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ORIGINAL ARTICLE

EXTRACELLULAR VESICLES OUTPERFORM ADIPOSE DERIVED-MESENCHYMAL STROMAL CELLS IN ALLEVIATING OSTEOARTHRITIS CHONDROCYTE INFLAMMATION IN VITRO WHEN INCORPORATED INTO PLATELET-DERIVED FIBRIN GEL

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ABSTRACT

Background: Orthobiologics are increasingly used in orthopedics, with platelet rich plasma (PRP) widely applied for its autologous origin and bioactive content. PRP fibrin gels allow local retention and sustained release but show limited ability to restore stable hyaline cartilage. Mesenchymal stromal cells (MSCs) and extracellular vesicles (EVs) contained in the secretome may stimulate hyaline cartilage formation and be combined with PRP gels, yet a direct comparison between cell- and EV-containing PRP gels is lacking.

Methods: PRP fibrin gels embedding adipose-derived stromal cells (ASCs) or ASC-derived EVs (ASC-EVs) were compared in vitro on osteoarthritic chondrocytes. A vesicle-equivalent design was used: gels containing 5 $\times 10^6$ ASCs/ml PRP, releasing ~10 $\times 10^9$ EVs in 48 h, were compared with gels incorporating the same EV dose. Gel properties and effects of the gel released particles in 48 hours were assessed on pathologic chondrocyte viability, stress and inflammatory/catabolic gene expression.

Results: ASC-EVs supplemented PRP gels allowed the generation of equivalent gel volumes from reduced plasma amounts. ASC-EVs release preserved chondrocyte viability and metabolic activity and induced a broader downregulation of OA-related inflammatory and catabolic genes. In contrast, ASC-supplemented gels induced cellular stress and showed weaker anti-inflammatory effects.

Conclusions: ASC-EVs based PRP gels elicited a more favorable and controlled biological response than cell-based formulations, despite representing only part of the ASC secretome. These results support EV-based PRP gels as a promising, more standardized alternative for cartilage repair, deserving further *in vivo* validation.

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KEYWORDS
Adipose, cartilage, orthobiologics, platelet rich plasma, extracellular vesicles

INTRODUCTION

In recent years, biologic materials, collectively termed orthobiologics, have gained increasing relevance in orthopedics[1]. Platelet-rich plasma (PRP) is widely used in clinical practice for its autologous origin and its content of bioactive factors involved in inflammation modulation and tissue repair[2-4]. PRP formulated as a fibrin gel enables local application and sustained release of these factors at the lesion site[5]. However, PRP has shown promising clinical benefit with symptoms and function improvement albeit a limited ability to promote stable regeneration of native hyaline cartilage[6], with gel having better results than the injectable formulation[7]. Thus, modifications of this technology, including supplementation or removal of factors or therapeutics, are under study to improve molecular mechanisms involved in the restoration of hyaline cartilage and the overall effectiveness of PRP[8].

Mesenchymal stromal cells (MSCs)-based products have been extensively investigated for cartilage repair[9-10], with clinical studies of expanded MSCs also reporting symptomatic improvement[11]. Increasing evidence indicates that their therapeutic effects are largely mediated by paracrine mechanisms[12]. In this context, MSC-derived extracellular vesicles (EVs) have emerged as key effectors[13], carrying anti-inflammatory[14] and cartilage-protective signals[15]. Preclinical studies have demonstrated their ability to modulate catabolic pathways and support hyaline cartilage homeostasis[16], fostering interest in EV-based, cell-free therapies for cartilage repair.

Both cell- and EV-based intra-articular treatments are limited by poor retention and rapid clearance from the joint[17-21]. To overcome these limitations, tissue engineering has opened the way to new therapeutic approaches employing orthobiologics combined with hydrogels[22,23]. In this view, PRP fibrin gel represents an attractive platform, as it is already used as scaffold option in orthopedic practice[24,25] and may synergize with delivered regenerative cues.

While either MSCs[26,27] or EVs[28,29] incorporation into PRP gels has been shown to be feasible and biologically active, with either factors from MSCs or pure EVs being able to be released from the gel over time, a direct comparison between these two formulations is lacking. Such a correlation is relevant from a translational perspective, as cell-based (MSCs) and cell-free (EVs) approaches differ in safety, standardization, and regulatory complexity[30].

The aim of this study was to compare the biological activity of PRP fibrin gels embedding ASCs with that of PRP gels embedding ASC-derived extracellular vesicles, focusing on their effects on osteoarthritic chondrocytes. This study represents a significant advancement over current knowledge by providing a direct, head-to-head comparison of MSC-based cellular versus cell-free delivery within a clinically-relevant PRP-fibrin scaffold, thereby identifying the most effective therapeutic configuration for the molecular restoration of damaged cartilage.

MATERIALS AND METHODS

Ethics approval and consent to participate

The study was conducted in accordance with the ethical standards established in the Declaration of Helsinki of 1946 and approved by the Ethics Committee of San Raffaele Hospital. Specifically, approval was obtained for the protocol entitled “Caratterizzazione e valutazione del potenziale rigenerativo delle cellule progenitrici tessuto-specifiche ottenute da tessuti muscoloscheletrici” (approved on 16 December 2020; registration no. 214/int/2020) concerning the use of surgical waste material, and for the protocol entitled “Utilizzo dei Patient-Reported Outcome Measures (PROMs), delle valutazioni cliniche oggettive e biomolecolari per il monitoraggio dei pazienti sottoposti a trattamenti di medicina rigenerativa presso il Centro REGAIN–Regenerative Galeazzi Institute” (approved on 8 March 2018; registration no. 6/int/2018) concerning regenerative medicine procedure–derived waste material. Written informed consent was obtained from all participants prior to inclusion in the study.

Adipose tissue collection, ASCs isolation and expansion

Adipose tissue waste material was obtained from the abdominal region of healthy female donors (mean age 37 ± 5 years) undergoing elective aesthetic procedures and processed as previously described[31]. Adipose-derived mesenchymal stromal cells (ASCs) were isolated based on plastic adherence and expanded in α-MEM supplemented with 10% fetal bovine serum (FBS) (both from Thermo Fisher Scientific, Waltham, MA, USA). No more than passage 3 was used for experimental procedures.

ASCs immunophenotype

ASCs were stained with the following antibodies according to the manufacturer’s instruction for 30 min at 4°C in the dark: anti-CD73-PE (code 130-111-908), CD90-FITC (code 130-114-859) and CD45-PC7 (130-110-634) (Miltenyi Biotec, Bergisch Gladbach, NRW, Germany). After one wash in FACS buffer, a minimum of 30,000 events were acquired with a CytoFLEX flow cytometer (Beckman Coulter, Fullerton, CA, USA) and processed with CytExpert v2.3 software (Beckman Coulter).

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Cartilage Collection, Chondrocytes Isolation and Expansion

Articular cartilage was obtained as surgical waste material from patients with knee osteoarthritis (OA; Kellgren–Lawrence grade III–IV) undergoing total knee arthroplasty. The donor cohort had a mean age of 70 ± 9 years and included six males and five females. Cartilage samples were enzymatically digested with 0.15% (w/v) type II collagenase (Worthington Biochemical, Lakewood, NJ, USA) at 37°C for 22 h. Isolated chondrocytes were collected by plastic adherence and cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) (both from Thermo Fisher Scientific). To maintain an OA-like inflammatory phenotype, chondrocytes were stimulated with interleukin-1β (IL-1β; 1 ng/mL) (Thermo Fisher Scientific).

PRP collection

Five platelet-rich plasma (PRP) samples were obtained using the Endoret® system (BTI, Vitoria, Álava, Spain) from patients undergoing PRP-based regenerative orthopedic procedures. The donor cohort had a mean age of 54 ± 10 years and included three males and two females.

ASCs' Secretome Production and EVs Isolation

ASCs at approximately 90% confluence were washed three times with phosphate-buffered saline (PBS), and serum-free α-MEM (12 ml per T175 culture flask) was added. After 48 h, conditioned media (secretomes) were collected, pooled, and extracellular vesicles (EVs) were isolated as previously described[32]. Briefly, conditioned media were centrifuged at 100,000 ×g for 3 h at 4°C using a 70Ti rotor (Beckman Coulter, Fullerton, CA, USA). ASC-EV pellets were washed with PBS and resuspended in serum-free DMEM/F12. EV particle concentration was determined using a NanoSight NS300 system (NanoSight Ltd., Amesbury, UK), and samples were adjusted to a final concentration of 1 ×1011 particles/ml. Aliquots containing 1 ×1010 EVs were stored at -80°C until use.

Transmission electron microscopy

After resuspension of the ASC-EV pellet in PBS, 5 µl of the suspension were deposited onto formvar- and carbon-coated grids and allowed to adsorb for 10 min at room temperature. Excess liquid was blotted with filter paper. Negative staining was then performed using 2% (w/v) aqueous uranyl acetate for 10 min, and the excess stain was removed with filter paper. Grids were air-dried at room temperature and examined using a TALOS L120C transmission electron microscope (Thermo Fisher Scientific, Waltham, MA, USA) operated at 120 kV.

ASC-EVs immunophenotype

ASC-EVs were characterized for surface marker expression using a bead-based assay with the MACSPlex EV Kit (Miltenyi Biotec, Auburn, CA, USA), according to the manufacturer's instructions. Briefly, EVs were incubated overnight at room temperature with antibody-coated capture beads targeting EV epitopes for the capture phase. Captured EVs were then incubated for 1 h with a cocktail of anti-tetraspanin antibodies (anti-CD9, anti-CD63, and anti-CD81), followed by washing. Samples were subsequently analyzed using a CytoFlex flow cytometer (Beckman Coulter).

Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) was performed using a NanoSight NS300 system (NanoSight Ltd.) on pooled ASC-EVs obtained from eight donors and diluted 1:3 in phosphate-buffered saline (PBS). For each sample, five technical replicates were acquired. Particle concentration and high-resolution size distribution profiles were generated using NTA software version 3.4.

Fibrin gel preparation and ASCs or ASC-EVs incorporation

Prior to activation, PRP samples were divided into two 1 mL aliquots and supplemented either with EVs (1 ×1010 EVs per ml of PRP) or with the number of ASCs releasing the equivalent number of EVs in 48 hours (5 ×10^6 ASCs). PRP samples were subsequently activated by the addition of $CaCl_2$ (final concentration 22.8 mM) and incubated for 2 h at 37°C to induce fibrin gel formation. The resulting fibrin gels were collected and wet weight was measured. For both ASCs- and EVs-supplemented gels, a double volume of DMEM/F12 without serum was added with respect to the wet weight in mg (e.g., 200 µl volume for 100 mg gel weight). After 48 hours the supernatant was removed and used immediately.

ASCs and ASC-EVs incorporation and ASC-EVs release detection in fibrin gel

To monitor ASCs incorporation in fibrin gels, ASCs were marked with 5(6)-carboxyfluorescein-diacetate-succinimidyl-ester (CFDA-SE, Sigma–Aldrich, St. Louis, MO, USA; 1 µM, 30 min at 37°C) and washed twice with PBS. To monitor ASC-EVs incorporation in fibrin gels, ASC-EVs in cell culture supernatant were stained with CFSE (10 µM, 30 min at 37°C) and then isolated by ultracentrifugation as previously described. For standard immunofluorescence analysis, after clot formation the supernatant was removed and gels, with or without ASCs or ASC-EVs, were fixed overnight in 3.7% formaldehyde. Samples were then dehydrated through a graded alcohol series, embedded in paraffin, and sectioned at 4 µm thickness for end-point immunofluorescence analysis. Fluorescent images were acquired using an IX71 inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan). For the detection of ASC-EVs release from gels, EVs samples before gel formation and after 48 hours gel release were analyzed with a CytoFlex flow cytometer (Beckman Coulter) in the FITC channel after FITC-fluorescent nanobeads of 100, 160, 200, 240, 300, 500 and 900 nm (Biocytex, Marseille, France) calibration.

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Fibrin gel released supernatant's effect on human chondrocytes

Passage 1 chondrocytes derived from OA patients were seeded at a density of 1 ×10^4 cells/cm^2. After 24 h, three experimental conditions were established: (i) control cultures maintained in DMEM/F12 supplemented with IL-1β (1 ng/ml) to preserve an OA-like inflammatory phenotype; (ii) cultures treated with 48 h-conditioned supernatant from fibrin gels containing ASCs, in the presence of IL-1β; and (iii) cultures treated with 48 h-conditioned supernatant from fibrin gels containing ASC-derived EVs, in the presence of IL-1β. All experimental conditions were supplemented with 10% foetal bovine serum (FBS) depleted of endogenous EVs by ultracentrifugation (100,000 ×g for 9 h at 4°C).

After 48 h of treatment, chondrocytes were harvested and total RNA was extracted using the miRNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. First-strand cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA), and quantitative real-time PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories) on a CFX Opus 96 Real-Time PCR System (Bio-Rad Laboratories). TATA-box binding protein (TBP) was used as the reference gene. Relative mRNA expression levels were calculated using the 2-ΔΔCt method and reported as fold change versus control.

In a parallel experiment, following 48 h of treatment, phase-contrast images of chondrocytes were acquired with an Olympus CKX53 microscope (Olympus Corporation) and cell viability was assessed using an automated cell counter (NucleoCounter® NC-3000™, ChemoMetec, Allerød, Denmark).

Clustering analyses

Hierarchical clustering was done on the fold changes values with respect to untreated pathologic chondrocytes with the statistics calculator numiqo (https://numiqo.com/statistics-calculator/cluster/hierarchical-cluster-analysis-calculator). The following parameters were used: i) Linkage method, complete; ii) Distance, Manhattan. Principal component analysis (PCA) was performed on the same dataset with the PCA calculator (https://www.statskingdom.com/pca-calculator.html).

Statistical analyses

Statistical analyses were performed using GraphPad Prism software version 8.0.2 (GraphPad Software, San Diego, CA, USA). Data distribution was assessed using the Shapiro-Wilk normality test (α = 0.01). For comparisons of absolute values, a two-tailed parametric Student's t-test was applied when data were normally distributed; otherwise, a two-tailed non-parametric Wilcoxon test was used. For fold changes and ratio data, a one-sample t-test was performed with the null hypothesis set to 1. For all analyses, statistical significance was defined as p ≤0.05. Data are presented as mean ± standard error of the mean (SEM).

RESULTS

ASCs and ASC-EVs phenotype

ASCs expressed the mesenchymal stromal cell lineage markers CD73 and CD90 and lacked expression of the hematopoietic marker CD45 *(Fig. 1A)*. Transmission electron microscopy revealed that isolated EVs displayed the characteristic cup-shaped morphology *(Fig. 1B)*. Nanoparticle tracking analysis (NTA) showed a mean EV diameter of 192 ± 45 nm (n = 5) *(Fig. 1B)*. The presence

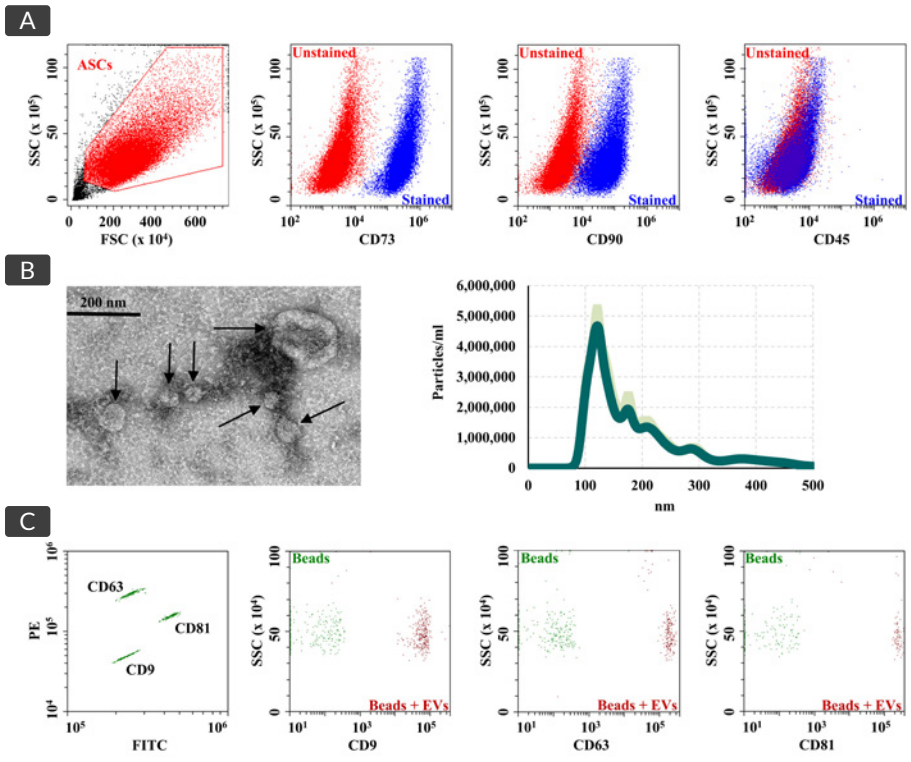


Figure 1. *ASCs and ASC-EVs features and immunophenotype. A) By flow cytometry, ASCs were positive for the MSC-lineage markers CD73 and CD90, while negative for the hematological marker CD45. One representative plot is shown. B) Transmission electron microscopy and Nanoparticle tracking analysis of purified EVs. C) By flow cytometry, ASC-EVs were positive for EV-lineage markers CD9, CD63 and CD81. One representative plot is shown.*

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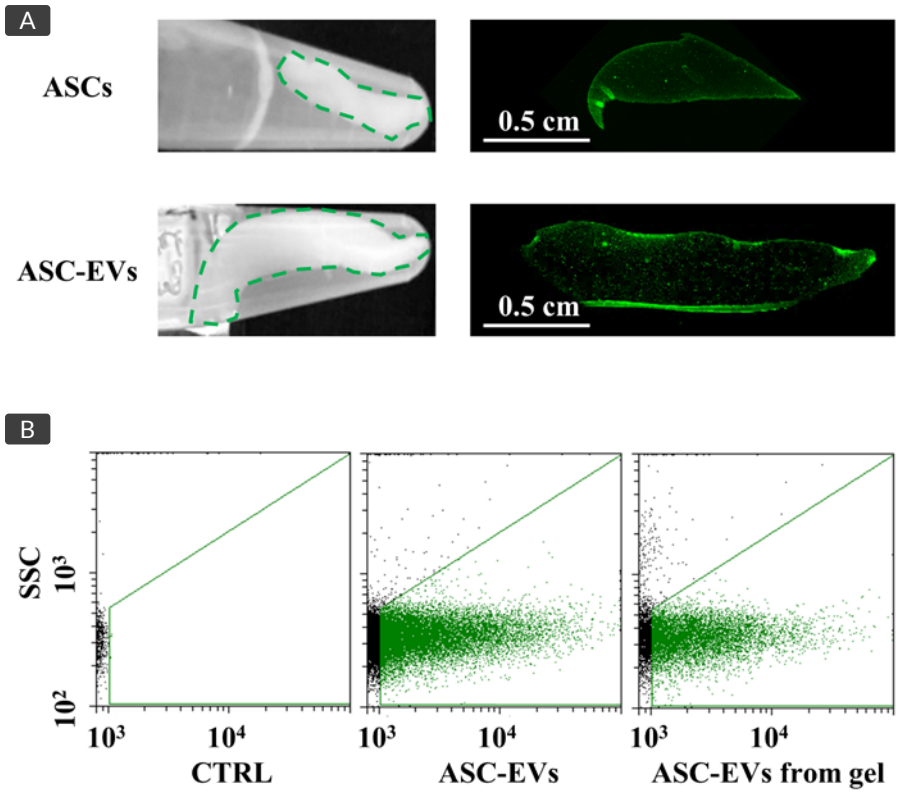


Figure 2. *ASCs and ASC-EVs incorporation in fibrin gel. A) Representative platelet gels with fluorescent ASCs or ASC-EVs. Representative pictures are shown. B) ASC-EVs detected by flow cytometry before addition to gel (compared to suspension solution without EVs, CTRL) and after 48 hours release. One representative plot is shown.*

of EV-associated markers CD9, CD63, and CD81 was confirmed *(Fig. 1C)*.

ASC-EVs supplementation affects fibrin gel size and weight with respect to ASC supplementation

ASCs and ASC-EVs were efficiently incorporated into fibrin gels *(Fig. 2A)*. Wet fibrin gels containing EVs were significantly larger and heavier than those with ASCs, exhibiting a weight ratio of 1.75 ± 0.11 (n = 5, p ≤0.01). After 48 hours, EVs released from gels were detected *(Fig. 2B)*.

The released of fibrin gels embedded with ASCs negatively impact chondrocytes morphology

Morphological changes in OA patient-derived chondrocytes were evaluated after 48 h of treatment with conditioned media released from ASCs or ASC-EVs gels *(Fig. 3)*. Compared with control cultures, chondrocytes treated with ASCs gel-derived supernatants exhibited a more dispersed growth pattern, reduced intercellular connectivity, and a granular cytoplasmic appearance. In contrast, chondrocytes exposed to ASC-EVs gel-derived supernatants maintained a morphology comparable to control cells, characterized by an even distribution across the culture surface and preserved cell-cell contacts, with no evident cytoplasmic features indicative of cellular stress.

PRP-EVs have a superior healing potential than PRP-ASCs

OA patient-derived chondrocytes were assessed for changes in the expression of genes related to OA-associated cytokines (interleukin-6 - IL6), chemokines (CCL5, CCL8), matrix metalloproteinases (MMP1, MMP3), and metabolic regulators (indoleamine 2,3-dioxygenase 1 - IDO1 - and prostaglandin-endoperoxide synthase 2 - COX2) *(Table 1)*. Treatment with the release of ASC-EVs gels significantly reduced (p ≤0.05) mRNA levels across all gene categories. In contrast, the release of ASCs gels induced a significant reduction in only a subset of these genes. Overall, the release of ASC-EVs gels consistently exerted a stronger effect than ASCs gels, reaching higher statistical

vs OA chondrocytes		
Gene	**+ ASCs gel**	**+ ASC-EVs gel**
IL6	0.05**	0.13***
CCL5	ns	0.21**
CCL8	0.05**	0.05***
IDO1	ns	0.42****
COX2	ns	0.50*
MMP1	0.10**	0.28**
MMP3	0.21*	0.33**
CDKN1A	ns	0.64*

Table 1. *Gene expression evaluation in OA chondrocytes after treatment with the release of either ASCs gels or ASC-EVs gels.*
Fold change mean ratios vs untreated chondrocytes are shown when significant.
*N = 3; ns for not significant; * for p value ≤0.05; ** ≤0.01; *** ≤0.001; **** ≤0.0001.*

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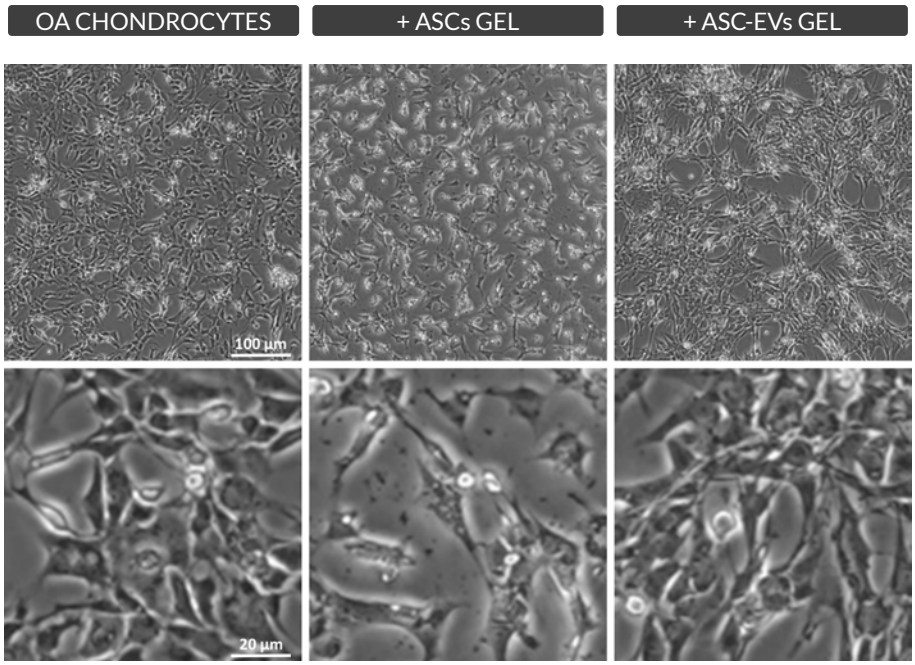


Figure 3. *Release of fibrin gel's effect on pathologic chondrocytes. Pathologic chondrocytes from osteoarthritis patients were treated with the 48 hours-release of fibrin gels with either ASCs or ASC-EVs for 48 hours. Alteration of morphology is visible with ASCs. Two magnifications (4× and 20×) are presented. One representative donor is shown.*

significance in three of the four gene categories. Notably, only the release of ASC-EVs gels effectively downregulated the stress- and apoptosis-related gene cyclin-dependent kinase inhibitor 1A (CDKN1A). Confirming these data, both PCA and hierarchical clustering analyses showed how both gels were able to reduce inflammation, albeit ASC-EVs had a better performance being more distant from pathologic OA HCH *(Fig. 4)*.

The release of PRP-ASCs gels impacts OA-chondrocytes viability

Given the compromised phenotype of OA chondrocytes and the absence of CDKN1A downregulation following treatment with ASCs gel-derived supernatants, cell viability was assessed. ASCs gel release caused a modest but statistically significant reduction in viability (mean reduction 5% ± 1; p ≤0.05, n = 3), whereas ASC-EVs gel release maintained viability levels comparable to control cells (p >0.7).

DISCUSSION

In this study, platelet-rich plasma (PRP) gels supplemented either with adipose-derived stromal cells (ASCs) or with ASC-derived extracellular vesicles (ASC-EVs) were directly compared, with ASC-EVs gel showing the best performance *in vitro* on pathologic chondrocytes.

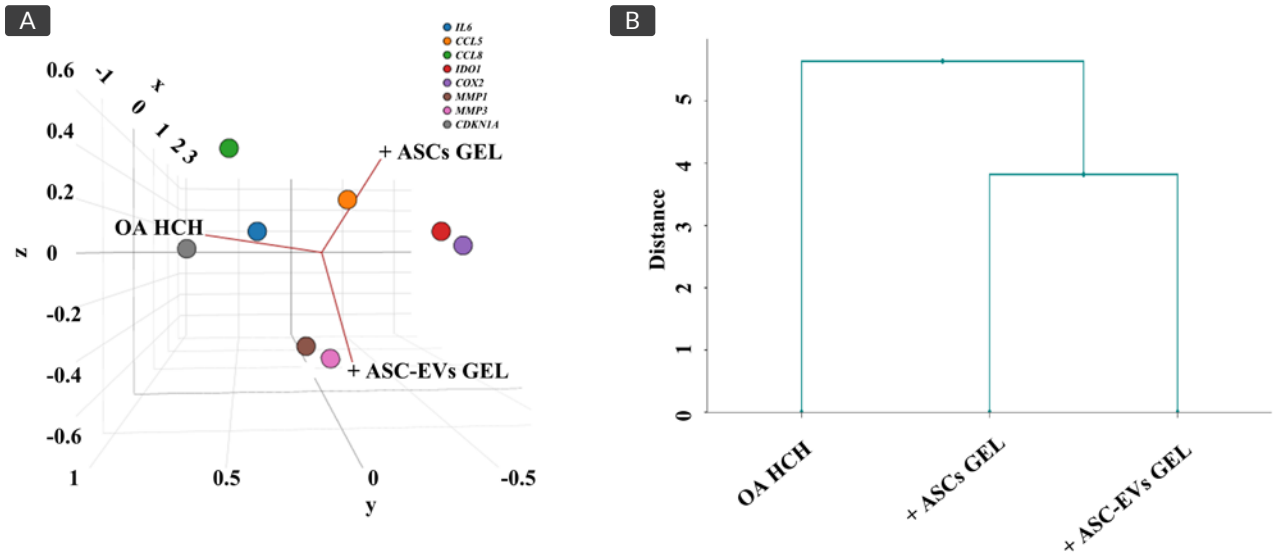


Figure 4. *Effect of gel release on pathologic chondrocytes. A) Principal component analysis of the fold change values for the analyzed genes in Table 1. The x, y and z axes represent principal components 1, 2 and 3, respectively. The original variance appears along the diagonal of the covariance matrix, while the principal components variance is represented by the eigenvalues. The total variance across all dimensions is 3. The original 3 dimensions, account for 100% of the variance, while the 3 principle components, explains 100% of the variance. Each marker positions each single gene in the 3D-environment. B) Hierachical clustering for the fold change values for the analyzed genes in Table 1. Both gels cluster separately from control cells (OA HCH), with ASC-EVs being the most distant.*

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A dose of 5 ×10^6 ASCs per 1 ml of liquid PRP was selected, as this was the cell number able to release approximately 10 ×10^9 EVs in 48 hours, corresponding to the dose incorporated into the same PRP volume prior to gelation, with both gels left for 48 hours to release particles (newly produced or embedded). This EVs number was selected based on previously published data from our group that showed its suitability to be gel-encapsulated at high efficiency and then, once released, effective in reducing inflammation in pathologic chondrocytes in vitro[28]. This experimental design allowed a controlled comparison between cell-based and cell-free formulations while maintaining comparable levels of vesicle-associated bioactivity. The formation of heavier gels with ASC-EVs is of practical relevance, as it enables the generation of the same gel volumes from a smaller amount of plasma. From a translational and economic standpoint, this may reduce the volume of blood required from the patient, minimize procedural invasiveness and improve product standardization without compromising clinical usability. As an example, the use of small-volume/vacuum sample collection tubes was proven to reduce hemolysis[33]. This is also a key point in the World Health Organization guidelines on drawing blood, that prompt to using an evacuated tube that collects a small volume (up to 5 ml) and has a low vacuum, thus helping to avoid collapse of the vein and decrease hemolysis[34]. Notably, hemolysis in PRP releases hemoglobin, iron and other substances that trigger inflammation, oxidative stress and immune responses, potentially hindering tissue repair, causing toxicity and interfering with lab results[35]. Moreover, often PRP kits are available in monolateral or bilateral formats, with the latter having increased costs for both the National Health System and the patients. The use of less blood may reduce the need of more expensive formats to collect a sufficient volume of plasma for the intended clinical applications.

Beyond these properties, marked differences emerged in the biological effects of the released products on pathologic chondrocytes. The release from ASCs gels induced increased cellular distress, suggesting impaired cellular homeostasis. Such stress responses may compromise chondrocyte viability and functionality, both of which are essential for maintaining cartilage integrity[36] and supporting regenerative processes[37]. Also, ASCs gel release displayed a moderate anti-inflammatory capacity, as reflected by the modest modulation of OA-related inflammatory and catabolic genes. In contrast, PRP gels supplemented with ASC-EVs exhibited a markedly more favorable biological profile. Importantly, ASC-EVs gel release did not induce cellular suffering in OA chondrocytes, preserving cell viability and metabolic competence. Moreover, EVs supplementation resulted in a broader and more pronounced downregulation of genes involved in inflammatory and catabolic pathways, indicating a stronger capacity to modulate the pathological processes underlying cartilage breakdown. These findings are particularly noteworthy given that EVs represent only a defined fraction of the ASC secretome[38], lacking many soluble factors released by viable cells. According to a simple subtraction hypothesis, a reduced therapeutic efficacy compared with the full cellular product could be expected. Instead, the enhanced and more controlled biological response elicited by EVs suggests that a defined and stable cell-free formulation may retain beneficial signals while avoiding possible detrimental or stress-inducing cues associated with living cells interaction with the environment. Future experiments are needed to identify molecules released by ASCs in presence of PRP gels that may reduce anti-inflammatory potential and target cell proliferation/viability.

Eventually, from a regulatory and economic perspective, these results further strengthen the attractiveness of EV-based approaches. Cell-based products typically face greater manufacturing complexity, batch-to-batch variability and stricter regulatory requirements, often being classified as advanced therapy medicinal products (ATMPs)[39]. In contrast, cell-free EV formulations may offer improved standardization, scalability and safety profiles, with potentially lower production costs and simplified regulatory pathways. In fact, The European Directive (Directive 2001/83/EC and Regulation 1394/2007/EC) states that EVs directly purified from cells are not considered ATMPs and are classified with biological specifications, having an easier regulatory pathway[40]. The absence of viable cells also reduces concerns related to cell survival, engraftment and long-term safety in vivo[41].

This study has some limitations. First, the present study was conducted using in vitro models, which, although highly controlled and mechanistically informative, cannot fully recapitulate the complexity of the osteoarthritic joint environment in vivo. Factors such as mechanical loading, synovial-cartilage crosstalk, immune cell infiltration and systemic influences may modulate the biological effects of PRP-based formulations and could impact the relative performance of cell-based versus cell-free approaches. Second, the comparison between ASCs and ASC-EV gels was based on an estimated equivalence in EV number released by ASCs rather than on a complete quantitative matching of the full ASC secretome. While this strategy allowed a rational and controlled comparison, it does not account for other ASC-derived soluble mediators that may contribute to biological effects and could behave differently under alternative dosing or release kinetics. Third, EVs were administered as a bulk population without stratification into specific EV subtypes (e.g., small EVs versus larger microvesicles). As different EV subpopulations may exert distinct biological activities, further refinement of EV composition could influence efficacy and reproducibility[42], particularly in the context of product standardization. Fourth, PRP composition itself is known to exhibit inter-donor variability in platelet concentration and growth factor content. Residual variability may still influence gel

properties and biological outcomes, potentially affecting the generalizability of the findings. Also, while the study highlights potential regulatory and economic advantages of cell-free EV-based formulations, formal assessments of manufacturing scalability, long-term stability, cost-effectiveness and regulatory classification were beyond the scope of this work. Future studies integrating preclinical in vivo validation and process development will be necessary to fully define the translational pathway of ASC-EV gels. Finally, while adipose tissue donors for ASCs were healthy young females and PRP was obtained from middle-aged orthopedic patients, it must be noted that specific exclusion criteria and donor-related variables, such as age, sex and underlying metabolic conditions, remain highly debated factors in the literature that can significantly influence the potency of both MSCs and platelet-derived products, as well as the response of cartilage and chondrocytes[43].

CONCLUSION

Overall, this comparative analysis highlights the in vitro therapeutic relevance of ASC-EVs gels for cartilage protection. The data support a paradigm shift from cell-based toward rationally designed cell-free strategies, reinforcing both the scientific novelty and translational potential of EV-based PRP formulations.

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