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Characterisation of Equine Synovial Fluid Derived Extracellular Vesicles from Young and Old Horses

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Equine osteoarthritis is a disease that impacts the welfare and performance of horses from all disciplines. Similar to its effect on human joints, osteoarthritis in horses is a painful joint condition that leads to lameness and decreased range of movement. This study compared the lubricating synovial joint fluid from eight young and seven old horses, specifically looking for differences in the extracellular vesicles (EVs). EVs are small nanoparticles present in the joint synovial fluid. EVs contain a type of ribonucleic acid (RNA) called microRNAs (miRNA), which can alter expression of genes and therefore influence the environment of a joint as some gene changes may promote or prevent osteoarthritic changes. This study was investigating whether there was a difference in the miRNAs present between old and young horses, as well as whether other characteristics of the EVs differ. Our study aimed to add to ongoing research into the role of EVs in the progression of osteoarthritis, and whether they could be used as biomarkers to diagnose joint changes. Our study did not find any significant differences in the size, concentration or miRNA expression of three miRNAs tested which suggests that their characteristics remain similar as a horse ages. However, a trend of decreased expression of these miRNAs was found, and while not statistically significant, this suggests that older horses may have lower levels of expression of certain osteoarthritis and inflammation related miRNAs. Additional work is required to confirm these findings.

Abstract

Equine osteoarthritis (OA) continues to be the most prevalent cause of lameness in horses. It exists in two entities: idiopathic and age-related. It has been hypothesized that extracellular vesicles (EVs) contribute towards the propagation of OA throughout the joint. It is fundamental to determine if age influences joint EV characteristics and their cargo (such as miRNAs) to understand if they facilitate disease development within the equine joint. Synovial fluid samples from the equine metacarpophalangeal joint were collected from young horses aged 0 - 5 years (n = 8) and old horses aged 14 - 21 years (n = 7). EVs were extracted using differential ultracentrifugation followed by characterisation of the EVs using nanoparticle tracking analysis, along with transmission electron microscopy. Rt-qPCR was performed to quantify the miRNA expression of Eca-mir199a-3p, Eca-mir-148a and Eca-mir-146a. No significant differences were found between the size (nm) and concentration (EVs/ml) of EVs in young and old horses. miRNA expression analysis showed no statistically significant evidence of altered expression, however, a trend of decreased expression in all miRNAs in older horses could be suggestive of impaired cartilage homeostasis with age. Limitations include a small sample size. Future work would look to include RNA sequencing in order to expand the miRNA panel to determine if there are other differentially expressed miRNAs between young and old EVs, which may inform OA pathophysiology and serve as biomarkers of disease or therapeutic targets.

Introduction

Diseases of the equine locomotor system continue to be the most prevalent cause of lameness in horses (Weeren & Brama, 2001). More specifically, osteoarthritis (OA) is regarded as being a significant burden on the equine industry due to lack of reversible therapeutic options and cost of treatment (McCoy, 2016). Estimations in the United States indicate that up to 60% of equine lameness is attributed or related to OA (United States Department of Agriculture, 2000). Degeneration and structural alterations of the articular cartilage within a joint is commonly considered to be the defining feature of OA, however the pathology spans much further than this (Goodrich & Nixon, 2006). Changes within the subchondral bone, synovial membrane, joint capsule and adjoining connective tissue structures are all fundamental components of the OA pathway (Goldring, 2006), which is described in Figure 1. Alterations in these structures changes the joint's key role of providing a smooth and nearly frictionless motion as the horse moves, absorbing and dispersing the loaded weight produced during movement (Weeren & Brama, 2001). These changes lead to clinically apparent lameness which can contribute to excessive loading of a contralateral limb (Neundorf *et al.,* 2019). Unfortunately, it is common to diagnose OA at the onset of clinical signs, by which stage irreversible damage has occurred and no treatment options are able to reliably reverse the disease (McCoy, 2016).

While clinical resolution is currently not a possibility, options for medical management are available. These include the use of non-steroidal anti-inflammatory medication, intra-articular steroids, and oral chondroprotectants (Goodrich & Nixon, 2006; Mirza *et al.,* 2016). In addition, recent treatments focussing on regenerative effects have become available, including platelet rich plasma, intra-articular hyaluronic acid (Niemelä *et al.,* 2016), and stem cell therapies (Broeckx, 2019). The effects of these novel therapies have been described to various extents in the literature, with a range of clinical outcomes. Their mechanisms of actions are not fully understood, and while they represent interesting alternatives, they are only able to slow disease progression as opposed to reversing it. In addition, these methods come with inherent risks, for example, the use of stem cells which involves introducing an uncontrollable cellular proliferation into a joint (Lee & Hui, 2006).

To improve treatments and diagnostics, ageing changes within the equine joint over time must be better

understood. Since their discovery approximately 50 years ago, extracellular vesicles (EVs) have gained growing recognition within the field of joint regulation and homeostasis (Boere, 2017). Microvesicles (0.1 – 1.0 µm), apoptotic bodies (1.0 - 5 µm) and exosomes (0.03 - 0.1 µm) are different subtypes of EVs, characterised by their size, contents and functions. The role of their molecular cargo and potential to be biological markers has achieved widespread interest in recent years (Théry, 2018). EVs participate in intracellular communications and among many other roles, are protective carriers for biologically active signalling molecules (Malda *et al.,* 2016). We know that EVs can act as biomarkers for the progression of disease states from the cells in which they originate from (Robbins, 2017), however we do not know how EVs change with ageing alterations in synovial maintenance and homeostasis.

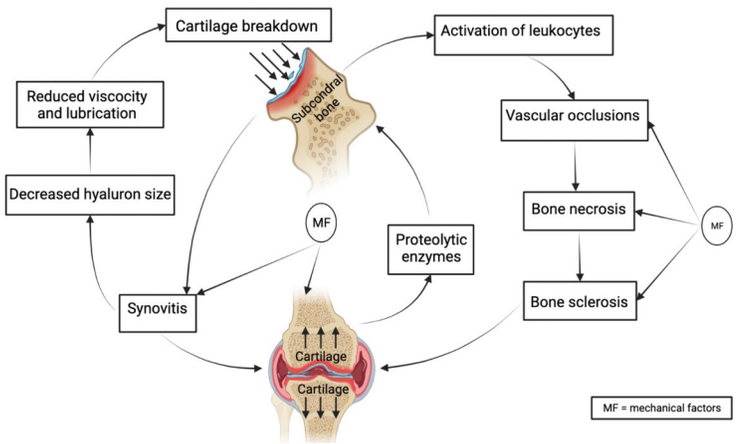

Figure 1. A schematic diagram adapted from Goodrich and Nixon, 2006. The figure depicts the pathological changes that can occur within arthritic joints. Each pathway can lead to loss of function. In more severe cases of osteoarthritis, a combination of pathways will occur at the same time, leading to more severe and debilitating changes.

MiRNAs are an abundant class of small, single stranded non-coding RNA molecules ranging from 18-24 nucleotides that negatively regulate gene expression at the post-transcriptional level and act as overarching regulators of gene expression (Gibson & Asahara, 2013). MiRNAs play key roles in many osteoarticular diseases, and while their complex network of interactions is still being defined, they are regarded as suitable biomarkers for pathological conditions (Desjardin *et al.,* 2014). The inhibition or over-expression of several miRNAs has been reported to affect expression of cartilage matrix genes (Gibson & Asahara, 2013). This study aimed to understand whether expression of the following miRNAs: Eca-mir199a-3p, Eca-mir-148a and Eca -mir-146a changes with age to gauge the best course of action when considering allogenic and autologous therapies. These specific miRNAs were chosen due to their contributions to cartilage metabolism and joint inflammatory pathways (Desjardin *et al*., 2014; Perrini *et al.,* 2016; Rakic *et al.,* 2017). Changes in the EVs with age progression would favour the use of allogenic therapies, impacting management processes.

We hypothesize that synovial fluid derived EV characteristics and cargo changes with age, which would have implications on OA development and therapeutics.

Methods & materials

Ethics approval

The collection of synovial fluid samples from The University of Liverpool's biobank was approved by the ethics committee of The University of Liverpool under reference number VREC561.

Sample cohorts and collection

The University of Liverpool Equine Musculoskeletal Biobank provided 15 synovial fluid samples from the metacarpophalangeal joint. Samples were collected from young horses between the ages of 0 - 5 (n = 8) and old horses between the ages of 14 - 21 (n = 7) and graded macroscopically based on the OARSI histology initiative (McIlwraith, 2010). Only samples receiving an OA score at the time of sample collection of three or less out of six were used. The sex and breed of the horses was not controlled for.

Sample preparation

Samples were thawed on ice and spun at 1400 RPM for 10 minutes (Thermo Scientific™ Medifuge™ Small Benchtop Centrifuge). The supernatant was removed, and 1 µg/ml of hyaluronidase (Bovine origin, Sigma Aldrich) added and treated at 37°C for 60 minutes. The sample was then spun at 1000 x g for five minutes, the supernatant removed and stored at -20°C overnight.

Differential ultracentrifugation

EV extraction was undertaken using differential ultracentrifugation. The samples were spun at 300 x g for 10 minutes, the supernatant collected and spun at 2000 x g for 10 minutes, the supernatant collected and spun at 10,000 x g for 30 minutes. Next, the supernatant was spun at 100,000 x g for 70 minutes, and the supernatant was removed, leaving a pellet which contained small microvesicles, exosomes and proteins. The pellet was washed with filtered phosphate buffered saline (PBS) and suspended in 50 µl of filtered PBS.

Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) (Nanosight NS300 (Malvern Analytics, UK)) was used to provide data regarding the size and concentration of the EVs in each

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sample. Properties of light scattering and Brownian motion provided the nanoparticle size distribution of the samples in liquid suspension. Analyses were performed by the same operator for all 15 samples. All samples were mixed by vortexing and diluted 1:50 in particle free PBS to a final volume of 1 ml to obtain a concentration within the recommended measurement range (1 - 10 x 10^8 particles/mL). The following settings were used in line with the manufacturer's software manual (NanoSight NS300 User Manual, MAN 0541-01-EN-00, 2017). Camera levels were adjusted until all particles were clearly visible and not in excess of 20 % signal saturation. Autofocus was adjusted to avoid indistinct particles. For each measurement, three one-minute videos were captured with the following conditions: 25°C cell temperature and 40 µl/s syringe speed.

RNA extraction and cDNA preparation

RNA was extracted using a miRNAeasy serum advanced kit following manufacturer's instructions (Qiagen, Crawley, UK) and RNA spike-in kit (Qiagen, Crawley, UK). RNA was eluted to a final volume of 18 ml using RNase-free water. We could not find a reliable housekeeper gene described in the literature so a spike-in for normalisation, was used in its place.

cDNA was synthesised immediately using miRCURY LNA RT kit (Qiagen, Crawley, UK). A mastermix containing the reaction buffer, RNAse free water, reverse-transcriptase enzyme and spike-in was prepared and incubated for 60 min at 42°C, followed by 5 min at 95°C to heat inactivate the reverse transcription and then immediately cooled to 4°C. This provided a template for real time qPCR, enabling accurate quantification of low levels of miRNA and discrimination between closely related miRNA sequences (Qiagen, 2019). The samples were stored at -20°C overnight.

Real time qPCR (qRT-PCR)

qRt-PCR was conducted using miRCURY LNA miRNA SYBR green PCR (Qiagen, Crawley, UK) and miRCURY LNA miRNA PCR assays (Qiagen) kits. 5 µl of mercury SYBR green mastermix, 1 µl of universal primer, 1 µl of RNase-free water and 3 µl of template cDNA were mixed using a Roche LightCycler 480 for 2 min at 95°C, 10 s at 95°C and 60 s at 56°C (45 cycles) and melting curve analysis at 60-95°C. Real time PCR was used to confirm the miRNA expression of Eca-mir199a-3p, Eca-mir-148a and Eca-mir-146a.

Statistical analysis

Each dataset was tested for Gaussian distribution using a Shapiro-Wilk normality test and then compared using unpaired t-tests ($p<0.05$) using GraphPad PRISMâ Version 9.2.0 (San Diego, CA, USA).

Results

NTA analysis: Measurement of mean, mode, D10:50:90 ratio and concentration of EVs/ml

No significant differences in the size (Figures 2a & 2b) or number (Figure 2c) of EVs were found between the datasets when unpaired, two-tailed t-tests were run. The D10:50:90 ratio compared differences in diameter (in nanometres) of the particle that is in the 10/50/90th percentile (Figure 2d). No significant differences were found (Table 1).

Table 1. NTA analysis of samples taken from young and old horses was evaluated using t-tests. P value results are illustrated for each measure considered.

Data Set	P Value	Significantly different ($p<0.05$)
Mean EV Size	0.8128	No
Mode EV Size	0.1199	No
Total Evs/ml	0.7746	No
D10	0.8339	No
D50	0.7634	No
D90	0.7104	No

Real-time qPCR analysis – miRNA expression

MiRNA expression analysis was performed and adequate melt curves demonstrated good primer specificity for the following miRNAs: Eca-mir199a-3p, Eca-mir-148a and Eca-mir-146a. There was no difference in expression. However a pattern of reduced expression in older horses was found for Eca-mir199a-3p (Figure 3a), Eca-mir-148a (Figure 3b) and Eca-mir-146a (Figure 3c). Figure 3d displays the decreased expression in all three miRNAs.

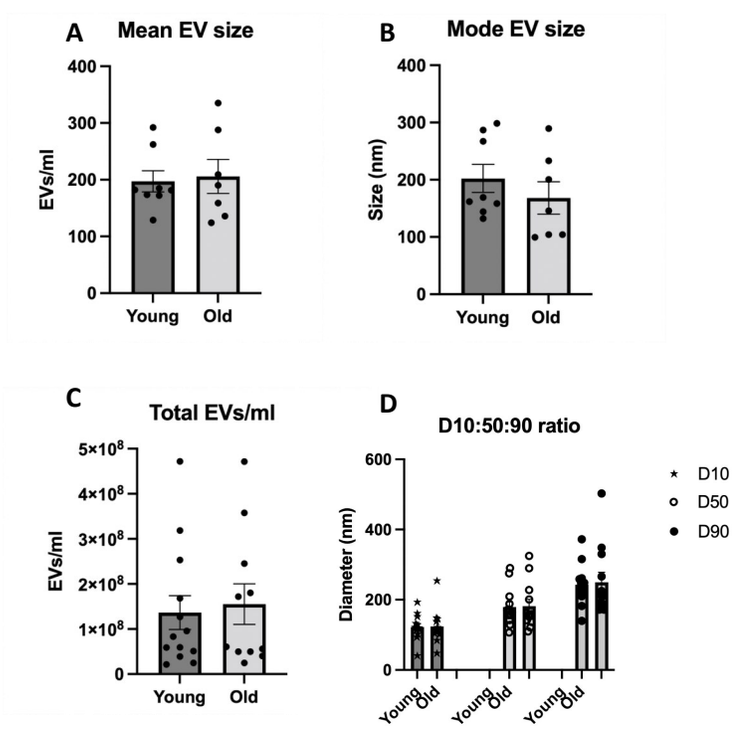

Figure 2. This study found no significant difference in the properties of EVs when young and old horses were compared. A) Displays the mean size of EVs in young and old horses. B) Displays the mode size of EVs in young and old horses. C) Displays the total concentration of EVs in young and old horses. D) Displays the D10:50:90 ratio in young and old horses which compares the difference in diameter (in nanometers) of the particle in the 10th/50th/90th percentile.

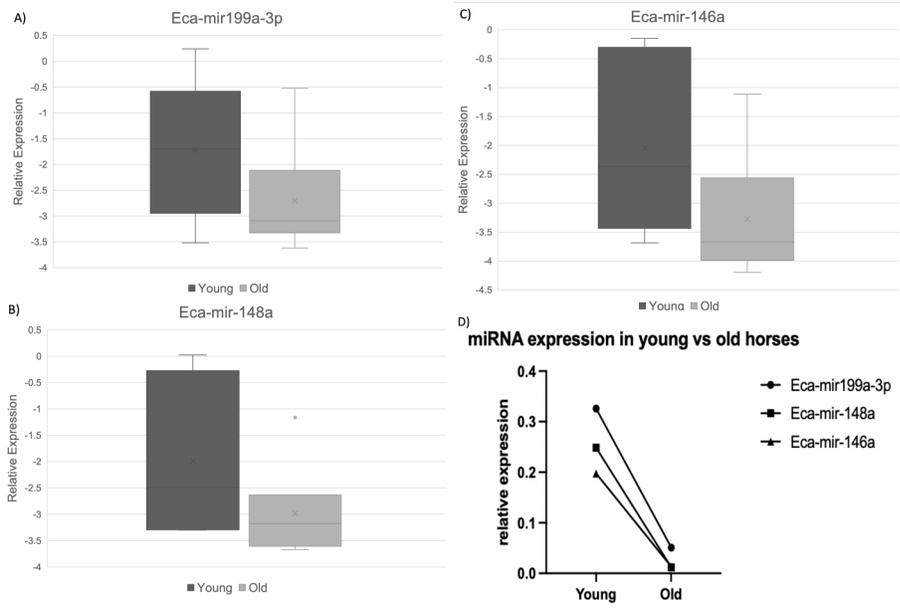

Figure 3. miRNA expression displayed using box and whisker plots. All three miRNAs (displayed in A, B and C) show no statistically significant difference between younger and older horses. D) Relative expression of the miRNAs in young versus old horses. While there was no statistical significance, there is a trend of decreased expression of all three miRNAs in older horses.

Discussion

This is the first study to our knowledge to characterise EVs derived from young and old equine synovial fluid. Samples were taken from horses with no gross OA in order to determine age effects only, which is important as OA is an age-related disease and we only wanted to assess age. In this study, the effects of age on the number, size and concentration of equine synovial derived EVs was evaluated. No statistically significant difference between the EV size, mode, D10:50:90 ratio and concentration of EVs was found using NTA. These findings indicate that in our samples, age does not effect these characteristics. The variation in data and small sample size may have contributed to these results. Additionally the age range was quite large and further studies in more defined age groups are warranted. Characteristics such as concentration (Eitan *et al*., 2017) and number (Akbar *et al.,* 2019) of plasma derived EVs have been shown in other publications to decrease with ageing due to being more readily internalised by leukocytes. None of our samples had advanced OA in the joints, and if changes occur most noticeably when a proliferation of white blood cells such as leukocytes invade the area, then changes in EV characteristics may solely be due to OA changes as apposed to ageing.

Real-time qPCR analysis found no significant difference in miRNA concentration between samples, but interestingly a pattern of lower expression in older horses in all three miRNAs was found.

Eca-mir199a-3p plays an important role in the maintenance of cartilage structure and regulating the balance between catabolic and anabolic processes (Desjardin *et al*., 2014). The over or under expression of Eca-mir199a-3p impacts the expression of genes crucial to cartilage matrix, and while their mechanisms of actions are still not clear, Eca-mir199a-3p is linked to genes COL2A1, cartilage oligomeric matrix protein and the cartilage transcription factor SOX9 (Gibson & Asahara, 2013; Desjardin *et al*., 2014). The extent of gene expression has been described in the literature as dependant upon the stage of development and region of cartilage. In synovial fluid derived EVs that are likely to come from principally synovium, but also cartilage and the circulation, it appears there may be a reduction in expression on EVs. However this hypothesis needs to be tested on a larger sample size.

Eca-mir-146a plays a role in tumour necrosis (Pauley *et al.,* 2008), osteoclastogenesis (Pauley & Cha, 2011) and inflammatory responses (Olivieri *et al.,* 2021), and in the last 10 years has been grouped into a subset of miRNAs called inflamma-miRs, owing to its ability to mediate inflammatory pathways. Eca-mir-146a levels change significantly in tissues and the bloodstream during ageing and have been shown previously to regulate inflammatory pathways, making it an eligible biomarker of inflammation related to ageing. Our data suggest that older horses may show lower expression of Eca-mir-146a. This correlates with the findings of Pauley & Cha (2011), who demonstrate the suppression of cartilage and bone degradation after intravenous infusion of the miRNA, essentially removing a protection mechanism that stops the bone and inflammatory changes associated with OA. In essence, we suggest that in the same way that the literature has shown OA to impact Eca-mir-146a expression, ageing may also impact its expression. This contrasts with findings from Zhang *et al*. (2017), who describe the inhibition of Eca-mir-146a in patients as being a potential therapeutic approach to improve OA. The wide-ranging findings in the literature highlights the need for a more profound understanding of the pathophysiological role of miRNAs in the ageing pathway.

While the functional role of Eca-mir-148a has not been studied, it is described as playing a significant role in a complex cartilage ageing pathway involving chondrocytes and tumour lines (Balaskas *et al.,* 2020). Over-expression of Eca-mir-148a protects cartilage from degradation and promotes its production (Vonk *et al*., 2014). We suggest that expression of Eca-mir-148a is decreased in older patients. In patients with OA changes, Vonk *et al*. (2014) suggested osteoarthritic changes may be influenced by the Eca-mir-148a that acts as a disease-modifying compound in older patients. When normal cartilage is compared to OA cartilage, OA cartilage shows decreased expression of Eca-mir-148a, as well as changes in the level of cartilage matrix degradation and production. It is interesting that our samples, which had very low gross OA scores, also show this trend of decreased expression in older patients.

Future studies with a larger power calculation and further miRNA primers included would be useful to tackle the limitations encountered by this study. Furthermore, samples taken from groups with a larger, more defined age gap (for example over 10 years difference) and with gross OA scores of only 0 would greatly increase the validity of the study and may provide more resounding data. Sex and breeds of the horses was not controlled for which in turn would lead to differing amounts of OA changes as a result of varied intensities of exercise. Future work includes understanding whether these factors affect the characteristics of EVs at different stages within a horse's life, and whether improving the optimisation process of RNA extraction to obtain a higher yield of RNA would provide supplementary data.

Conclusion

Research on the role of EVs in OA-related pathology has attracted much attention in recent years due to their potential to act as biomarkers and influence equine therapeutics. This paper has successfully compared the characteristic of EVs in young compared to older horses for the first time and found there to be no difference. A pattern of decreased expression in older horses has been found in the following miRNAs: Eca-mir199a-3p, Eca-mir-148a and Eca-mir-146a. More research is needed to quantify the therapeutic potential of these miRNAs and whether they can be used as a biomarker for disease within the equine joint.

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