Invited lecture/Research

Extracellular Particles from Equine Milk

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Abstract:
Equine and bovine milk extracellular particles were investigated by scanning electron microscopy, interferometric light microscopy and flow cytometry. Scanning electron microscopy revealed micro-sized globular particles and numerous nano-sized particles. Higher concentration of micro and nano-sized particles was found in skimmed milk when compared to the whole milk and the differences were statistically significant.

Keywords: Extracellular vesicles; Milk; Interferometric light microscopy; Flow cytometry; Scanning electron microscopy; Concentration of particles

1. Introduction

1.1. Milk as a complete food

Milk is a complete food with all nutritionally important components required for a newborn mammal – it comes in liquid form and contains proteins (corpuscular casein and dissolved whey proteins), fat (fat globules) and carbohydrates (milk sugar, i.e., lactose) (Musaev et al., 2021, Malacarne et al., 2002).

1.2. Milk as a source of a variety of particles

Milk is also a source of a variety of small particles including molecules, their complexes, and extracellular particles (Ong SL et al., 2021). It contains bioactive components derived from various cellular sources (Musaev, A et al., 2021) and immunologically active cells (Palmeira P et al., 2016). Here, we investigated the extracellular particles from equine milk. The concentration was measured by interferometric light microscopy and flow cytometry. To observe the morphology of particles, scanning electron microscopy micrographs were obtained from equine milk.

2. Methods

2.1. Milk Sampling

For flow cytometry and interferometric light microscopy animal was milked by hand from a Posavje (breed) mare on 91st day of lactation. Milk 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 milk, whole milk was centrifuged at 300 g for 15 min and the cream was removed from the top using a pipette with the tip shortened for 2 mm by scissors. The procedure was repeated twice. For scanning electron microscopy, the milk samples were obtained at different days of lactation from Posavje (breed) mare.

2.2. Scanning electron microscopy (SEM)

The milk samples were incubated with added 1% OsO_4 for 1 hour, washed 3 times for 10 minutes with dH_2O, dehydrated in graded ethanol (EtOH): 30%, 50%, 70%, 80% and 90% for 10 minutes at each concentration and in absolute EtOH 2-times for 10 minutes, incubated with added absolute EtOH and HMDS (ratio 3:7) for 10 minutes, incubated in added absolute EtOH and HMDS (ratio 1:1) for 10 minutes, incubated in added 100% HMDS for 10 minutes and depleted of HMDS by evaporation in exicator with silica gel for 12 hours. After fixation, samples were gold-sputtered and observed by the scanning electron microscope (SEM, JEOL JSM-6500F).

2.3 Flow Cytometry (FCM)

The particle numbers in samples from equine and bovine milk were estimated by flow cytometry, using a MACSQuant Analyzer flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) and the related software. Before measurement, the milk was diluted by saline to obtain a measurable dilution (200x bovine and equine whole and skimmed milk). The following instrument settings were employed: FSC: 458 V; SSC: 467 V with a trigger set to 1.48; B3: 300 V; and R1: 360 V. Particles were detected from the forward (FSC) and side scatter parameters (SSC).

2.4 Interferometric Light Microscopy (ILM)

The average hydrodynamic diameter (D_h) and the number density of small particles in milk were determined by interferometric light microscopy using Videodrop (Myriade, Paris, France). Before measurement the milk was diluted by saline for injections (Braun, Melsungen, Germany) to obtain a measurable dilution. The range of dilution was 8x - 500x for bovine whole milk, 32x - 500x bovine skimmed milk and 16 x - 50x for equine whole and skimmed milk. 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 high 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, $\langle d^2(\Delta t)\rangle = \langle 4D\ \Delta t\rangle$ while D_h was estimated by assuming that the particles were spherical and using the Stokes-Einstein relation $D_h = kT/3\pi\eta D$. 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).

2.5. 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 software. The value p = 0.05 was taken as a threshold for statistical significance.

3. Results

Concentration and size of the particles in the equine milk was obtained by FCM and ILM **(Table 1).**

SEM micrographs revealed presence of globular particles' morphology (**Figure 1**).

Table 1. Concentration and size of particles in the equine whole and skimmed milk determined by FCM and ILM. The measurement was done on the 91st day of lactation. Average ± Standard Deviation assessed from 3 measurements are given.

Sample	ILM (size range 80 nm - 400 nm)	ILM	FCM
	Number density ($\times 10^9$/mL)	D_h (nm)	Number density ($\times 10^9$ /mL)
91st day whole milk	28.89 ± 2.14	308 ± 12	0.23 ± 0.06
91st day skimmed milk	36.80 ± 2.00	292 ± 7	2.14 ± 0.41
p (t-test)	0.009	0.12	<0.00001

4. Discussion

According to Walstra (1969), in the bovine milk there is a subpopulation of milk fat globules with a diameter smaller than 1 μm. They seem to represent 80% of all milk fat globules but only approximately 5 % of the volume of the milk fat (Walstra, 1969).We found significantly higher concentration of EPs sized 80 – 500 nm in the skimmed milk than in the whole milk indicating that fat globules occupy larger space but are not very numerous. Equine milk fat globules were reported to be relatively small, their average volume-surface diameter being about 2.75 μm (Welsch et al., 1988). In the cow's milk, a population of medium globules (ranging from 1 to 8 μm) was measured; it was estimated that these globules constitute approximately 94% of the fat volume (Walstra, 1969). However, when the average size of EPs in the equine milk was assessed by ILM, there was no significant difference between the whole and skimmed milk, which might be explained by the low fat content in the equine milk.

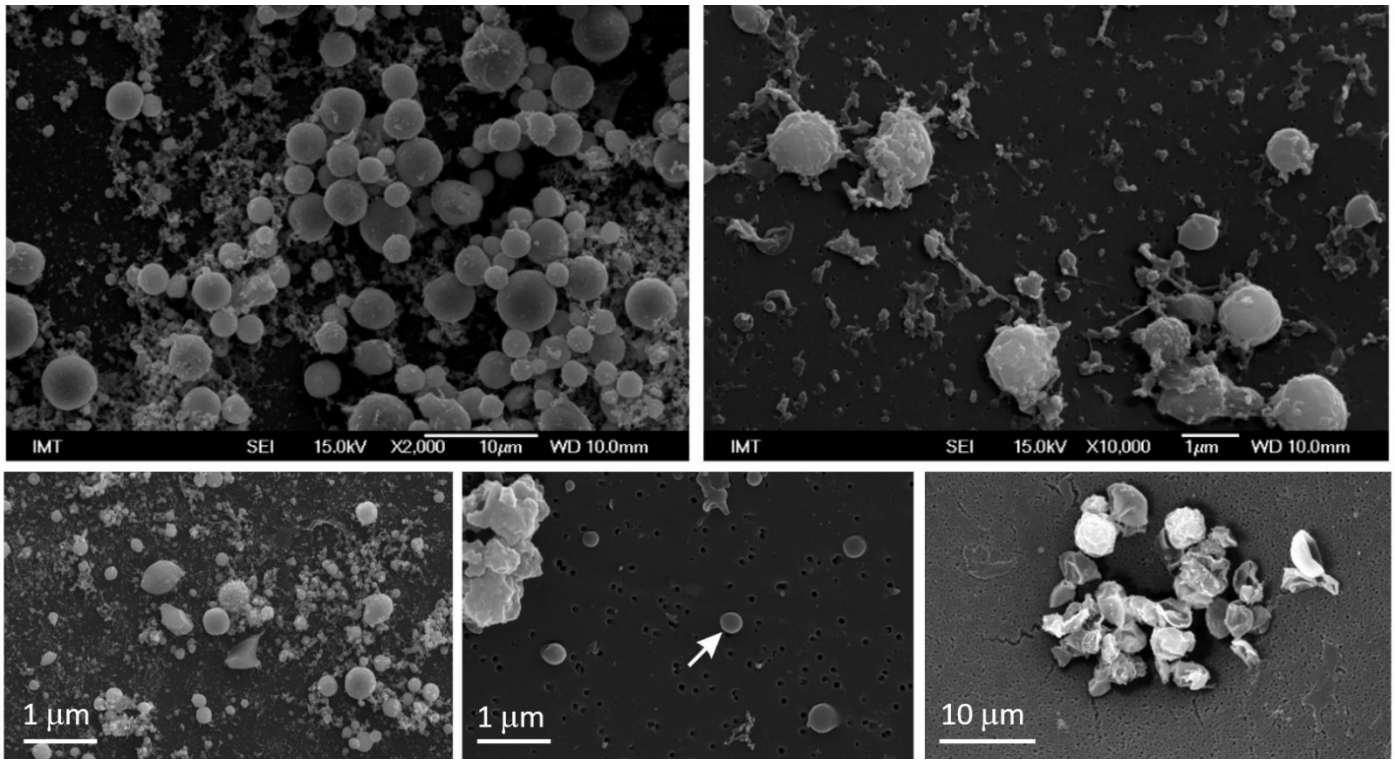

Figure 1. SEM micrographs of equine milk: A – Equine milk on the 1st day after parturition, B – Equine milk on the 1st day after parturition, C – Equine milk on the 2nd day after parturition, D – Equine milk on the 3rd day after parturition, E – Equine cream.

The content of fat in the equine milk was found lower (1,21%) than in the bovine milk (3,61%) and in the human milk (3,64%) (Malacarne M, et al., 2002). Similarly, the concentration of particles measured by FCM was significantly higher in the skimmed milk and lower in the whole milk. The average size of the EPs measured by ILM was significantly higher in the whole milk which might be due to milk fat globules (present in the whole milk) and casein micelles (present in the whole and skimmed milk).

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

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