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Impact of adipose-derived mesenchymal stem cells and their secretome on osteoarthritis in a rat model

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Abstract

Background Osteoarthritis is a common degenerative joint disease marked by cartilage degeneration and inflammation. This study investigates the therapeutic potential of adipose-derived stromal cells (ASCs) and their secretome in a rat model of osteoarthritis.

Methods ASCs were extracted from human adipose tissue, cultured, and primed with human platelet lysate. The secretome was collected after 48 h of serum-free culture. Osteoarthritis was induced in rats using monosodium iodoacetate, and after 14 days, they were treated with saline solution, ASCs, or secretome. Over five weeks, body weight and histopathological changes were monitored.

Results No clinical complications arose post-treatment, and all rats gained weight similarly. ASC treatment increased histopathological changes associated with osteoarthritis, including severe cartilage necrosis and bone remodeling. Conversely, the secretome treatment resulted in mild to moderate cartilage degeneration, similar to that observed in the control group. These findings suggest that ASCs may contribute to disease progression in this model, while the secretome did not show significant effects on cartilage histology compared to the control group. Further studies are needed to determine whether optimizing the secretome composition or dosing could enhance its therapeutic potential.

Conclusions This study highlights the complexity of ASC interactions with the immune system, while secretome may be a well-tolerated treatment, further studies are needed to determine its potential therapeutic benefits.

Keywords Adipose-derived mesenchymal stem cells, Secretome, Human platelet lysate, Osteoarthritis, Rat model, Immune response

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Background

Osteoarthritis (OA) is a highly progressive and debilitating joint disease characterized by the gradual loss of articular cartilage, damage to the subchondral bone, and impairment of the surrounding soft tissue [1]. Physical and mechanical factors significantly influence the susceptibility and severity of OA [2]. Additionally, inflammation contributes to both the symptoms and progression of OA. Recent research has identified a critical link between inflammation in OA and the progression of structural changes. Pro-inflammatory cytokines secreted by the synovial membrane stimulate chondrocytes to synthesize matrix metalloproteases and other catabolic genes, leading to extracellular matrix degradation [3]. Cartilage has a very limited capacity for self-regeneration, and untreated lesions typically require surgical intervention [4]. Current adjuvant treatments include pharmacologic management, patient education, strengthening exercises, and weight loss [2]. However, these strategies are generally ineffective for long-term repair, underscoring the need for novel therapeutic approaches [4]. Regenerative therapy using mesenchymal stem cells (MSCs) represents a promising alternative for OA treatment, potentially overcoming the limitations of current treatments while restoring the tissue's unique biological and functional properties [2]. MSCs can home to injury sites, where they secrete a variety of bioactive factors that perform several key functions, including modulating the immune response by reducing inflammation, promoting the survival and proliferation of tissue-specific resident stem cells, enhancing angiogenesis, and facilitating extracellular matrix remodeling, all of which contribute to the repair and regeneration of damaged tissues.

Preclinical and clinical studies have extensively investigated the efficacy of adipose-derived mesenchymal stem cells (ASCs) in OA treatment. ASCs, when intra-articularly injected, have demonstrated the ability to modulate inflammation, reduce cartilage degradation, and improve joint function [5, 6]. Clinical trials have confirmed that ASC-based therapies can provide symptomatic relief and cartilage regeneration, as evidenced by MRI imaging and improved pain scores [7]. However, factors such as cell preparation, injection protocols, and host immune response may influence therapeutic outcomes.

Studies tracking ASCs post-injection indicate that their engraftment in the joint is limited, and their benefits are largely paracrine-mediated, rather than resulting from direct differentiation into chondrocytes [8–10]. To harness these paracrine effects in a cell-free approach, recent efforts have focused on the use of ASC-derived secretome or extracellular vesicles, which retain the regenerative potential of ASCs while potentially reducing immune-related complications [11]. Warmink et al. found that extracellular vesicles derived from MSCs had no therapeutic effect in metabolic OA models; MSC injections actually worsened OA outcomes [12]. Additionally, Li et al. demonstrated that human ASCs persist locally in the joint for up to 10 weeks post-injection, suggesting that their effects are likely dependent on both their transient presence and secreted bioactive factors [13]. Similarly, Horie et al. reported that intra-articular MSC injections promote cartilage repair through paracrine signaling, leading to increased collagen type II synthesis and inhibition of OA progression [11]. These findings suggest that ASC-derived products, including conditioned media, secretome, and extracellular vesicles, could be promising alternatives to whole-cell therapies. Human platelet lysate (hPL), a pooled product, allows for thorough characterization and can be used allogeneically. In vitro, hPL supports cellular viability, enhances proliferation, delays senescence, ensures cellular genomic stability, and preserves cellular immunophenotype [14]. Additionally, cells cultured with hPL exhibit increased secretory activity [15]. These promising characteristics make hPL an ideal biological adjuvant.

Building on this knowledge, the present study aims to investigate the effects of hPL-primed ASCs and their secretome (SEC) in a rat model of OA. We hypothesize that hPL conditioning enhances ASC paracrine activity, potentially improving their therapeutic efficacy. Furthermore, by evaluating both whole ASCs and their SEC, we aim to determine whether cell-free therapy could offer comparable or superior outcomes, with improved safety and scalability for clinical translation.

Human PL has been shown to increase the secretion of growth factors from stromal cells, leading to the hypothesis that hPL could enhance the release of trophic and anti-inflammatory factors from ASCs, thereby improving tissue regeneration and modulating inflammation. Based on this hypothesis, hPL could be intra-articularly injected with ASCs to achieve superior therapeutic efficacy. Alternatively, hPL could be used to treat ASCs in vitro to promote their secretory activity, resulting in an improved ASC-conditioned medium, known as secretome, that could be directly delivered to patients, thus addressing the challenges associated with cell-based therapies.

Methods

In vitro preparation of cell therapy

Isolation of stromal vascular fraction (SVF)

SVF was extracted from subcutaneous adipose tissue obtained as discarded material from abdominoplasty surgeries performed on five healthy women (with an average age of 38.6 ± 8.5 years) under written consent at IRCCS Ospedale Galeazzi-Sant'Ambrogio in Milan, Italy, following Ethics Committee approval (Comitato etico Ospedale San Raffaele, #125/int/2019, 2019/07/18, NCT 04225481, clinicaltrial.gov). The adipose tissue was initially cleansed

twice with phosphate buffer solution (PBS, Gibco) containing 5000 IU/mL of penicillin-streptomycin (Gibco) to eliminate excess blood and rinse any contaminating debris. Subsequently, the tissue was mechanically minced and enzymatically digested with 0.15% (w/v) animal-free collagenase type I (2 mL/g tissue, Worthington Biochemical Corporation) at 37 °C for 1 h in agitation. The collagenase action was inactivated by adding an equal volume of DMEM high glucose (Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone). The resulting solution was filtered through a 100 μm filter to eliminate undigested tissue. Following three washes with PBS, the cell pellet was suspended in Complete Medium composed of DMEM high glucose supplemented with 10% FBS, 2 mM L-Glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Gibco), and cultured in cell-treated Petri dishes at 37 °C with 5% CO_2. The medium was replaced after 24 h to remove unattached cells, and subsequently changed every 3–4 days until reaching cell confluence, thus obtaining selected ASCs. After reaching confluence, ASCs were characterized based on their adherence to plastic, fibroblast-like morphology, and ability to proliferate. While no additional purification steps were applied to specifically deplete non-MSC populations, ASCs were expanded under standard culture conditions that have been shown to favor MSC proliferation while minimizing the persistence of hematopoietic cells, including macrophages.

Preparation of ASCs for animal treatment

Before administering ASCs to the animals, cells cultured in Complete Medium were transitioned to a medium enriched with 5% hPL (Sextone Biotechnologies), along with 2 mM L-Glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. These cells were primed for 48 h and subsequently harvested. They were then suspended and cryopreserved with 10% DMSO and 90% FBS in liquid nitrogen. Before treatment, cells were thawed, washed to remove DMSO and FBS, and sub-cultured in DMEM high glucose supplemented with 5% hPL, 2 mM L-Glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin to allow recovery from cryopreservation stress. Cells were maintained at 37 °C with 5% CO_2 and expanded for one passage before being harvested for treatment. ASCs were used at passage 3 for all in vivo experiments, and 1×10^6 ASCs were suspended in 50 μL of sterile saline solution for injection into the knee joint.

Preparation of secretome for animal treatment

For the preparation of the secretome (SEC), ASCs that had been primed with hPL for 48 h were washed with PBS to remove residual hPL and subsequently cultured in a serum-free medium, consisting of DMEM high glucose supplemented with 2 mM L-Glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Gibco) for an additional 48 h. This step was conducted to stimulate the secretion of growth factors and bioactive molecules. The cells were maintained in a standard incubator at 37 °C with 5% CO_2 and 95% humidity during the entire culture process. After the 48-hour serum-free culture period, the conditioned medium containing the secretome was carefully collected. To eliminate cellular debris and ensure a pure secretome preparation, the medium was centrifuged at 350 xg for 5 min at room temperature. The supernatant, containing the secretome, was then aliquoted and stored at -80 °C until use in animal treatment experiments.

In vivo experiments

Ethics statement

Ethical approval for the experimental procedures was granted by the Animal Care and Use Committee (IACUC) of the Mario Negri Institute for Pharmacological Research (IRFMN) (Permit number 544-2019-PR). The rats were managed by EU legislation (Council of the EC Directive 2010/63/EU) and Italian law (D. legs 26/2014). All protocols were carried out in strict compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and this paper has been written in line with the ARRIVE statement. Specifically, 24 male Sprague-Dawley rats (12 weeks old, 317 ± 13 g), obtained from Envigo-Inotiv (Bresso, Milan, Italy), were used in this study. The rats were kept on a 12-hour light/dark cycle with unrestricted access to food and water. The decision to use male rats in this study was made to minimize variability due to hormonal fluctuations associated with the estrous cycle, which can influence pain perception and inflammation. However, we acknowledge that OA is more prevalent in females, and the exclusion of female subjects may limit the generalizability of our findings.

Induction of OA and treatments

The rats were anesthetized using 1.5% isoflurane. They received injections of 2 mg of monosodium iodoacetate (MIA I2512, Sigma-Aldrich, Italy) dissolved in 50 μL of sterile 0.9% NaCl saline solution. MIA was delivered into the left femorotibial joint using a 29-gauge needle through the patellar tendon. After the injection, the limb was stretched and flexed to permit the distribution of MIA. After the procedure, buprenorphine (0.1 mg/kg SC, Temgesic, Schering Plough) was administered, and then the rats were allowed to recover in their home cages. After 14 days, rats were randomly divided into 3 treatment groups ($n = 8$ each): sham control group (CTRL) receiving 50 μL of sterile 0.9% NaCl saline solution; Secretome group (SEC) receiving 50 μL of secretome; and ASC therapy group (ASCs) receiving 1×10^6 ASCs/50 μL

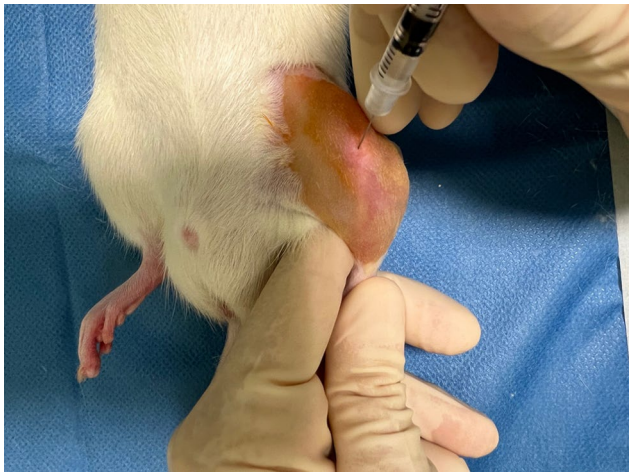

Fig. 1 Intraarticular injection. 50 µL of solutions based on the selected groups were injected into the articular joint

of sterile saline solution (Fig. 1). The contralateral limb was used as healthy control. The animals' welfare and general health were assessed daily throughout the study. Observations included monitoring for clinical signs of infection, lameness, weight-bearing ability, swelling, local hyperemia, and any signs of pain or distress. Additionally, changes in body weight were recorded weekly. After five weeks, the rats were euthanized using CO_2 as per established protocols. Subsequently, histological analyses were performed on explanted limbs to assess the articular cartilage and subchondral bone to quantify the severity and progression of OA in the treated groups.

Body weight

Body weight (b.w.) was measured on the day of MIA injection (D0) and weekly up to the day of explantation (D49) and reported as relative b.w. increase on the D0 baseline.

Histological and immunohistochemical analyses

The histological process for studying knee OA in rats involves the collection, preparation, and examination of knee joint tissues to assess the structural changes associated with OA. After rat euthanasia, knee joints were collected and fixed in 10% neutral buffered formalin (Bio Optica, Milan, Italy) for 48 h. Formalin-fixed knees were decalcified in a 20% solution of Formic acid 98% PA-ACS (ITW Reagents Panreac, Barcelona, Spain) diluted in Neutral buffered formalin 10%. The decalcification process lasted 7 days: at the end of the third day, the decalcifying solution was replaced, while on the seventh day, the knees were placed in ethanol 70% before the inclusion process. Specifically, the specimens were dehydrated, embedded in paraffin, and cut into 5 µm sections. Tissue morphology was blindly evaluated by hematoxylin and eosin (H&E) and Toluidine blue staining was performed for the cartilage matrix. Histology determined the degree of OA between experimental groups based on the histological score according to the OARSI-modified score system reported in Table 1.

Table 1 Histological score

Score	Features
0	No lesions
1	Articular cartilage with variable GAG loss and/or chondrocyte hypertrophy and/or chondrocyte proliferation. Capsule is normal. No inflammation.
2	Articular cartilage with diffuse GAG loss +/- necrosis up to the tidemark; possible fibrillation or superficial erosion. Capsule is normal. No inflammation.
3	Articular cartilage with diffuse necrosis up to the tide mark + focal up to subchondral bone with bone remodeling. Capsule can be thickened with GAG deposition.
4	Articular cartilage with diffuse necrosis up to subchondral bone of at least one cartilage with diffuse bone remodeling. Capsule can be thickened with GAG deposition/osteophytes.
5	Complete loss/destruction of at least one articular cartilage, covered by fibrovascular tissue, entrapping also degenerated meniscus. Capsule can be thickened with GAG deposition/osteophytes.

The presence of human cells in the rat joints was assessed using immunohistochemical analysis. For immunohistochemistry (IHC), sections were stained automatically using the Thermo Scientific™ Autostainer 480 S System (Thermo Fisher Scientific). Dewaxing of tissue sections and heat-induced epitope retrieval (HIER) were simultaneously performed using the Dewax and HIER Buffer H pH 9 (Thermo Fisher Scientific) at a boiling temperature for 40 min. Endogenous peroxidase was blocked with 3% H_2O_2 for 10 min at room temperature. Nonspecific protein binding was prevented with 10% goat serum for 30 min at room temperature. Sections were incubated for 1 h at room temperature with the following primary antibody: rabbit anti-human MHCI 1:600 (Abcam, ab52922) for human-derived cells. Sections were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, BA-1000) diluted 1:200. Labeling was performed with Vectastain® Elite ABC-Peroxidase kit (Vector Laboratories, PK-6100) diluted 1:150, and the reaction was visualized with Peroxidase ImmPACT® DAB Substrate (Vector Laboratories, SK-4105). Sections were counterstained with Mayer's hematoxylin and mounted with Micromount (Diapath). For each marker, adequate positive controls were included in the immunostaining runs.

Statistical analysis

The sample size was calculated based on the "resource equation" method [16]. Statistical analyses were carried out with GraphPad Prism 5 software (GraphPad Software, San Diego, California, USA). For comparisons

between groups, statistical significance was assessed using one-way ANOVA with Tukey's post hoc test for normally distributed data and the Kruskal-Wallis test followed by Dunn's post hoc test for non-normally distributed data. Normality was assessed using the Shapiro-Wilk test. Data are reported as means ± standard deviation (SD) and as median and range for the histological score. The level of significance was $p < 0.05$.

Results

Clinical examination

No clinical complications were observed following the MIA injection or subsequent treatments. Four rats randomly distributed in the 3 groups showed swelling and local erythema but maintained full weight-bearing capacity on the MIA-injected joint 24 h after injection. These findings were solved within 48 h in all the subjects. No other clinical signs of inflammation and/or infection were observed. However, we acknowledge that joint function and pain perception were not directly assessed through gait analysis or weight-bearing tests. Therefore, our conclusions regarding ASC effects are based primarily on histopathological changes rather than functional impairment.

Body weight

The relative increase in b.w. from the baseline (D0) is indicated in Fig. 2. All rats in all groups showed a gradual increase in body weight over time, consistent with their growth curve, without significant differences among groups. The treatments had the same effect on body weight at all values of time without significant differences.

Histopathology and immunohistochemistry

The histological score showed a score of 0 for all joints in the healthy knee group, indicating no detectable cartilage degeneration or pathological changes. Regarding the groups, there was no statistically significant difference between the CTRL and SEC groups, with a median value of 1.9 (Fig. 3). Both groups showed similar scores, suggesting comparable levels of cartilage degeneration.

Given the high range of values, it is not possible to conclude that SEC treatment led to an improvement in pathology compared to CTRL (Fig. 3). Overall, in CTRL and SEC-treated joints, the articular cartilage was characterized by GAG loss, minimal to mild necrosis up to the tide mark, and fibrillation or superficial erosion. The capsule was normal in the CTRL and SEC-treated joints (Fig. 4). On the contrary, the ASCs group showed more severe OA changes, with a median value of 4.5 (Fig. 3), characterized by marked GAG depletion, cartilage

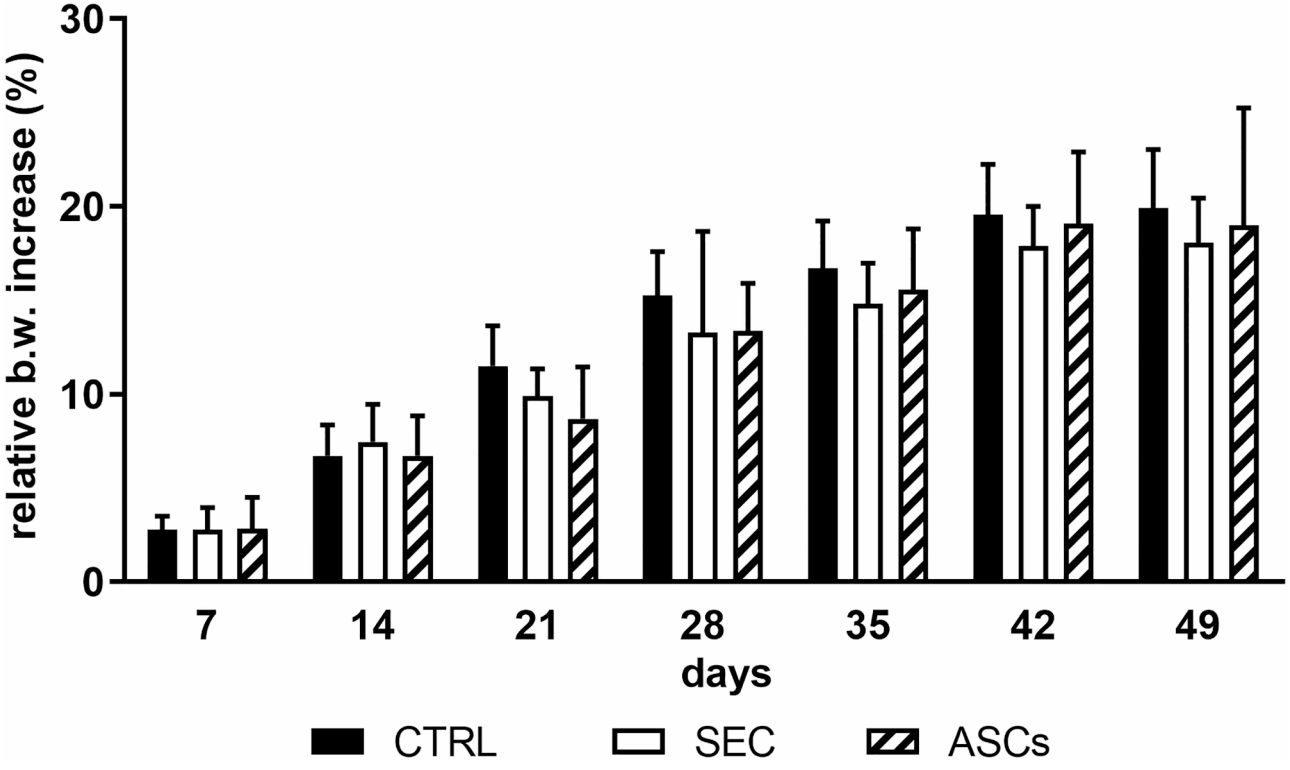

Fig. 2 Body weight. The histogram depicts the relative body weight increase in the experimental groups over time ($n = 8$/each group). Comparisons between groups and time points were analyzed with two-way ANOVA and Bonferroni's post hoc test. No significant differences were found

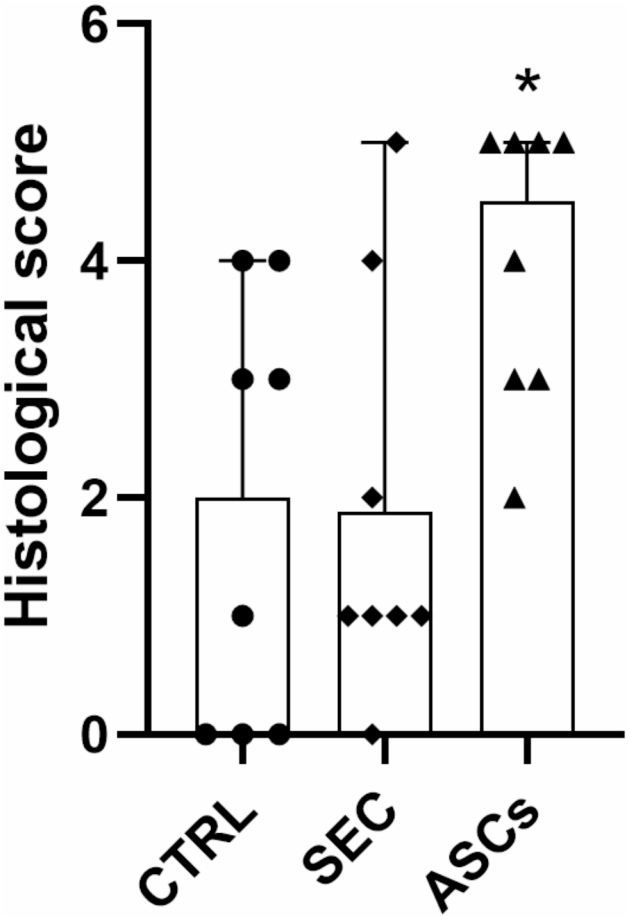

Fig. 3 Histopathological score. The histogram depicts the results of the histopathological semi-quantitative score in the experimental groups. Comparisons between groups were analyzed with the Kruskal-Wallis test. Data are presented as median ± range. (*) $P < 0.05$

fibrillation, cartilage necrosis, and subchondral bone remodeling (Fig. 4). The capsule was thickened with occasional GAG deposition and presence of osteophytes (Fig. 4). No human MHCI-positive cells were detected in the knee joints of control and ASC-treated animals (data not shown).

Discussion

The principal finding of this study is that, contrary to the anticipated regenerative and immunomodulatory effects, intra-articular administration of human ASCs in a rat model of MIA-induced OA was associated with more severe histopathological changes. This suggests that the presence of xenogeneic ASCs in an immunocompetent rat joint may have contributed to the disease progression rather than cartilage protection. Conversely, the SEC did not exacerbate OA, supporting its potential as a safer, cell-free alternative. The therapeutic potential of ASC-based therapies in regenerative medicine is significant, owing to their ability to not only differentiate into various cell types but also modulate the immune response, making them a versatile tool for tissue repair and the treatment of inflammatory and autoimmune diseases, or conditions like OA [17]. Considering the therapeutic potential of ASCs, the present study assessed the activity of ASCs and their secretome cultured in hPL to control the development and/or progression of the OA degenerative process in a rat model of OA. The MIA model of OA in rats is a widely used experimental model to study the pathogenesis and treatment of OA, leading to cartilage degeneration and changes that mimic those seen in humans. This model is particularly valued for its reproducibility, rapid induction of OA-like symptoms, and the ability to study nociceptive behavior and joint pathology [18, 19]. The immunocompetent rat model has been used elsewhere to understand the complex interplay between human ASCs and host immune response in OA, in which the immunomodulatory effects of ASCs could reduce inflammation and slow the progression of OA [8–10]. The principal observation of this study was that, contrary to the anticipated regenerative and anti-inflammatory effects, the administration of human ASCs in a rat model

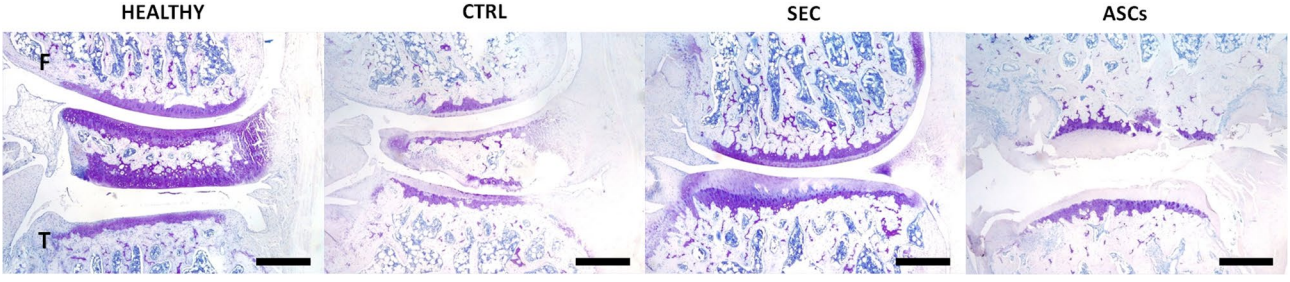

Fig. 4 Representative histopathological sections of knee joint cartilage from different treatment groups. Magnification 4X, scale bar 500 µm. Toluidine blue staining highlights proteoglycan content within the cartilage matrix. The CTRL and SEC groups display mild cartilage degeneration with GAG loss and superficial fibrillation. The ASC-treated group exhibits severe cartilage necrosis (visible as loss of viable cells) and subchondral bone remodeling. The histological sections include heterogeneous joint regions, with some sections displaying the central zone of the menisci, which stains more intensely due to higher proteoglycan content, while others show fibrous regions that stain less intensely with Toluidine blue. These differences correspond to areas subjected to varying loading conditions and may influence the interpretation of staining intensity

of MIA-induced OA was associated with more severe histopathological changes. These findings suggest that the presence of human ASCs in an immunocompetent rat joint may have contributed to an adverse biological response, potentially linked to an immune-mediated mechanism. Although overt clinical signs of inflammation were not observed, histopathological analysis revealed occasional minimal inflammation in the ASC-treated joints. While this response was not widespread, it may have influenced disease progression. Notably, the SEC treatment did not elicit similar pathological changes, suggesting that the presence of whole cells, rather than their paracrine factors, played a key role in this effect. In the present study, human-derived ASCs were not detected by immunohistochemistry with anti-human MHCI, but this was in part expected since the animals were examined 5 weeks after the treatment, and it is conceivable that inoculated cells were no longer present. Although there are studies demonstrating the feasibility of using human cells in studies conducted on immunocompetent rats [10, 20, 21], and while the joint is often considered an immune-privileged site, evidence suggests that implanted cells can still be recognized and eliminated by host immune cells, particularly in the presence of ongoing joint degeneration. This may explain the observed exacerbation of cartilage damage following ASC administration, which does not occur with SEC. While ASCs hold regenerative promise, based on our findings, their application in xenogeneic models may provoke adverse immune reactions, which can mask any therapeutic effect. On the other hand, the secretome is administered as an acellular treatment, avoiding inflammatory responses. While the ASC treatment in our study led to increased pathological changes, other studies have reported varying outcomes depending on the cell preparation and experimental conditions. For instance, Yang et al. [22] evaluated the effects of both stromal vascular fraction and ASCs in a rat model of OA and observed cartilage regeneration with both treatments. Unlike our study, they used rat-derived ASCs, which likely avoided the immune response that may have influenced our results. Similarly, Pers et al. [8] reported positive effects of ASCs in an immunocompetent model, but their study involved autologous ASCs, eliminating the risk of xenogeneic immune reactions. Other investigations have shown that the therapeutic potential of ASCs can be influenced by their culture conditions and mode of delivery. For example, Lin et al. [10] observed significant improvements in OA symptoms using human ASC spheres cultured on calcium-alginate scaffolds. However, their 3D culture system likely provided a protective microenvironment that reduced immunogenicity. In contrast, our study administered ASCs as a cell suspension without a scaffold, which may have increased their recognition by the host immune system. Several other studies have investigated xenogeneic MSC transplantation in immunocompetent rats, demonstrating beneficial and adverse outcomes depending on the model and cell preparation used. Horie et al. [11] found that intra-articular injection of human MSCs promoted meniscal regeneration in a rat model through paracrine signaling, increasing type II collagen expression while showing no signs of an immune reaction. Similarly, Li et al. [13] demonstrated that human ASCs persisted for up to 10 weeks after intra-articular injection in a rat OA model, with evidence of engraftment and proliferation. However, Warmink et al. [12] observed that intra-articular human MSC injection worsened OA outcomes in a metabolic OA rat model, suggesting a possible adverse immune reaction. Our findings are more aligned with Warmink et al. [12], as ASCs did not provide cartilage protection and instead correlated with increased OA severity. Conversely, the SEC treatment in our study, which involves cell-derived factors without cells, did not exacerbate OA, highlighting the role of cellular components in immune activation [21]. Previous studies have also demonstrated that xenogeneic cell transplantation in immunocompetent hosts can trigger immune recognition, which may lead to local inflammation, leading to increased cytokine and chemokine production and recruitment of inflammatory cells to the joint [23]. This contrasts with the expected immunomodulatory role of ASCs, which has been shown to reduce inflammation in other contexts [8]. Although ASCs have been shown to possess regenerative and immunomodulatory properties, the heterogeneous nature of the cell population means that certain cell subsets could potentially exert pro-inflammatory effects. While our initial hypothesis considered the possibility of an immune-mediated reaction to xenogeneic ASCs, we did not observe clear histological evidence of inflammation in the joint tissues. Minimal inflammatory changes were observed sporadically, but they were insufficient to draw definitive conclusions about an immune-mediated reaction. Therefore, we acknowledge that the observed cartilage degeneration may have resulted from factors other than overt inflammation, such as host-microenvironment incompatibility, ASC stress-induced senescence or impurity, or failed integration. The absence of detectable human cells at 5 weeks post-injection suggests that ASCs were likely cleared by the host immune system. This aligns with findings by Warmink et al. [12], where human MSCs were not detectable in rat joints after a similar time frame. In contrast, Li et al. [13] showed longer ASC persistence, potentially due to differences in cell preparation or the rat model used. In our study, the lack of ASC engraftment raised the question of whether cell viability, culture conditions, or immunogenicity played a role in the observed outcomes. The interaction between

the transplanted human ASCs and the existing pathological environment in the rat model could also play a critical role in the observed outcomes. The OA joint environment, characterized by ongoing inflammation, tissue degradation, and altered cellular signaling, may not be conducive to the proper integration and function of human cells. Instead of facilitating repair and regeneration, ASCs might undergo stress-induced senescence or dysfunctional differentiation, contributing to the disease's progression rather than amelioration [24]. Immunocompetent models are capable of recognizing and reacting to foreign human antigens presented by the transplanted cells, leading to an immune-mediated attack. This response can exacerbate inflammation within the joint, further deteriorating the OA condition [25]. Such reactions are not only limited to the immediate rejection of transplanted cells but can also stimulate a broader inflammatory response, aggravating the OA pathology. The SEC-treated group did not show disease worsening, suggesting that paracrine factors rather than whole cells may be responsible for the beneficial effects of ASCs. This is consistent with the growing research supporting the use of extracellular vesicles or secretome-based therapies as an alternative to whole-cell MSC transplantation [11]. The paracrine signaling hypothesis suggests that ASC-derived factors modulate inflammation and promote tissue repair, making acellular therapies potentially safer and more scalable. While acknowledging the limitations of the current study, the data presented provide valuable insights into the potential challenges of using human ASCs in xenogeneic models. A limitation of this study is the lack of ASC characterization. Without these data, we cannot exclude the possibility that the ASCs used retained some immunogenic properties, potentially contributing to the observed effects. Additionally, as the selection was based solely on plastic adherence, cellular heterogeneity may have influenced the results. While immunohistochemical analysis was used to detect human cells in the rat joints, PCR-based detection would have offered greater sensitivity and quantitative assessment, providing more insight into the persistence of human cells. Future studies should include detailed immunophenotyping and molecular tracking methods to better understand the mechanisms underlying the observed effects. Moreover, future research should also focus on optimizing hPL-primed ASCs in immunodeficient animals to verify their therapeutic potential while avoiding the xenogeneic reaction. In our study, SEC was collected from ASCs cultured under specific conditions designed to enhance their secretory activity. While the secretome used in the experiments was ensured to derive from a consistent number of ASCs, the exact correspondence between the amount of SEC and the number of cells that produced it was not directly quantified. This is an important consideration due to the variations in cell secretory activity that could impact the concentration of bioactive factors within the secretome. While the results obtained with SEC exhibited inter-subject variability, this variability was also observed in the control group, suggesting that it is not specific to the SEC treatment but rather reflects inherent differences in the biological response among subjects. Future studies should aim to further investigate the factors contributing to this variability, including individual differences in OA progression and response to treatment, as well as optimize SEC preparation to enhance its consistency and therapeutic potential.

Conclusions

This study explores the therapeutic potential of ASCs and their SEC in a rat model of OA. The SEC treatment did not exacerbate OA damage and was not associated with increased inflammation, unlike the ASC treatment. However, it did not demonstrate significant regenerative effects compared to the control group. These findings suggest that while SEC may be a well-tolerated treatment, further studies are needed to determine its potential therapeutic benefits. These findings also suggest that ASCs, though potentially beneficial in tissue regeneration, may provoke adverse immune reactions in this model, whereas SEC could serve as a safer, cell-free alternative for OA treatment. This research highlights the complexity of ASC-based therapies in xenogeneic models and the need for further investigation into the role of SEC. Future studies should focus on optimizing its preparation, characterizing its active components, and assessing its efficacy in models with reduced biological variability.

Abbreviations

ASCs	Adipose-derived stromal cells
CTRL	Sham control group
FBS	Fetal bovine serum
HIER	Heat-induced epitope retrieval
hPL	Human platelet lysate
MIA	Monosodium iodoacetate
MSCs	Mesenchymal stem cells
OA	Osteoarthritis
PBS	Phosphate-buffered saline
PRP	Platelet-rich plasma
SEC	Secretome
SVF	Stromal vascular fraction

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Author contributions

S.P., S.L., M.M. and A.B.L. conceptualized the study; S.P., S.L., C.R., S.C. and A.B.L. were responsible for methodology, investigations data curation; S.P., C.R., S.C. and A.B.L. wrote the original draft preparation; S.P. and A.B.L. revised and edited the manuscript; S.P., S.L., C.R., M.M. and A.B.L. supervised the study; S.P. and M.M. provided with the project administration; S.P. and M.M. provided

with funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Data availability
original data presented in the study are openly available in Zenodo at https://zenodo.org/records/12781067.

Declarations

Ethics approval and consent to participate
The study was conducted following the Declaration of Helsinki, and approved by the Ethics Committee of COMITATO ETICO OSPEDALE SAN RAFFAELE (#125/int/2019, 2019/07/18, NCT 04225481, clinicaltrial.gov). *Consent to participate statement*: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patients to publish this paper. The current study is not registered on specific portals, as it is not a pharmacological study nor does it involve unmarked medical devices. According to this, a specific clinical trial number is not applicable. The animal study protocol was approved by the Animal Care and Use Committee (IACUC) of the MARIO NEGRI INSTITUTE FOR PHARMACOLOGICAL RESEARCH (IRFMN) (Permit number 544-2019-PR, 2019/07/23).

Competing interests
The authors declare no competing interests.

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