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CARTILAGE

Comparative effect of platelet- and mesenchymal stromal cell-derived extracellular vesicles on human cartilage explants using an ex vivo inflammatory osteoarthritis model

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Aims

Extracellular vesicles (EVs) are nanoparticles secreted by all cells, enriched in proteins, lipids, and nucleic acids related to cell-to-cell communication and vital components of cell-based therapies. Mesenchymal stromal cell (MSC)-derived EVs have been studied as an alternative for osteoarthritis (OA) treatment. However, their clinical translation is hindered by industrial and regulatory challenges. In contrast, platelet-derived EVs might reach clinics faster since platelet concentrates, such as platelet lysates (PL), are already used in therapeutics. Hence, we aimed to test the therapeutic potential of PL-derived extracellular vesicles (pEVs) as a new treatment for OA, which is a degenerative joint disease of articular cartilage and does not have any curative or regenerative treatment, by comparing its effects to those of human umbilical cord MSC-derived EVs (cEVs) on an ex vivo OA-induced model using human cartilage explants.

Methods

pEVs and cEVs were isolated by size exclusion chromatography (SEC) and physically characterized by nanoparticle tracking analysis (NTA), protein content, and purity. OA conditions were induced in human cartilage explants (10 ng/ml oncostatin M and 2 ng/ml tumour necrosis factor alpha (TNFα)) and treated with 1 × 10^9 particles of pEVs or cEVs for 14 days. Then, DNA, glycosaminoglycans (GAG), and collagen content were quantified, and a histological study was performed. EV uptake was monitored using PKH26 labelled EVs.

Results

Significantly higher content of DNA and collagen was observed for the pEV-treated group compared to control and cEV groups. No differences were found in GAG quantification nor in EVs uptake within any treated group.

Conclusion

In conclusion, pEVs showed better performance than cEVs in our in vitro OA model. Although further studies are needed, pEVs are shown as a potential alternative to cEVs for cell-free regenerative medicine.

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Keywords: Platelet lysate, Osteoarthritis, Extracellular vesicles, Regenerative medicine, Cartilage repair, Human umbilical cord mesenchymal stromal cells

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Article focus

- Comparative regenerative potential of extracellular vesicles (EVs) isolated from different sources.

Key messages

- The effects of pEVs on collagen content makes them a promising treatment candidate for cartilage regeneration.

Strengths and limitations

- This study shows how EVs improve cartilage integrity in an ex vivo osteoarthritis (OA)-induced model.
- Further studies in a whole joint scenario, such as in vivo studies, are necessary to fully comprehend how EVs affect OA.

Introduction

Extracellular vesicles (EVs) are phospholipid bilayer membranous bodies secreted by all cells, containing proteins, lipids, and nucleic acids that facilitate cell-to-cell communication, and have therapeutic applications in regenerative medicine.[1,2] EVs have similar functions to their parental cells and offer advantages over them, such as being less tumorigenic, less immunogenic, sterilizable, and being easier to store, manipulate, and characterize.[3,4] EVs have regenerative effects in various medical conditions, and some EV-derived products are already in clinical trials with more being developed for regenerative medicine.[5,6]

EVs are a promising treatment for osteoarthritis (OA) as they regulate cell-to-cell communication.[7,8] OA is a joint disease where the extracellular matrix (ECM) in articular cartilage is progressively destroyed, leading to inflammation, pain, and discomfort.[9-11] Chondrocytes repair and maintain cartilage, but age and traumatic injuries at the articular cartilage deteriorate this function.[12] Current treatments for OA are palliative, limited, and can cause adverse effects.[13] Autologous chondrocyte implantation (ACI)[14] has limitations such as limited chondrocytes, high cost, and complexity in surgery. Therefore, there is a need to develop a new therapeutic strategy to prevent OA and regenerate injured cartilage. Platelet-rich plasma (PRP) injection, which is a minimally invasive method and rich in growth factors such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor beta-1 (TGF-β1), fibroblast growth factor (FGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF) or insulin-like growth factor-1 (IGF-1), and cytokines, promoting proliferation and differentiation, has shown efficacy in treating mild degenerative chondral lesions, musculoskeletal injuries, OA, ligament injuries, and tendinopathies.[15-17]

An alternative to PRP is the use of platelet lysate (PL), which has some advantages in terms of storage as it can be frozen once taken from the donor, which allows its allogenic use in patients. Compared to PRP, PL seems to be more beneficial on tendon tissue regeneration,[18] and EVs present in PL appear to be responsible for its regenerative effect.[19] PL-derived EVs (pEVs) show the same advantages as EVs and could be studied as an alternative treatment for OA, since they also have the capacity to cross tissue barriers and reach target cells that platelets could not otherwise reach, and that way can exert their effect.[20] Moreover, in vitro osteogenic and chondrogenic studies with PL have shown great results, and different groups have studied the use of pEVs in tissue regeneration.[1,21,22]

Whereas pEVs are a promising study field, MSCs are extensively studied in regenerative medicine, including OA and damaged cartilage.[23,24] However, using MSCs may have some disadvantages, such as the need for cell culture and potential adverse effects of supplements or differentiation factors.[25,26] Nevertheless, bioproducts derived from platelet concentrates are already being collected under Good Manufacturing Practice (GMP) and used for treating platelet deficiencies or dysfunction, and are recognized by the World Health Organization (WHO),[27] which could facilitate clinical translation use of pEVs as treatment in therapeutics. Thus, this study aimed to evaluate the effectiveness of pEVs compared to human umbilical cord mesenchymal stromal cell (hUC-MSC)-derived EVs (cEVs) in an ex vivo inflammatory OA model using human cartilage explants.

Methods

Human platelet lysate processing. Buffy coats were obtained from the IdISBa Biobank with approval from the Ethics Committee of the Balearic Islands (CEI-IB) (IB 1995/12 BIO), following ethical approval of the project by the CEI-IB (IB 3656118 PI). From these buffy coats, platelet concentrates were prepared following the conventional procedure used in blood banks with minor modifications. Briefly, six fresh buffy coats containing 25% to 40% residual plasma were pooled without any consideration of blood group, sex, and/or age. When possible, buffy coats with platelet concentration above 200 × 10^9 platelets/l were used. Buffy coats from donors who had taken non-steroidal anti-inflammatory drugs (NSAIDs) were excluded.

Selected buffy coat bags were washed with 0.9% NaCl and centrifuged at 650 ×g for ten minutes. Then, leucocytes were filtrated to finally obtain a platelet concentrate. Platelet concentration was determined and adjusted at 1,200 to 1,800 × 10^9 platelets/l. To obtain PL, ten bags of six buffy coats were pooled to obtain a macro-pool, and three freeze/thaw cycles (-80°C/37°C) were performed to lyse more than 80% of platelets. Then, a centrifugation at 5,050 ×g for 20 minutes at room temperature discarded cell debris and supernatant was filtered by 40 µm pore size membrane (GVS Filter Technology, Italy). Then, microbiological control assays were performed by using the BACT/ALERT system (bioMérieux, France) for aerobic and anaerobic growth. Finally, the whole batch was aliquoted in 50 ml tubes and stored at -20°C until use.

Then, to eliminate small cell debris, PL was centrifuged at 1,500 ×g for 15 minutes at 4°C and supernatant was filtered through 0.8 µm pore size membrane (Sartorius, Germany). Afterwards, PL that was to be used for EV isolation by size exclusion chromatography (SEC) was aliquoted in 5 ml aliquots and stored at -20°C until use.

Mesenchymal stromal cell culture, EV production, and conditioned medium processing. Human umbilical cord mesenchymal stromal cells (hUC-MSCs) were obtained from the IdISBa Biobank with the approval of the CEI-IB (IB 1995/12 BIO), following ethical approval of the

project by the CEI-IB (IB 3656118 PI). For cEV production, 100,000 hUC-MSCs between passages 6 and 14 were grown in 175 cm^2 flasks with proliferative medium – Dulbecco's Modified Eagle Medium (DMEM)-low glucose supplemented with 20% embryonic stem cell tested fetal bovine serum (FBS) and 1% penicillin-streptomycin (all Biowest, France) – until 60% of confluence. Then, cells were washed twice with phosphate-buffered saline (PBS) and medium was replaced by DMEM-low glucose supplemented with 1% penicillin-streptomycin without FBS to obtain conditioned media.

After 48 hours of incubation, conditioned media were collected and centrifuged at 1,500 ×g for 15 minutes at 4°C to remove cell debris. The supernatants were filtered through a 0.22 µm porous membrane and centrifuged at 10,000 ×g for 30 minutes at 4°C to remove the largest EVs. Supernatants, 500 ml, were concentrated by a 100K tangential flow filtration system (TFF; Pall Corporation, USA) until 5 ml of concentrated medium was obtained, which was then ready for EV isolation by SEC.

EV isolation by size exclusion chromatography. EVs from PL (pEVs) or from hUC-MSC conditioned medium (cEVs) were isolated by SEC. Briefly, 5 ml of each sample were loaded in a HiPrep Sephacryl S-400 HR precast column (GE Healthcare, USA) connected to an AKTA purification system coupled with a collector Frac 950 (GE Healthcare). Flow rate settings for column equilibration were settled at 0.5 ml/min, and the column cleaning procedure was displayed according to the manufacturer's manual. Sample was eluted with PBS and 5 ml fractions were collected. In addition, a chromatogram was obtained by measuring the 280 nm ultraviolet (UV) absorbance. pEVs and cEVs containing fractions (fraction number 9 in each case) were collected according to this chromatogram and EV presence in that fraction was confirmed by western blot (Supplementary Figure b). Once EVs were obtained, they were aliquoted and stored at -80°C until use.

Total protein quantification. Total protein content in EV samples was measured by absorbance at λ = 280 nm with NanoDrop spectrophotometer (NanoDrop Technologies, USA).

Nanoparticle tracking analysis and purity ratio. A Nanosight NS300 (Malvern Instruments, UK) was used to determine the particle concentration of EV samples. Samples were diluted 1/1,000 and analyzed as previously described.[28] Three different videos were captured, and 1,498 frames were analyzed by the in-build NanoSight Software NTA 3.2 Dev Build 3.2.16 (Malvern Instruments) with a detection threshold of 3.

The purity ratio was calculated as described by Webber and Clayton[29] using the following equation:

$$\text{Purity}\left(\frac{\text{Particles}}{\mu g}\right) = \frac{\frac{\text{Particle}}{\text{mL}}}{\frac{\mu g}{\text{mL}}}$$

Human cartilage explant culture. Human articular cartilage explants were obtained from the IdISBa Biobank, and their use for the current project was approved by the CEI-IB (IB 1995/12 BIO), following overall ethical approval of the project by the CEI-IB (IB 3656118 PI). Healthy human cartilage explants from talus and calcaneus bones were surgically removed during a conventional tissue procurement procedure from cadaveric donors, washed with PBS, and excised using a 3 mm diameter biopsy punch (Scandidact, Denmark). Explants were then cultured in a 96-well plate with DMEM/F12, supplemented with 1% penicillin-streptomycin at 37°C, and stored in 5% CO_2 overnight. The next day, medium was changed and explants were either supplemented with 10 ng/µl Oncostatin M (OSM; MilliporeSigma, USA) and 2 ng/µl tumour necrosis factor alpha (TNF-α) (R&D Systems, USA), resuspended in PBS-0.01% bovine serum albumin (BSA) (to induce OA-like conditions),[30] or supplemented with vehicle PBS-0.01% BSA (MilliporeSigma) (control group).

Two different treatments were tested: 1 × 10^9 particles/well of EVs from PL (pEVs); and 1 × 10^9 particles/well of EVs from hUC-MSC conditioned medium (cEVs). Dose was selected according to preliminary studies (Supplementary Figure c). Thus, in our experiment four groups were evaluated, a control group without inflammation and three groups under OA-like conditions: a non-treated group (OA); a group treated with EVs derived from platelets (pEVs); and a group treated with EVs derived from cells (cEVs).

Medium with inflammatory supplements and treatments was refreshed every other day until day 14, and spent media at days 1, 7, and 14 were stored at -20°C. After 14 days, explants were washed twice with PBS, histology explants were processed to fix and paraffinize, and the rest were weighed and stored at -20°C until digestion.

Cartilage explant digestion. Cartilage explants stored at -20°C were placed in microcentrifuge tubes and digested overnight at 60°C with papain digestion buffer containing 125 µg/ml papain (MilliporeSigma), 0.2 M NaH_2PO_4 (PanReac, Spain), 0.1 M EDTA (PanReac), and 0.01 M cysteine (PanReac) at pH 6.5. After digestion, samples were centrifuged and supernatants collected to quantify DNA content immediately by Hoechst assay. The rest of the supernatants were stored at -80°C until glycosaminoglycan (GAG) and collagen quantification.

DNA quantification: Hoechst 33258 assay. Digested cartilage explant solutions were diluted 1/4 and mixed in a 96-well plate with 0.2 µg/ml Hoechst 33258 (MilliporeSigma). Plates were read in a Cary Eclipse Fluorescence Spectrophotometer (Agilent, USA) at the following wavelength: $\lambda_{excitation}$ = 356 nm and $\lambda_{emission}$ = 458 nm. A 12 mg/ml DNA stock solution (Herring sperm DNA; MilliporeSigma) was used to build a standard curve with a range from 0 to 1,250 ng DNA. Raw data were normalized by explants' weight and expressed as ng DNA/mg tissue. The procedure was performed each time at 4°C, and the 96-well plate and Hoechst 33258 were protected from light when possible.

Glycosaminoglycan quantification: 1,9-Dimethylmethylene blue assay. GAG quantification was performed from

digested cartilage explants and also from spent culture media collected at days 1, 7, and 14. Samples were diluted 1/40 (digested explants) or 1/5 (culture media), placed in 96-well plates, and DMMB solution (1.6% 1,9-Dimethylmethylene Blue (MilliporeSigma), 0.3% Glycine (MilliporeSigma), 0.16% NaCl (Scharlab, Spain), 0.9% acetic acid 0.1 M (PanReac) in distilled water at pH 3) was added. The plate was shaken for ten seconds and absorbance was immediately read at λ = 525 nm. Chondroitin-6-sulfate (MilliporeSigma) diluted with Milli-Q water was used to build a standard curve from 0 to 50 µg/ml. Raw data were normalized by explants' weight and represented as µg GAG/mg tissue for explants or normalized by ml and represented as µg GAG/ml media.

Collagen quantification. Collagen content was quantified using an adapted Sirius Red assay.[31] Digested cartilage supernatants were centrifuged, diluted 1/4, and mixed with 0.5 µM Sirius Red F3BA (MilliporeSigma) in 0.5 M acetic acid (PanReac). After incubation at room temperature, samples were centrifuged and supernatants were placed in a 96-well plate and read at λ = 540 nm. A standard curve (from 0 to 50 µg collagen/ml) was built using skin calf collagen in 0.5 M acetic acid. Raw data were normalized by explants' weight and represented as µg/mg tissue.

Histology. Cartilage explants were fixed for three hours with 4% paraformaldehyde, embedded in paraffin, and sectioned into 6 µm slices. Tissue sections were deparaffinized, rehydrated, and stained with Sirius Red F3BA (MilliporeSigma) for collagen staining[32] or toluidine blue (MilliporeSigma) for GAG staining. Stained sections were visualized by BX60 microscope (Olympus, Japan). Images were taken at 100× with Nikon D5600 camera (Nikon, Japan) at 1/80 shutter speed and ISO 20,000 under polarized light for Sirius Red F3BA staining or 1/250 shutter speed and ISO 2,500 under bright field for toluidine blue staining.

PKH26-EV labelling. EV samples were labelled with the lipophilic dye PKH26 Red Fluorescent Cell Linker Kit (MilliporeSigma) as previously described.[33] Briefly, EV samples were concentrated until almost dry and resuspended with diluent C from PKH26 kit. Then, PKH26 was added to a final concentration of 4 µM and a 5% BSA-PBS solution (1:1) was added to prevent unspecific dye interactions. Labelled EVs were washed twice through an Exo-spin mini-HD column (Cell Guidance Systems, UK) to obtain a final fraction of PKH26 labelled-EVs (PKH-pEVs and PKH-cEVs). The same protocol was followed with a sample without EVs (control group).

PKH26 labelled-EVs uptake. EVs with PKH26 (PKH26-pEV and PKH26-cEV) were used to treat cartilage explants under OA-like conditions as previously described. In addition, PKH26 without EVs was used for the control group. Ex vivo cartilage explants were cultured as described before.[33] However, labelled-EV uptake was monitored at zero, one, two, three, four, and five hours. At each time, explants were washed with PBS, fixed, and processed to paraffinize and obtain 6 µm tissue sections as described in the histology section to later stain with 4′,6-diamidino-2-phenylindole (DAPI).

DAPI staining. Tissue sections were rehydrated and permeabilized with 0.1% Triton X-100 (MilliporeSigma)-0.1% sodium citrate (Scharlab) solution. Then, these were washed with PBS and a drop of mounting Fluoroshield with DAPI (MilliporeSigma) was added. They were then covered with a cover glass, kept at room temperature overnight protected from light, and stored at 4°C until visualization with a confocal microscope (Leica Microsystems, Germany). Images were taken at 40× and analyzed using ImageJ software version 1.8.0 (National Institutes of Health, USA). PKH26 labelled EV uptake was quantified, and raw data were normalized by background fluorescence intensity of the control group.

Statistical analysis. A total of nine donors were used for the study with human cartilage explants in different independent experiments. For each donor, different replicates were used depending on the assay and the total sample availability. For pEV isolation two macro-pools were processed, and for cEV isolation four different hUC-MSC donors were used. Values represent mean (standard error of the mean (SEM)). The Kolmogorov-Smirnov test was used to assume parametric or non-parametric distribution of the data. Differences between groups with parametric distribution were assessed by analysis of variance (ANOVA) followed by Bonferroni test as a post-hoc when necessary. When data were non-parametric, the Kruskal-Wallis test was assessed followed by Mann-Whitney U test. When comparing two independent groups, an independent-samples *t*-test was performed. Results were considered statistically significant at $p < 0.05$. SPSS programme for Windows, version 25.0 (IBM, USA) was used to perform statistical analysis, and GraphPad Prism version 7.0 (GraphPad Software, USA) was used for data representation.

Data availability. We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV230588).[34] Raw data were also deposited in the Figshare repository (doi: 10.6084 / m9.figshare.23225528).[35]

Results

Characterization of pEVs and cEVs isolated by SEC. Once EVs were isolated, they were characterized in terms of particle size and concentration by nanoparticle tracking analysis (NTA) and in terms of protein concentration (Table I). pEVs presented higher particle concentration, smaller particle size, and significantly higher protein concentration when compared to cEVs. However, both samples presented similar purity. Other characteristics, such as morphology or surface markers, were routinely checked with no major differences (Supplementary Material).

EVs as treatment for human cartilage explants under OA-like conditions. Once OA-like conditions were induced and after 14 days of treatment, DNA content (Figure 1a)

Table I. Extracellular vesicle characterization in terms of quantity, size, protein concentration, and purity.

Type	EV concentration, particles/ml (SEM)	EV size, nm (SEM)	Protein concentration, µg/µl (SEM)	Purity, particles/µg
pEV	1.0×10^{11} (7.5×10^{10})	121 (17)	0.269 (0.102)	3.1×10^{8}
cEV	2.7×10^{10} (9.0×10^{9})	150 (15)	0.035 (0.015)*	9.7×10^{8}

Results were statistically compared using an independent-samples *t*-test.
*$p < 0.05$ vs pEV.
cEV, human umbilical cord mesenchymal stromal cell-derived extracellular vesicle; EV, extracellular vesicle; pEV, platelet lysate-derived extracellular vesicle; SEM, standard error of the mean.

was significantly higher in the pEVs treated group compared to the control group and the cEVs treated group, and so it was observed when tissue sections were stained with DAPI (Figure 1b). However, no significant differences were found in GAG quantification nor in toluidine blue images among groups for cartilage explants (Figures 1c and 1e). However, there was a general decrease of GAG content in the groups cultured under OA-like conditions compared to the non-inflamed control group, which indicated that OA-like conditions were affecting the ECM components in a similar way to the natural OA process.

Degraded GAG released to the media was also quantified. Figure 1d shows that after one day of OA induction, spent media from all the inflamed explants presented higher degraded GAG levels than the non-inflamed control group. However, after seven days of treatment, significantly decreased levels of degraded GAG were quantified for the pEV-treated group compared to the OA group, reaching similar levels to the non-inflamed control at day 14. This protective effect was not observed for the cEV-treated group at any of the days evaluated, which showed significantly higher levels of GAG released to cell culture media than the control at all the timepoints evaluated, and even significantly higher levels than the pEV-treated group at day 7.

Differences were also observed in collagen quantification and Sirius Red F3BA staining (Figures 1f and 1g), where the pEVs group showed higher collagen content compared to the other inflamed groups (OA and cEVs) and images also showed visually higher content of large collagen fibres (yellow) in pEVs.

EV uptake by human cartilage explants under OA-like conditions. An EV uptake assay was set up to confirm that EVs reached chondrocytes and exerted their effect on them. PKH26 labelled EVs were used as treatment in inflamed explants for five hours. Each hour progression of labelled EVs was monitored by confocal images, and presence of these EVs was quantified (Figures 2a and 2b). After one hour of incubation, the different groups of labelled EVs clearly reached chondrocytes; after five hours, a slightly higher presence of PKH-pEVs around chondrocytes than PKH-cEVs was observed.

Discussion

Use of EVs in therapy must meet quality and safety standards for clinical translation. Most regenerative medicine studies focus on MSC-derived EVs, which require in vitro/ex vivo cell expansion that needs optimization for EV safety and industrial/regulatory challenges.[27] We suggest using platelet-derived EVs since they outperformed cEVs in our ex vivo inflammatory OA model on human cartilage explants.

Autologous therapy minimizes the risk of rejection and eliminates the need for immunosuppression, but it may present challenges in terms of cell availability and manufacturing. It also requires a longer turnaround time, as cells must be collected, processed, and reintroduced to the patient. By contrast, allogenic therapy involves using cells or tissues from a donor to provide therapeutic benefits to the recipe; however, they also present disadvantages such as the need for donor matching, risk of graft rejections, or limited availability of donors, which limits the access to treatment for patients in need. The use of EVs is advantageous in terms of wider availability, as it relies on pre-existing cell banks or tissue donors.[36–38] Platelet concentrates from conventional blood donations present advantages over MSCs: they have a high EV production capacity for either autologous or allogenic therapy; present standardized procedures under GMP for blood collection; and are recognized as therapeutic bioproducts for treating platelet-related disorders by the WHO.[27] These features suggest that platelets are a great candidate for EV isolation. Platelets do not need to be in vitro expanded for EV isolation. Platelets and their derivatives (PRP and PL) from allogenic platelets are already produced at clinical grade, but pEVs have an advantage: they can cross tissue barriers and migrate to target cells, making them a safer and more efficient candidate for regenerative medicine and tissue repair compared to other blood cell products.[39] In fact, we have previously demonstrated enhanced pEV effects compared to its source, probably due to a protective encapsulation of the active biomolecules offered by the vesicles themselves.[28] Here, we compare pEVs to cEVs as treatment in our OA-like ex vivo model, since most studies in regenerative medicine focus on MSCs-derived EVs.

SEC is the preferred EV isolation method as it yields purer EVs, as demonstrated by the purity ratio of both samples, when compared to other methods such as ultracentrifugation,[40] which was once considered the gold-standard method for EV isolation. This is consistent with previous studies using SEC as an isolation method.[41] In fact, some studies report that the sequential use of two or more isolation methods improves the depletion of lipoproteins and protein contaminants, but ultimately the choice of the EV isolation method should depend on the starting material, its volume, and its intended use.[42]

To prove the regenerative potential of these EVs as OA treatment, we first established an ex vivo OA model according to

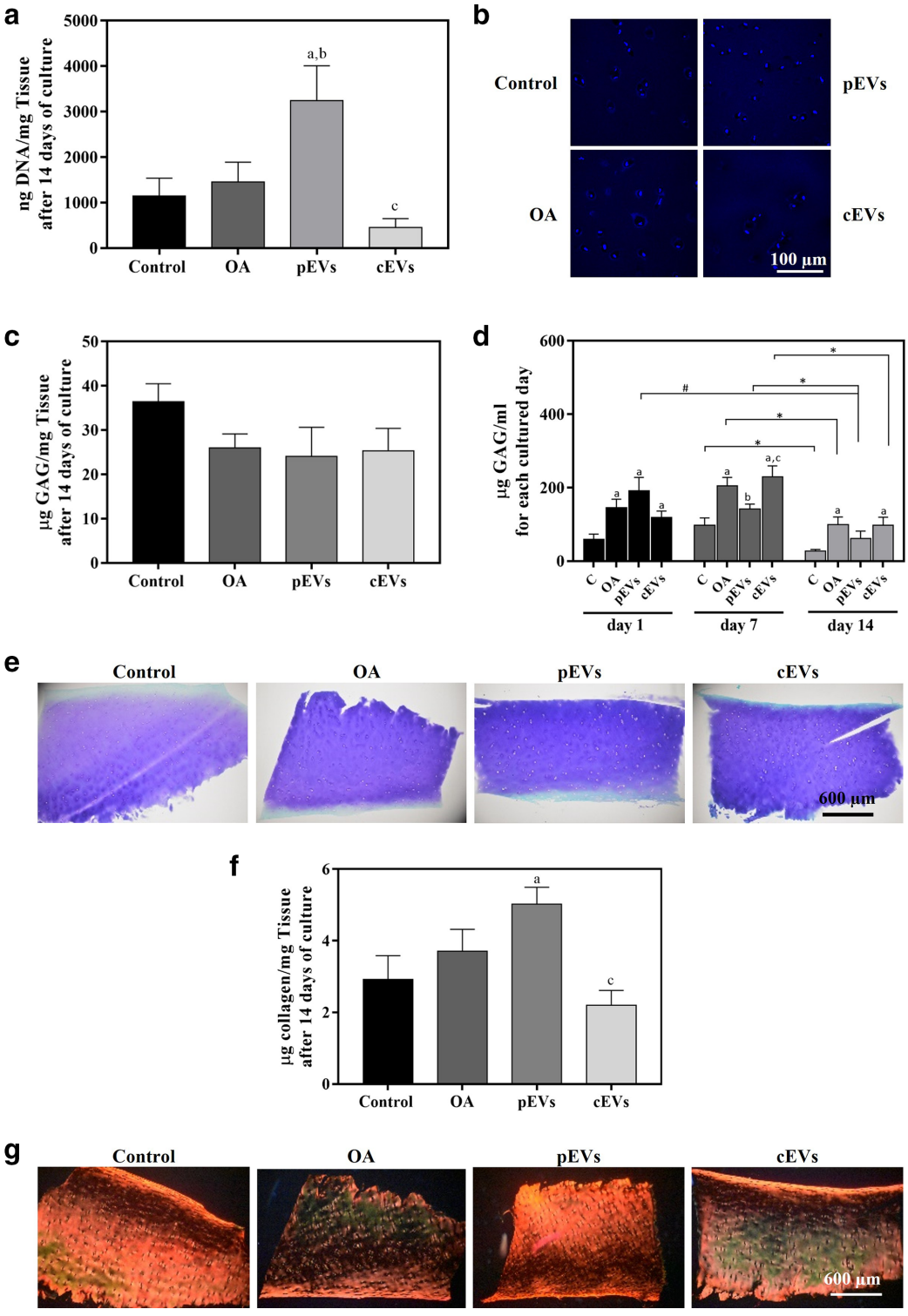

Fig. 1

Cartilage explants under osteoarthritis (OA)-like conditions after 14 days of treatment with platelet lysate-derived extracellular vesicles (pEVs) or human umbilical cord mesenchymal stromal cell-derived EVs (cEVs). a) DNA, c) glycosaminoglycan (GAG), and f) collagen quantification of cartilage explants after 14 days of in vitro culture under inflammatory stimulus to induce OA-like conditions and treated with 1 × 10^9 particles of pEVs or cEVs. d) GAG quantification of spent media at days 1, 7, and 14 of explants cultured under OA-like conditions and treated with pEVs or cEVs. Representative images of 6 µm tissue sections stained with b) 4′,6-diamidino-2-phenylindole (DAPI) for nuclei identification (blue), e) toluidine blue for GAG staining (deep purple), or g) Sirius Red F3BA for collagen staining. Larger collagen fibres were detected as bright yellow or orange under polarized light microscopy, while the thinner ones including reticular fibres were detected as green. Scale bar represented on images. Nine different donors were used and the experiments were performed in triplicate. Results were compared for statistical significance using Kruskal-Wallis test with Mann-Whitney U test for DNA, collagen, and GAG in spent media quantification and using analysis of variance for GAG quantification in digested explants. [a]p < 0.05 vs control, [b]p < 0.05 vs OA, [c]p < 0.05 vs pEVs. *p < 0.05 vs day 1, [#]p < 0.05 vs day 7. C, control.

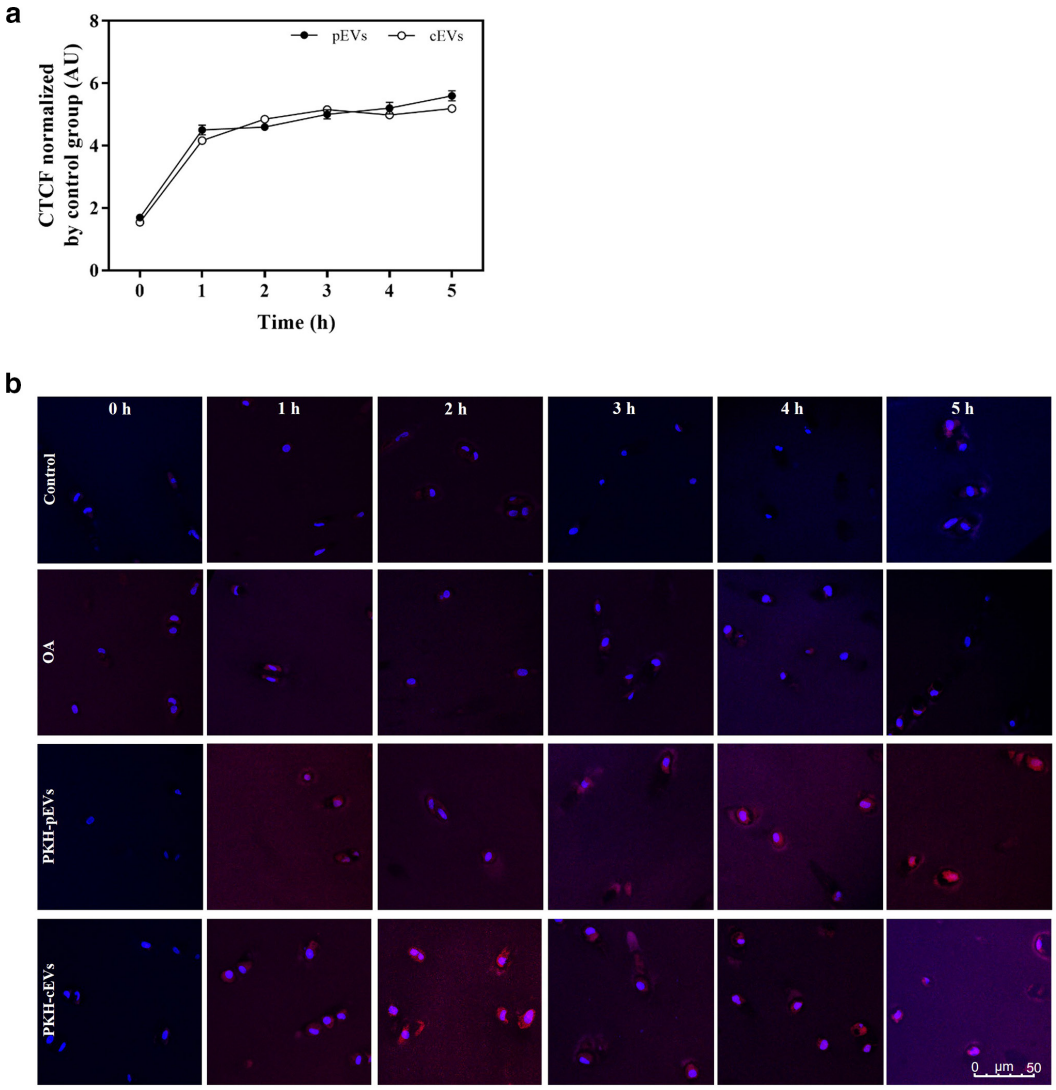

Fig. 2

PKH26 labelled-extracellular vesicle (EV) uptake by cartilage explants under osteoarthritis (OA)-like conditions at different time points. a) Corrected total cell fluorescence (CTCF) of cartilage explants under inflammatory stimulus to induce OA-like conditions, treated with PKH26 labelled-EVs (1×10^9 particles of PKH-pEVs or PKH-cEVs) and monitored at one, two, three, four, and five hours. Results were normalized by the mean background signal of the control and OA groups, which were treated with PKH26 without EVs. b) Representative confocal images of 6 µm tissue sections of cartilage explants treated, as previously described,[33] at the different timepoints. Cell nuclei marked with 4′,6-diamidino-2-phenylindole (DAPI) (blue) and up-taken EVs labelled with PKH26 (red). Images were taken at 40× with their representative scale bar. Different areas from the same tissue section were photographed, and a total of 116 images were analyzed for PKH-pEV treatment and 174 images for PKH-cEV treatment. One donor was used for this experiment performing a triplicate for each treatment and time, but for the control one sample was used for each control and time. cEV, human umbilical cord mesenchymal stromal cell-derived extracellular vesicle; pEV, platelet lysate-derived extracellular vesicle. AU, arbitrary units.

previous reports.[30] Typical OA presents different stages that differ in terms of osteophyte formation and cartilage degradation, which compromise the ECM structure and composition.[43] GAG content, a major component of ECM, was found to decrease in explants under OA-like conditions, confirming the establishment of OA model. This was expected, as the early stage of OA involves the proteolytic breakdown of ECM components and reduced production of ECM molecules by chondrocytes.[44,45]

The higher DNA quantification in the pEVs group suggests that chondrocytes were more susceptible to growth and duplicate than those in the cEVs group. GAG content decreased in all OA-induced groups, indicating that the OA process was properly set up. However, both treatments failed to induce GAG regeneration during the evaluated time, but the pEV-treated group showed reduced GAG degradation over time, consistent with collagen quantification results. GAG plays an important role in cartilage lubrication, cellular differentiation, and morphogenesis by helping proteins to aggregate and bind together with type II collagen fibres. Its reduction suggests that pEVs may slow down the OA process and restore ECM integrity or, at least, reduce its degradation.[46,47]

Sirius Red F3BA staining allowed us to observe the collagen fibres present on the explants through images under polarized light microscopy. pEV-treated explants presented a visually higher content of larger fibres related to collagen type II (yellow), the most abundant in articular cartilage.[48] Collagen type II is crosslinked to proteoglycans in the matrix by collagen type IX. This forms randomly distributed fibril-associated collagens, which give strength and compressibility to the matrix and allow its resistance to deformation in shape and shock absorption. Moreover, the pEVs treated group also presented significantly higher levels of total collagen compared to the other groups. Moreover, the pEV-treated group presented significantly higher levels of total collagen compared to the other groups.

Collagen type III, in association with collagen type V, forms reticular fibres (green) found typically in reticular tissues such as bone marrow, among others.[49] Collagen type III deposition increases during OA,[50] which is concerning since during OA, ECM components are damaged, and newly synthesized collagen may vary from type II to III or X, leading to a reduction in ECM components in the matrix.[45,46] In fact, Otahal et al[51] showed that EVs from PRP increased the expression of collagen type II and aggrecan, as well as matrix metalloproteinase 3. Additionally, their study demonstrated that blood-derived EVs were capable of inducing chondrogenic gene expression changes in OA chondrocytes and preventing cytokine release.[51] Similar findings were observed by Cosenza et al[52] with murine bone marrow MSC-derived EVs, which also reinduced the expression of collagen type II and aggrecan and inhibited catabolic and inflammatory markers. However, these studies were performed on chondrocytes cultured in a monolayer, which is a simpler model for OA, but still demonstrates the direct effect of EVs on cells once they interact with chondrocytes.

pEVs have a key advantage over platelets in regenerative medicine due to their ability to cross tissue barriers and reach areas inaccessible to platelets, to exert platelet effect or as treatment on other cells.[20,27] This is also the case with synovial fluid, where pEVs are identified contrary to what is expected for platelets, which are rarely found in synovial fluid.[20,53] To prove that these effects were indeed triggered by EVs reaching chondrocytes and modulating their response, an uptake assay was performed. Both pEVs and cEVs reached chondrocytes during the first hour of tissue culture incubation and reached a plateau after two hours. This indicates that differences in chondrocyte response to the treatments are due to the composition and cargo of EVs, as no other physical characteristics showed major differences between the two treatments.

Using an OA ex vivo model with explants to test the effectiveness of EVs as a regenerative treatment has certain limitations. Firstly, ex vivo models do not fully replicate the complex in vivo environment, including the dynamic interactions between different cell types, the systemic circulation, and the immune response. This leads to the second limitation, which is the difficulty of establishing a particular OA stage during the ex vivo inflammation process. Additionally, the duration of the ex vivo experiments is short-term (14 days), which may not allow for the assessment of long-term effects or the evaluation of the sustained therapeutic potential of EVs. Lastly, the translatability of results from an ex vivo model to clinical applications may be challenging. Factors such as scale-up for production, delivery methods, and safety considerations need to be thoroughly evaluated in preclinical and clinical studies. Thus, further investigations using in vivo models and clinical trials are necessary to validate the therapeutic efficacy and safety before widespread clinical application. Hence, further studies in EV composition are also needed, such as performing metabolomics, microRNA-omics, and proteomics that would deepen the understanding of EVs but are beyond the scope of the present study, as well as the use of EVs in a more complex OA model, such as a rat in vivo model.

In conclusion, diverse EV sources have been proposed in the bibliography in the context of cartilage regeneration, including cEVs for osteochondral or OA regeneration.[26,54] However, cell-derived EVs entail some drawbacks for their clinical translation. Here, we propose pEVs as a candidate to be used as cell-free treatment to regenerate osteoarthritic cartilage, and our results comparing pEVs to cEVs as treatments for OA postulate pEVs to be a promising treatment in cartilage regeneration. Their effect upon collagen content in cartilage explants cultured in OA-like conditions indicates that pEVs should be further investigated as a possible regenerative treatment for OA.

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Supplementary material

Supplementary text describing the methods used in the study. Figures showing extensive extracellular vesicle (EV) characterization by transmission electron microscopy and western blot, as well as dose-response EV effect on the ex vivo cartilage explants when treated with different doses of platelet lysate-derived EVs and human umbilical cord mesenchymal stromal cell-derived EVs.

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Data sharing:

- The data for this study ara publicly available at the EV-TRACK knowledgebase (EV-TRACK ID: EV230588). Raw data were also deposited in the Figshare repository (http://www.https://figshare.com/); doi: 10.6084/m9.figshare.23225528.

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