miRNA Expression in Equine Follicular Fluid Secretome is Affected by the Clinical Condition and Age of the Mare

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The ovulation of a mature and competent oocyte is the result of intricate molecular interactions between follicular cells and the oocyte. In horses, *in vitro* maturation (IVM) is essential for *in vitro* embryo production, but success rates remain limited. The supplementation of IVM media with follicular fluid secretome has been proposed to enhance oocyte maturation rates. Significant progress has been made in unraveling the complete molecular signature of the intrafollicular milieu. However, we have yet to fully comprehend whether specific factors within the follicular fluid (FF) secretome could serve as biomarkers for predicting oocyte maturation rates, subsequent blastocyst formation, and ultimately, pregnancy rates.

To analyze if miRNA expression in equine FF secretome is affected by the clinical condition and age of the mare, preovulatory FF was collected from 8 mares aged between 7 and 18 years, 32 hours post hCG stimulation. For secretome isolation, the FF from each mare was diluted 1:3 with PBS and filtered through 10 kDa ultra-centrifugal units at 4000×g for 1 hour at 4°C. miRNA extraction was then conducted, followed by polyadenylation, reverse transcription reactions, and qPCR analysis to examine the expression of a panel of 10 miRNAs (miR-19b, miR-21, miR-23a, miR-24, miR-27b, miR-92a, miR-132, miR-372, miR-378, and miR-382) associated with follicle growth and oocyte maturation. qPCR products were quantified by using the $2^{-\Delta\Delta Ct}$ method, normalizing against U6 snRNA expression. Data for relative miRNA abundance were compared using the unpaired *t*-test. Furthermore, equine proteins within the FF secretome were identified using hybrid trapped ion mobility spectrometry coupled with liquid chromatography. miRNet 2.0 was used to predict the interactions between secretome miRNAs and proteins.

Our analysis revealed individual variability in miRNA expression, which correlated with the clinical condition of the mares and their age. Notably, one mare was healthy but aged (17 years), one was clinically obese (7 years), while another suffered from laminitis and was of advanced age (18 years). We identified six upregulated miRNAs (miR-19b, miR-21, miR-27b, miR-92a, miR-132, miR-382, $p<0.05$) in the group of unhealthy and older mares compared to the healthy and younger ones.

Based on our network analysis, the upregulated miRNAs were found to play crucial roles in various biological processes, including autophagy, angiogenesis, chromatin remodeling, and carbohydrate metabolism. Furthermore, these miRNAs were observed to interact with 30 protein-coding genes identified in the proteomic analysis. Interestingly, these targeted genes were implicated in Reactome pathways, such as extracellular matrix organization and the immune system. Despite the potential for the FF secretome composition to reflect the clinical condition and age of the mare, our

analysis did not reveal any differences in terms of follicle count or oocyte collection during ovum pick up (OPU).

Our study found six upregulated miRNAs in the secretome of unhealthy and old mares, indicating their potential use as biomarkers to predict FF secretome quality and oocyte maturation rates. However, further functional studies are required to elucidate their precise role in oocyte maturation.

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