

Exploring the Influence of Extracellular Vesicle Supplementation on the Equine Oocyte Fertilization Rate after ICSI

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The efficiency of *in vitro* embryo production in horses is limited compared to other animal species. This is due to the lack of an effective superovulation method, low availability of oocyte-cumulus complexes (COC), along with the limited rates of *in vitro* oocyte maturation (IVM) and poor fertilization and blastocyst rate.

Extracellular vesicles (EVs) are nanoparticles secreted by all body cells, playing a significant role in intercellular communication within the ovarian microenvironment, due to their ability to transport bioactive components. They are used in various studies as potential biomarkers and supplementary ingredients that allow to imitate the physiological conditions during IVM and, therefore, enhance the oocyte developmental potential.

The hypothesis of the conducted research was that EVs obtained from small ovarian follicles (< 20 mm) could improve the fertilization rate in mares. Follicular fluid was aspirated *post mortem* from ovaries lacking a corpus luteum, and small EVs were extracted using ultracentrifugation and characterized. The IVM process was performed in the commercial EQ-IVM medium (IVF-BioScience) with the addition of EVs (200 µg of EV protein/ml) or without supplementation (control). Additionally, EVs internalization during IVM was examined using fluorescent particle labeling and confocal microscopy. *In vitro* fertilization was performed by intracytoplasmic sperm injection (ICSI) of oocytes and presumptive zygotes were cultured *in vitro* in TCM 199 (Earl's salts, with the addition of FBS and gentamicin) for 5-7 days in the time-lapse system.

Confocal microscopy confirmed the internalization of EVs by both cumulus cells and the oocyte. Nanoparticle tracking analysis showed that the average size of EVs obtained from follicular fluid was 110.7 nm, and flow cytometry confirmed the presence of the surface markers, tetraspanin CD81

and CD63 in 56.5% and 22.4% of the particles, respectively. Using transmission electron microscopy, the disk shape characteristic of EVs was observed in the isolates. Total of 532 COCs designated for IVM were obtained from 75 slaughtered mares. After IVM, 196 oocytes (36.84%) exhibited the presence of a first polar body, with the experimental and control groups showing IVM rates of 39.76% and 34.28% respectively. A higher fertilization rate (34.7% vs. 20.2%) ($p < 0.05$), a slightly lower degeneration and improvement in the cleavage efficiency ($p < 0.1$) were observed in the experimental group compared to the control. Although in both groups the embryonic development arrested at the early stages, the results indicate the potential of follicular fluid-derived EVs as a factor supporting IVF in horses.

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