.68	24.83
334 bta-miR-1306	25.49	26.77	26.04	26.00	24.76	25.91	26.03	25.73	26.87	26.18	27.61	25.57
335 bta-miR-1260b	18.10	17.38	18.80	19.25	16.70	18.72	18.71	18.31	18.69	18.50	18.70	18.69
336 bta-miR-1307	19.98	21.05	20.97	23.13	17.78	21.02	20.77	19.96	21.00	21.04	21.50	20.83
337 bta-miR-1271	29.81	32.85	29.74	28.88	28.58	28.76	30.69	30.71	30.93	29.96	30.53	28.51
338 bta-miR-1343-3p	24.58	25.97	24.81	25.29	23.84	24.69	25.22	24.81	25.62	25.39	26.54	24.49
339 bta-miR-1343-5p	23.07	24.03	23.66	23.79	18.83	23.21	23.79	23.37	23.39	23.76	23.42	23.44
340 bta-miR-1281	23.35	25.70	23.56	25.30	22.63	24.35	24.11	24.22	25.16	25.61	25.68	23.79
341 bta-miR-1388-3p	26.00	27.90	26.66	26.55	23.22	26.63	26.47	26.78	26.67	26.73	27.91	25.81
342 bta-miR-1282	31.08	34.49	31.27	33.90	.	32.69	.	31.82	33.58	35.00	34.58	31.81
343 bta-miR-1284	29.50	31.00	.	.	30.15	29.45	.	.	31.90	32.84	33.42	29.84
344 bta-miR-1287	28.79	31.75	29.77	31.84	29.54	30.42	31.38	30.76	30.79	32.04	32.48	30.18
345 bta-miR-1291	21.56	23.34	22.87	22.73	19.24	21.75	22.77	21.73	21.85	22.20	.	22.48

Source: MAZZARELLA, R. et al. Changes in Oviductal Cells and Small Extracellular Vesicles miRNAs in Pregnant Cows. **Frontiers in Veterinary Science**, v. 8, n. March, p. 1–14, 4 mar. 2021

APPENDIX G – Full list of biological pathways with significant p-value ($p < 0.05$) predicted as modulated by 6 miRNAs (bta-miR-133b, bta-miR-205, bta-miR-584, bta-miR-551a, bta-miR-1193 and bta-miR-1225-3p) up-regulated in OECs from non-pregnant compared to pregnant cows.

Pathways	Number of genes	P-value
bta04360 Axon guidance	70	0
bta04014 Ras signaling pathway	91	0
bta04010 MAPK signaling pathway	105	0
bta05212 Pancreatic cancer	37	0.0001
bta04062 Chemokine signaling pathway	67	0.0003
bta05220 Chronic myeloid leukemia	34	0.0005
bta04810 Regulation of actin cytoskeleton	71	0.0008
bta04068 FoxO signaling pathway	49	0.0008
bta05214 Glioma	33	0.0009
bta05200 Pathways in cancer	155	0.001
bta04530 Tight junction	61	0.0011
bta05205 Proteoglycans in cancer	68	0.0014
bta00564 Glycerophospholipid metabolism	40	0.0015
bta04722 Neurotrophin signaling pathway	45	0.0016
bta04072 Phospholipase D signaling pathway	53	0.0018
bta04660 T cell receptor signaling pathway	40	0.0023
bta01522 Endocrine resistance	36	0.0027
bta04921 Oxytocin signaling pathway	52	0.0027
bta04012 ErbB signaling pathway	33	0.0028
bta04514 Cell adhesion molecules (CAMs)	53	0.0035
bta04912 GnRH signaling pathway	35	0.0038
bta04928 Parathyroid hormone synthesis. secretion and action	38	0.0039
bta05215 Prostate cancer	36	0.0046
bta04550 Signaling pathways regulating pluripotency of stem cells	48	0.0046
bta04710 Circadian rhythm	16	0.0047
bta04310 Wnt signaling pathway	53	0.0052
bta04611 Platelet activation	42	0.0053
bta05211 Renal cell carcinoma	28	0.0054
bta04925 Aldosterone synthesis and secretion	35	0.0056
bta04020 Calcium signaling pathway	63	0.0061
bta05163 Human cytomegalovirus infection	74	0.0061
bta04934 Cushing syndrome	51	0.0061
bta04071 Sphingolipid signaling pathway	41	0.0072
bta04137 Mitophagy	26	0.0072
bta05225 Hepatocellular carcinoma	55	0.0079
bta04144 Endocytosis	73	0.0082

bta05223 Non-small cell lung cancer	26	0.0083
bta04150 mTOR signaling pathway	50	0.0097
bta04152 AMPK signaling pathway	41	0.0099
bta05224 Breast cancer	48	0.0103
bta04920 Adipocytokine signaling pathway	27	0.0103
bta04261 Adrenergic signaling in cardiomyocytes	48	0.0103
bta04211 Longevity regulating pathway	32	0.0103
bta04024 cAMP signaling pathway	68	0.011
bta04061 Viral protein interaction with cytokine and cytokine receptor	33	0.012
bta05219 Bladder cancer	18	0.0122
bta04919 Thyroid hormone signaling pathway	39	0.0128
bta04668 TNF signaling pathway	39	0.0128
bta05226 Gastric cancer	48	0.0135
bta04926 Relaxin signaling pathway	42	0.0136
bta01100 Metabolic pathways	372	0.014
bta04130 SNARE interactions in vesicular transport	15	0.0143
bta04961 Endocrine and other factor-regulated calcium reabsorption	20	0.0149
bta04390 Hippo signaling pathway	48	0.0176
bta04931 Insulin resistance	36	0.0181
bta04015 Rap1 signaling pathway	63	0.0182
bta05218 Melanoma	26	0.0188
bta04728 Dopaminergic synapse	42	0.0198
bta04520 Adherens junction	25	0.0204
bta05210 Colorectal cancer	30	0.0216
bta04371 Apelin signaling pathway	43	0.0239
bta04659 Th17 cell differentiation	36	0.0244
bta05160 Hepatitis C	49	0.0245
bta04917 Prolactin signaling pathway	28	0.0255
bta04625 C-type lectin receptor signaling pathway	34	0.0261
bta04022 cGMP-PKG signaling pathway	50	0.0265
bta04070 Phosphatidylinositol signaling system	32	0.0279
bta04140 Autophagy	43	0.0282
bta04922 Glucagon signaling pathway	33	0.0283
bta04923 Regulation of lipolysis in adipocytes	21	0.0283
bta00310 Lysine degradation	23	0.0308
bta04911 Insulin secretion	28	0.0317
bta00561 Glycerolipid metabolism	23	0.0348
bta05166 Human T-cell leukemia virus 1 infection	65	0.035
bta04510 Focal adhesion	56	0.0371
bta05202 Transcriptional misregulation in cancer	54	0.0401
bta04933 AGE-RAGE signaling pathway in diabetic complications	32	0.0408
bta05412 Arrhythmogenic right ventricular cardiomyopathy (ARVC)	25	0.041
bta04713 Circadian entrainment	31	0.0444
bta05100 Bacterial invasion of epithelial cells	24	0.0447
bta00562 Inositol phosphate metabolism	24	0.0447
bta04370 VEGF signaling pathway	20	0.0451
bta04910 Insulin signaling pathway	41	0.0454

bta04340 Hedgehog signaling pathway	18	0.0475
bta04350 TGF-beta signaling pathway	29	0.0479
bta04670 Leukocyte transendothelial migration	34	0.0491

Source: MAZZARELLA, R. et al. Changes in Oviductal Cells and Small Extracellular Vesicles miRNAs in Pregnant Cows. **Frontiers in Veterinary Science**, v. 8, n. March, p. 1–14, 4 mar. 2021.