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Short communication

Extracellular vesicles in synovial fluid from juvenile horses: No age-related changes in the quantitative profile

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ABSTRACT

Extracellular vesicle (EV) concentration, characteristics and function in equine synovial fluid (SF) during normal growth and development has not previously been studied. Isolation of EVs was performed in SF from three healthy foals and two adult horses by differential ultracentrifugation (10,000*g* and 200,000*g*); EVs were purified by sucrose density gradient floatation and analysed by high-resolution flow cytometry (FCM), buoyant density and western blotting. Additionally, repeated biomarker analysis of sulphated glycosaminoglycans (GAG), matrix metalloproteinase (MMP), C-terminal crosslinked telopeptide type II collagen (CTX-II), collagenase cleaved neopeptide type II collagen (C2C) was performed in SF from 10 foals and six adult horses. In contrast with the quantitative EV profile, the biomarker profile in SF from juvenile joints was substantially different from that in SF from adult animals. However, there were qualitative differences in the high-resolution FCM scatter plots. Future in-depth functional analyses may reveal differences between juvenile and mature EVs in SF.

Biomarker studies have shown that the immature joint is characterised by quickly changing anabolic and catabolic parameters, because of the high metabolic activity of juvenile cartilage (Brama et al., 1998; van den Boom et al., 2004).

There is an increasing interest in the role of extracellular vesicles (EVs) in health and disease. These EVs facilitate intercellular communication by, among other mechanisms, stable transfer of proteins, lipids and nucleic acids (El Andaloussi et al., 2013) and have been identified in synovial fluid (SF; Boere et al., 2016). Extracellular vesicles are important actors in joint disease (Buzas et al., 2014) and EVs from embryonic stem cells have been shown to promote osteochondral regeneration in vivo (Zhang et al., 2016). Hence, EVs have been postulated as a possible new tool for cartilage and bone repair (Malda et al., 2016), but there is still a lack of fundamental knowledge about these structures in the joint.

We hypothesised that basic EV parameters, including EV concentration, would reflect the developmental stage of the articular tissues. Therefore, basic EV parameters in SF from healthy foals and adult horses were investigated and compared to reference profiles of biomarkers known to reflect growth and development.

Synovial fluid samples (See Appendix: Supplementary Table 1) were collected in vivo from ten healthy immature Haflinger horses, aged between 12 days and 13 months (repeated sampling; total $n = 22$) and post-mortem from six adult Warmblood horses, aged between 6 and 15 years.

Samples were analysed for proteoglycans by measuring sulphated glycosaminoglycan (GAG) concentration using the 1,9-dimethyl-methylene blue (DMMB) assay and for overall matrix metalloproteinase (MMP) activity based on cleavage of the fluorogenic peptide substrate FS-6. Quantitative determination of two degradation products of type II collagen, C-terminal crosslinked telopeptide type II collagen (CTX-II) and collagenase cleaved neopeptide type II collagen (C2C) was performed using commercial ELISA kits.

Extracellular vesicles were isolated by means of (ultra) centrifugation steps resulting in 10,000*g* (10 K) and 200,000*g* (200 K) EV-pellets, and subsequently analysed by high-resolution FCM in SF samples from subgroups of three foals (aged 12 days–7 months, repeated in vivo sampling) and two adult horses (aged 8 years, post-mortem sampling), as described earlier (Boere et al. 2016). Western blotting was used for protein identification.

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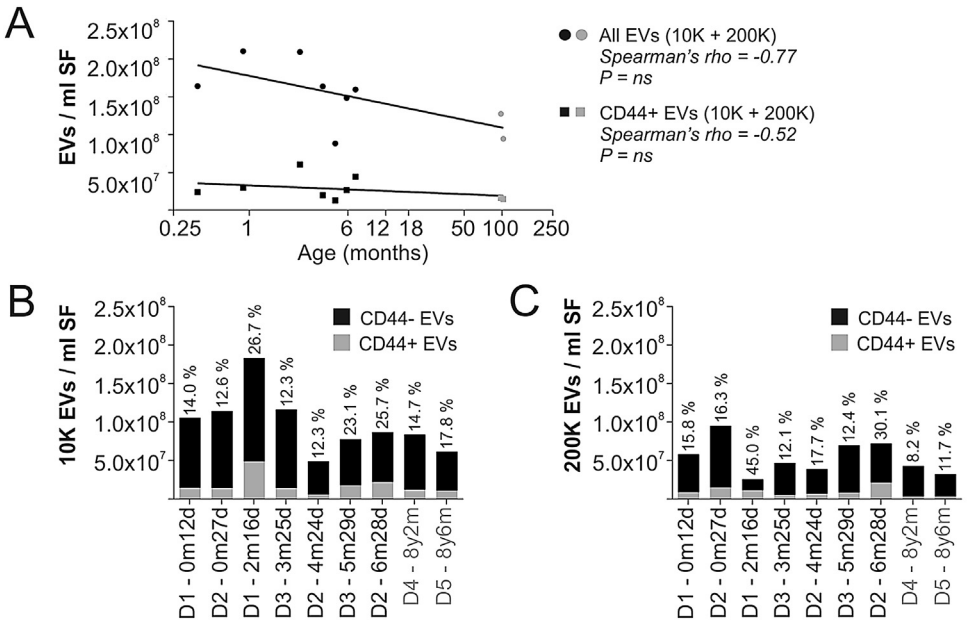

Fig. 1. Analysis of extracellular vesicle (EV) concentrations in synovial fluid (SF) from three foals (aged 12 days–7 months, repeated measurements for donors D1, D2 and D3; black dots/squares) and two adult horses (aged 8 years; donors D4 and D5; grey dots/squares). Data were calculated from high resolution flow cytometry (FCM) analysis of EVs in sucrose density gradient fractions. Prior to measurement, EVs were labelled with PKH67 (generic labelling of all EVs) and anti-CD44 antibody (labelling CD44+ EVs). (A) Weighted Spearman rank correlations for concentration of total EVs and CD44+ EVs (10K + 200 K) from immature to mature age on logarithmic scale. No significant correlation with age was found in the first 13 months of life (foals only). (B) CD44+ and CD44− 10 K EV concentrations for individual animals from Fig. 4A. (C) CD44+ and CD44− 200 K EV concentrations for individual animals from Fig. 4A. Bars represent the sum of CD44+ EVs (grey) and CD44− EVs (black). Percentages CD44+ EVs are indicated above bars. Ns, not significant.

All data were analysed in R v3.3.2.[1] Full details of the procedure can be found in the Supplementary information (See Appendix: Supplementary material).

Synovial fluid concentrations of GAGs (Spearman's rho −0.52, $P < 0.01$), MMP activity (Spearman's rho −0.70, $P < 0.001$) and concentration of the non-collagenase dependent marker of collagen type II breakdown CTX-II (Spearman's rho −0.52, $P < 0.01$) significantly decreased with age until adulthood. The concentration of the catabolic collagenase-dependent collagen marker C2C increased marginally, but significantly (Spearman's rho 0.40, $P < 0.05$; See Appendix: Supplementary Figs. 1–3).

Western blotting demonstrated that all foal samples contained EV markers CD9 and CD44, but the relative intensity of the bands was variable and the detection of CD44 was relatively weak (See Appendix: Supplementary Fig. 4). Overall, CD44 was more abundant in 200 K EVs.

High-resolution FCM showed higher total numbers of EVs and increased CD44+ EVs in foals (Fig. 1A), but there was not a significant correlation between the decrease in EV concentration and age, even not when specifically focusing at the first 7 months of life. Separate evaluation of 10 K EVs (Fig. 1B) or 200 K EVs (Fig. 1C) did not reveal major changes between age groups. Concentrations of total 10 K or 200 K EVs, and the percentages of CD44+ EVs, were variable between animals and there was not a significant correlation with age.

cBuoyant density data of EV-containing sucrose fractions in foals and in adult horses showed that 10 K EVs floated at slightly higher density (1.12–1.16 g/mL) compared to 200 K EVs (1.10–1.14 g/mL; Fig. 2), which could reflect other cell debris, or apoptotic bodies, or loss of EVs through the process.

Differences in the high-resolution FCM scatter plots (rwFSCxPKH67; See Appendix: Supplementary Fig. 5) revealed that possible differences in EV composition between EVs from foals and adult horses cannot be excluded.

The SF biomarker analysis confirmed patterns from earlier studies with a sharp and highly significant decrease in MMP activity, reflecting the declining intensity of cartilage metabolism with decelerating growth rate (Brama et al., 1998). The same, though less pronounced, was observed for the concentration of GAGs. Levels of C2C remained virtually constant, in agreement with our earlier findings in a different cohort of foals (de Grauw et al. 2011a). High levels of CTX-II have been linked to growth plate activity (Duclos et al., 2010); the decrease concurs with the slowing down and ultimately cessation of the process of endochondral ossification.

The hypothesis that general EV quantitative profile in SF would reflect joint development was not supported. Although the number of samples for EV analysis from SF was limited, samples were taken from a cohort of healthy foals of similar breed, housing, feeding, and exercise regime in the most dynamic period of joint development, which in our opinion justifies this conclusion. The lack of a significant effect of age on EV concentration in healthy foals and adult horses is in sharp contrast with the effect of inflammation, since in synovial fluid from human patients with rheumatoid arthritis, inflammation caused substantial changes in the SF EV quantitative profile (Marcoux et al., 2016). Hence, the physiological process of growth apparently does not affect the quantitative EV profile, although more subtle differences may exist. Based on differences in the high-resolution FCM scatter plots, we cannot exclude that SF-derived EVs in foals and adult horses are (functionally) different. These findings prompt the analysis of more samples, as well as in-depth functional analyses of EVs, including their cargo and signalling function.

The current study reveals that in the horse, healthy joint development is not reflected by the number of EVs present in SF or by changes in the CD44+ subset. However, there may still be functional differences between EVs from young and mature animals. Indications for such differences were found in the high resolution FCM scatter plots. This is a topic for future in-depth investigation.

[1] See: http://www.R-project.org (accessed 7 December 2018).

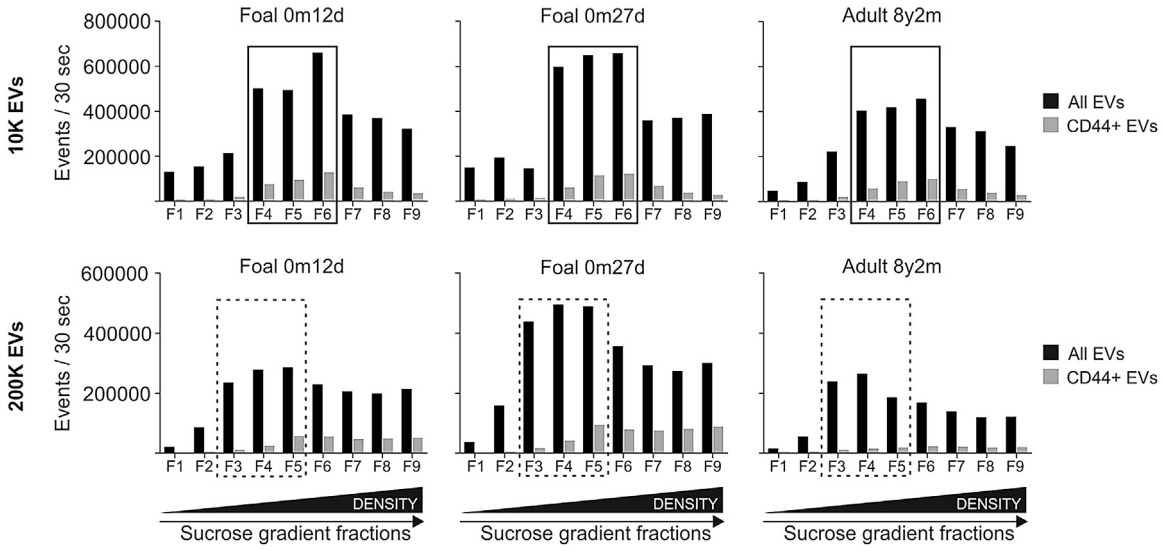

Fig. 2. Quantitative analysis (high-resolution flow cytometry; FCM) of extracellular vesicles (EVs) in individual sucrose fractions with different buoyant density. Prior to analysis, EVs were labelled with PKH67 (labelling all EVs) and anti-CD44 antibody (labelling CD44+ EVs). Results are shown for two foals (age <1 month) and one adult horse (aged 8 years). Black and dotted boxes indicate the fractions with highest concentrations of EVs (most events/30s), for 10 K and 200 K EVs respectively. Densities of individual fractions (g/mL) are as follows: F1 = 1.06, F2 = 1.08, F3 = 1.10, F4 = 1.12, F5 = 1.14, F6 = 1.16, F7 = 1.18, F8 = 1.20, F9 = 1.21.

Conflict of interest statement

During this study, the Wauben research group, Utrecht University, Faculty of Veterinary Medicine, Department of Biochemistry and Cell Biology, had a collaborative research agreement with BD Biosciences Europe to optimise the analysis of EVs using the BD Influx flow cytometer. None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.tvjl.2018.12.010.

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