Research

Extracellular Vesicles in the Udder Fluid from the Mare with Juvenile Galactorrhea

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Abstract: Extracellular particles (EPs) were investigated by interferometric light microscopy (ILM) in the udder fluid from the mare with juvenile galactorrhea. Number density n and hydrodynamic diameter D_h of EPs were measured. The samples were taken from a 1 year old Posavje mare. Higher number density (n) of nano - sized EPs were found in in the whole fluid in comparison with the skimmed fluid, the difference was statistically significant which is the opposite from the mare's mature milk or colostrum which was shown in our previous investigation when mature mare milk and colostrum were investigated. While the value of the udder fluid n was considerably lower than in the mature mare milk, the values of D_h in the respective samples were within the error of the method. The value of D_h was higher in the skimmed fluid in comparison with the whole fluid, and the difference was statistically significant.

Keywords: Extracellular vesicles; Light scattering; Vesicle characterization; Mare; Udder fluid; Milk; Nanovesicles

1. Introduction

1.1. Milk as a complete food

Mare's milk is the most important nutritional resource for foals during the first months of life. It contains proteins (corpuscular casein and dissolved whey proteins), lipids (fat globules), carbohydrates (milk sugar - lactose), vitamins and minerals ($Ca2^+$, P^+, K^+, Na^+). It has antimicrobial activity and antiviral activity. (Musaev et al., 2021; Malacarne et al., 2002). Human and cow milk contains extracellular particles like corpuscular casein micelles, fat globules and extracellular vesicles. (Hu et al., 2021). Here, we investigated the extracellular particles from the fluid of the mare with juvenile galactorrhea. The concentration was measured by interferometric light microscopy.

1.2. What is juvenile galactorrhea in the mare?

Juvenile galactorrhea in the mare refers to milk secretion in a young female horse (mare or filly) that has not been bred or is not pregnant. It is an inappropriate lactation in a non-pregnant, immature mare, usually due to hormonal dysregulation. In a normal situation, milk production in mares is triggered late in pregnancy under the influence of hormones like prolactin and estrogen. In juvenile galactorrhea, this process happens abnormally early, typically in immature or prepubertal fillies. It occurs without pregnancy or foaling (McCue & Sitters, 2011; Chavatte, 1997). Mares or fillies have enlarged udder (mammary gland) with milk or milk-like discharge – "fluid" from the teats.

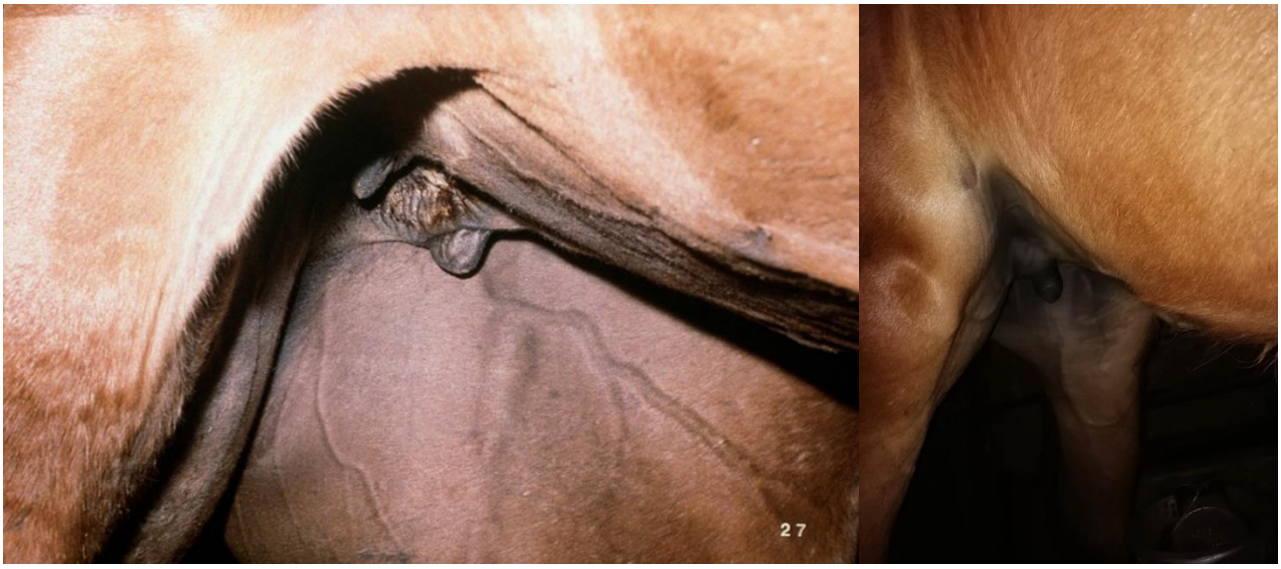

Figure 1. The udder of a maiden mare is shown on the left image (Image from: https://wellcomecollection.org/works/a2j9qt79/images?id=brwex7sh). On the right image the udder of a maiden mare with juvenile galactorrhea is shown. An enlarged teat is clearly visible.

2. Material and Methods

2.1. Milk Sampling

For interferometric light microscopy either udder fluid was milked by hand from an 1 year old Posavje (breed) mare. The fluid was collected into tubes VACUETTE® TUBE 3 ml Z No Additive 13x75 white cap-black ring, non-ridged (Greiner AG, Kremsmünster, Austria). To obtain skimmed fluid, whole fluid was centrifuged at 300 g for 15 min. This procedure was repeated twice. The "cream" was removed from the top using a pipette with the tip shortened for 2 mm by scissors. The tubes with colostrum and milk are shown in **Figure 2**.

Figure 2. The left tube contains udder fluid of the mare with juvenile galactorrhea and the right tube the normal mare milk.

2.2. *Interferometric Light Microscopy (ILM)*

The average hydrodynamic diameter (Dh) and the number density of small particles in milk were determined by interferometric light microscopy using Videodrop (Myriade, Paris, France) as described previously (Romolo et al., 2022). Before measurement the milk was diluted 100 × by saline for injections (Braun, Melsungen, Germany) to obtain a measurable dilution. Signals of the saline were under the detection limit. The threshold value 4.2 was used. 7 µL of sample was placed between cover glasses and illuminated by 2W blue LED light. The light scattered on the particle was imaged by a bright-field microscope objective and allowed to interfere with the incoming light. The image was recorded by a complementary metal–oxide–semiconductor high resolution speed camera. The obtained pattern that includes contrasting black and white spots was recognized as a particle and its position in the sample was assessed. Number density of the particles is the number of the detected particles within the detected volume (e.g. 15 pL). D_h was determined by tracking the position of the imaged particle within the recorded movie. It was assumed that particles undergo Brownian motion due to collisions with surrounding particles. The diffusion coefficient D of the motion of the particle is taken to be proportional to the mean square displacement d of the particle between two consecutive frames taken in the time interval Δt, $\langle d^2(\Delta t)\rangle = \langle 4D\,\Delta t\rangle$, where t is time, and D is diffusion coefficient. D_h was estimated by assuming that the particles were spherical and using the Stokes-Einstein relation $D_h = k_B T/3\pi\eta D$, where k_B is the Boltzmann coefficient, T is temperature and η is viscosity coefficient. Each particle that was included in the analysis was tracked and processed individually and the respective incident light signal was subtracted from each image. Processing of the images and of the movies was performed by using the associated software QVIR 2.6.0 (Myriade, Paris, France) (Romolo et al., 2022).

2.3. *Light microscopy*

Samples were placed into observation chamber, created between two cover glasses delimited by silicon grease and observed by the Nikon Eclipse 2000-S inverted optical microscope.

2.4. *Statistical analysis*

All measurements were performed in triplicates and presented by the average values and standard deviations. The differences were evaluated by the t-test using the Excel sofware. The value p = 0.05 was taken as a threshold for statistical significance.

3. Results

Higher number density (n) of nano - sized EPs were found in in the whole fluid in comparison with the skimmed fluid, the difference was statistically significant **(Figure 3)**. The average hydrodynamic diameter D_h was larger in the skimmed fluid in comparison with the whole fluid, the difference was statistically significant **(Figure 3)**. The difference between the average hydrodynamic diameters Dh was within the error of the method.

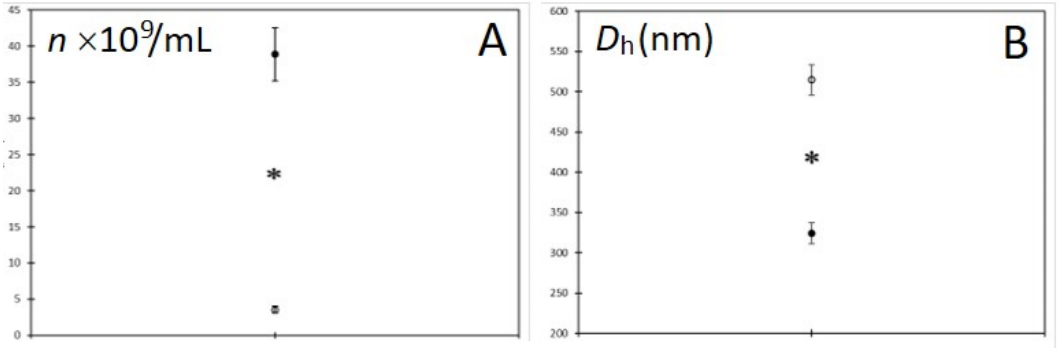

Figure 3. Average number density n (left) and average hydrodynamic diameter D_h of EPs in the mare whole (full circles) and skimmed (empty circles) udder fluid. The error bars represent standard deviations. Asterisk (*) represents statistically significant difference between the whole and the skimmed milk results.

Figure 4. Optical microscope images of : whole fluid (left image), skimmed fluid (middle image) and fluid supernatant – "cream" (right image). Magnification 600x.

4. Discussion

The number density of EPs n was higher in the whole fluid in comparison with the skimmed fluid, the difference was statistically significant (**Figure 3**). In a previous study of mare's colostrum and milk, the number density of EPs was higher in the colostrum than in milk (Arko et al., 2024b). The value of n in the "udder fluid" was much lower than in the mature mare milk from our previous investigation.

D_h was larger in the skimmed fluid in comparison with the whole fluid, the difference was statistically significant (**Figure 3**). A similar featue was observed in the mare's mature milk at day 7 after parturition (Arko et al., 2024b). The number density of micro and nano-sized particles n in skimmed mature equine milk measured by interferometric light microscopy and flow cytometry was observed when compared to the whole mature equine milk, and the differences were statistically significant (Arko et al. 2024a). Higher number density of EPs was found in the skimmed equine colostrum and milk when compared with the whole milk, but the difference was statistically significant only on day 7 after the parturition (Arko et al., 2024b).

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki.

Conflicts of Interest: The authors declare no conflict of interest.

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