Comparative Analyses of Small Extracellular Vesicles Isolation Strategies in Equine Plasma

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Introduction

Small extracellular vesicles (sEV) are a heterogeneous group of nanometric-sized particles (50 - 200 nm) of cellular origin and bounded by a membrane. They play a crucial role in cell communication by carrying various biologically active molecules (proteins, lipids, nucleic acids, etc.). Many studies have demonstrated their presence in biofluids and their role in a multitude of physiological and pathological processes, thus revealing their potential as diagnostic biomarkers. Many diseases are not well-defined yet in horses, and the use of sEV as biomarkers could confer a better understanding these pathological mechanisms.

The standard method for isolating sEV is the ultracentrifugation-based protocol developed by Théry et al. 2006. However, to date, there is no published protocol on the isolation and analysis of sEV from equine plasma. To obtain a reproducible protocol for isolating and characterizing sEV from horse plasma, three isolation methods (Ultracentrifugation [**UC**], Size Exclusion Chromatography [**SEC**] and a Commercial Kit [**CK**]) were compared based on the quantity and quality of recovered sEV. Additionally, the impact of the time (**0h**, **12h** and **24h**) between collection and storage of the sEV was also evaluated to analyze its effect on sEV integrity.

Materials and Methods

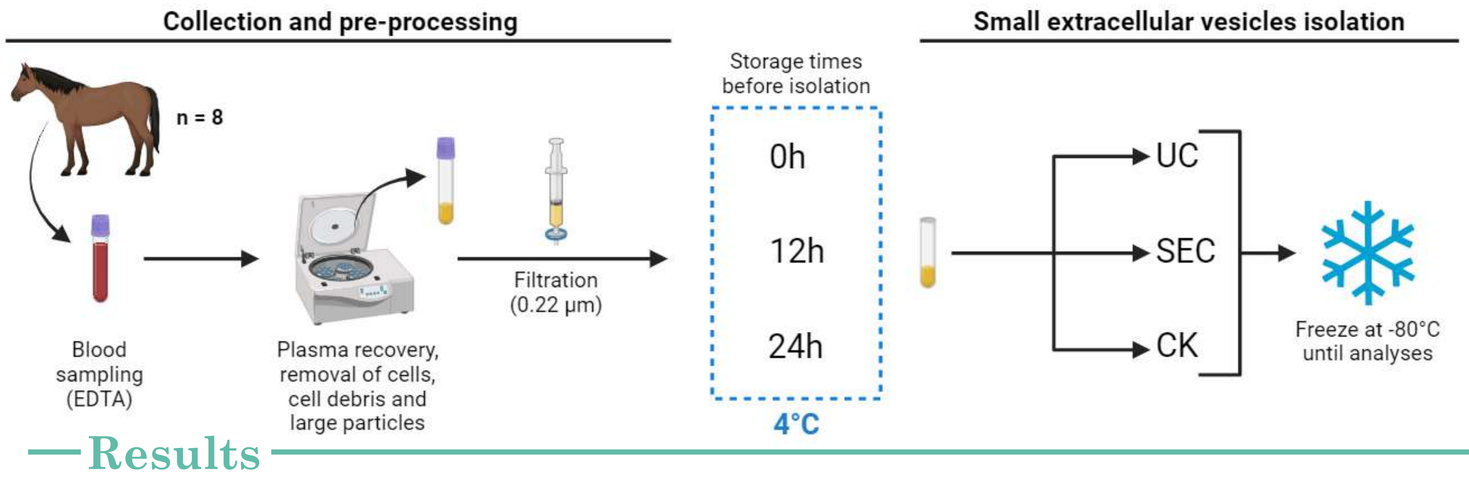

Figure 1. Experimental process for isolating sEV from horse plasma. Eight samples of horse blood were collected, then centrifuged and filtered to collect the plasma, remove cells, and large extracellular particles (>1µm). The plasma was then divided into three lots (0h, 12h, and 24h) to study the effect of time on sEV integrity. sEV were isolated from each plasma lot using three isolation methods (UC, SEC, and CK), aliquoted, and stored at -80°C for subsequent analyses.

Results

Expression of sEV specific markers

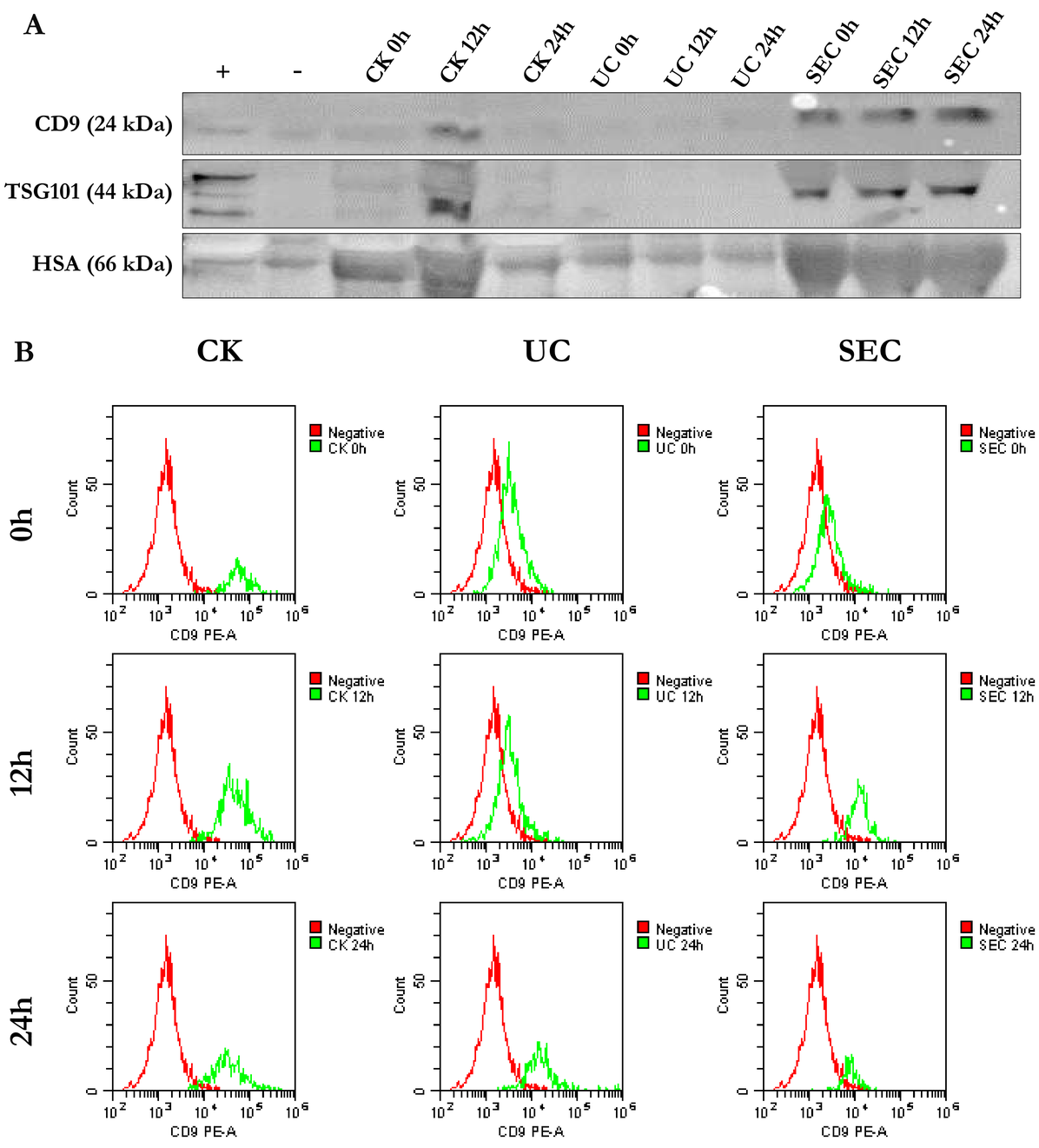

Figure 2. Characterization of sEV markers. (**A**) Western blots were performed to confirm the expression of several proteins associated with sEV (CD9, TSG101). Horse serum albumin (HSA) was used as a purity marker. (**B**) sEV were detected by flow cytometry using the Immunostep kit (CD63 beads and CD9 antibody).

- **All methods enabled the isolation of particles expressing the markers CD9, CD63, and TSG101.**
- **No effect of time was observed on the detection and levels of sEV markers.**

Physical, morphological and purity characterization

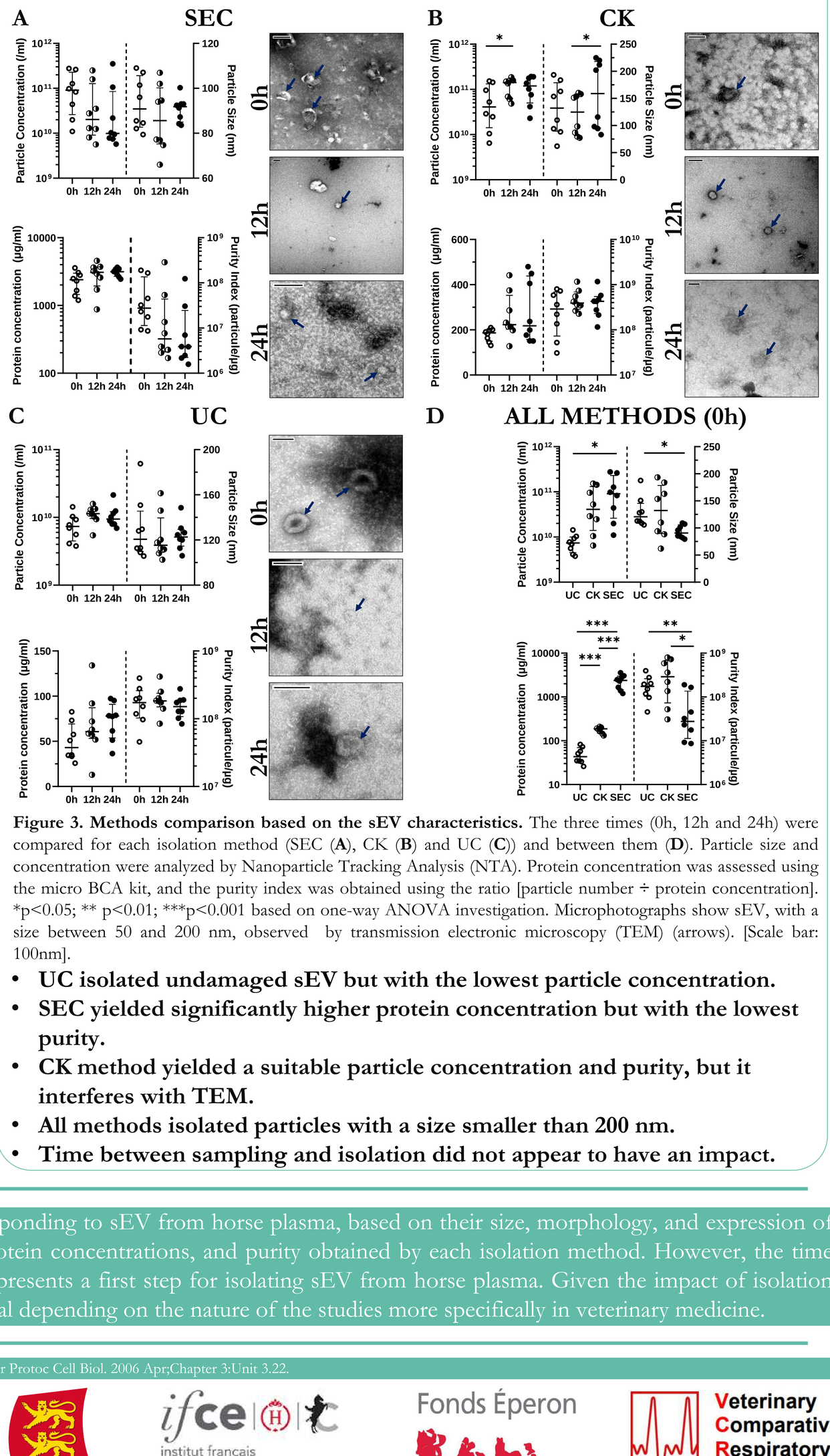

Figure 3. Methods comparison based on the sEV characteristics. The three times (0h, 12h and 24h) were compared for each isolation method (SEC (**A**), CK (**B**) and UC (**C**)) and between them (**D**). Particle size and concentration were analyzed by Nanoparticle Tracking Analysis (NTA). Protein concentration was assessed using the micro BCA kit, and the purity index was obtained using the ratio [particle number ÷ protein concentration]. *p<0.05; ** p<0.01; ***p<0.001 based on one-way ANOVA investigation. Microphotographs show sEV, with a size between 50 and 200 nm, observed by transmission electronic microscopy (TEM) (arrows). [Scale bar: 100nm].

- **UC isolated undamaged sEV but with the lowest particle concentration.**
- **SEC yielded significantly higher protein concentration but with the lowest purity.**
- **CK method yielded a suitable particle concentration and purity, but it interferes with TEM.**
- **All methods isolated particles with a size smaller than 200 nm.**
- **Time between sampling and isolation did not appear to have an impact.**

Conclusion

Our study demonstrates that all isolation methods enabled the recovery of particles corresponding to sEV from horse plasma, based on their size, morphology, and expression of specific markers. Furthermore, significant differences were observed in particle yields, protein concentrations, and purity obtained by each isolation method. However, the time between sampling and isolation did not influence these parameters. To date, our study represents a first step for isolating sEV from horse plasma. Given the impact of isolation method on sEV integrity, yield and purity, careful selection of the isolation method is crucial depending on the nature of the studies more specifically in veterinary medicine.

References

Théry C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol. 2006 Apr;Chapter 3:Unit 3.22.

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