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Short Report

Extracellular vesicles released by hair follicle and adipose mesenchymal stromal cells induce comparable neuroprotective and anti-inflammatory effects in primary neuronal and microglial cultures

Sara Hernando[1,2,3], Manoli Igartua[1,2,3], Edorta Santos-Vizcaino[1,2,3,*], Rosa Maria Hernandez [1,2,3,**]

[1] NanoBioCel Research Group, Laboratory of Pharmaceutics, School of Pharmacy, University of the Basque Country (UPV/EHU), Vitoria Gasteiz, Spain
[2] CIBER-BBN, ISCIII
[3] Bioaraba, NanoBioCel Research Group, Vitoria Gasteiz, Spain

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ABSTRACT

Background aims: Despite intensive research, to date, there is no effective treatment for neurodegenerative diseases. Among the different therapeutic approaches, recently, the use of extracellular vesicles (EVs) derived from mesenchymal stromal cells (MSCs) has gained attention.
Methods: In the present work, we focused on medium/large extracellular vesicles (m/lEVs) derived from hair follicle––derived (HF) MSCs, comparing their potential neuroprotective and anti-inflammatory effect against adipose tissue (AT)-MSC–derived m/lEVs.
Results: The obtained m/lEVs were similar in size with comparable expression of surface protein markers. The neuroprotective effect of both HF-m/lEVs and AT-m/lEVs was statistically significant in dopaminergic primary cell cultures, increasing cell viability after the incubation with 6-hidroxydopamine neurotoxin. Moreover, the administration of HF-m/lEVs and AT-m/lEVs counteracted the lipopolysaccharide-induced inflammation in primary microglial cell cultures, decreasing the levels of pro-inflammatory cytokines, tumor necrosis factor-α and interleukin-1β.
Conclusions: Taken together, HF-m/lEVs demonstrated comparable potential with that of AT-m/lEVs as multifaceted biopharmaceuticals for neurodegenerative disease treatment.

Introduction

Neurodegenerative diseases (NDs) such as Alzheimer disease (AD) and Parkinson disease (PD) are complex neurological conditions resulting in progressive neuronal loss within the affected brain areas [1]. Despite intensive research, the cause of neuronal loss is not fully understood. However, several molecular and cellular changes have been identified as key factors in neurodegeneration, which include abnormal protein handling, oxidative stress, mitochondrial dysfunction or neuroinflammation as well as microglia response to local damage [2]. Despite their significant burden in public health systems, to date, all new drug candidates have failed in phase 2 and 3 clinical trials, without meeting the minimal clinical requirements [3,4].

Indeed, various approaches including anti-inflammatory drugs and neurotrophic factors are being tested in preclinical models as novel treatments for PD. [5]. In line with these increasing efforts to improve the efficacy of PD treatment, cell-based therapy has been raised as a promising alternative approach. Among all of them, mesenchymal stromal cell (MSC)-based therapy is by far the most-used cell therapy. MSCs exhibit immunomodulatory effects, migratory ability and regenerative potential due to the secretion of cytokines, neurotrophins and angiogenesis regulatory factors [6]. Moreover, extensive scientific evidence points to the paracrine action of their secretome, which includes extracellular vesicles (EVs), as the main responsible agent for the therapeutic effects observed [7]. These nanometer-sized lipid membrane-enclosed vesicles are secreted by most cells and contain lipids, proteins and various nucleic acid species depending on the source cell [8]. Following the MISEV 2018 guidelines, EVs cannot be assigned to a particular biogenesis pathway due to the lack of consensus on specific markers for EV subtypes. Therefore, EV subtypes are classified in terms of (i) physical

* Correspondence: Edorta Santos-Vizcaino, NanoBioCel Research Group, Laboratory of Pharmaceutics, School of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria Gasteiz, Spain.
** Rosa Maria Hernandez, NanoBioCel Research Group, Laboratory of Pharmaceutics, School of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria Gasteiz, Spain.
E-mail addresses: edorta.santos@ehu.eus (E. Santos-Vizcaino), rosa.hernandez@ehu.es (R.M. Hernandez).

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characteristic (size or density), (ii) biochemical composition (presence of some proteins i.e., CD63+/CD81+ annexin) or (iii) descriptions of culture conditions or cell of origin (i.e., hypoxic EVs or apoptotic bodies). In the present research article, we classify the EVs according to their size; therefore, small extracellular vesicles (sEVs) will be those presenting range values of <100 nm or <200 nm and medium/large extracellular vesicles (m/lEVs) of >200 nm [9].

Among the different tissue sources to obtain MSCs, adipose tissue–derived (AT)-MSCs have been commonly used for cell-therapy purposes. However, some of the procedures to harvest these cells are still relatively harmful, painful and invasive for the patient. As the result of these drawbacks, more easily accessible sources for MSC collection have been investigated. In this regard, Hernaez-Estrada *et al.* [10] fully evaluated the immunomodulatory function of hair follicle-derived (HF) MSCs with results comparable with AT-MSC. Moreover, recent research by Las Heras *et al.* [11] concluded that the EVs derived from HF-MSC (HF-EVs) exhibited comparable results with the EVs released from AT-MSC (AT-EVs) in terms of characterization and functional assays for wound healing. Moreover, the previously mentioned research article showed comparable results between m/lEVs and sEVs with the same therapeutic action for wound healing.

With regard to ND treatments, various studies have evaluated the therapeutic potential of MSC-derived EVs [12]. In 2013, Katsuda *et al.* [13] were among the first to assess the efficacy of AT-EVs in AD pathology using mouse neuroblastoma N2a cells. More recently, Narbute et *al.* [14] confirmed the therapeutic potential of EVs derived from human teeth stem cells in a 6-hydroxydopamine (6-OHDA)-induced animal model of PD. However, in all those research papers, the authors focus on sEVs, discarding the m/lEVs fraction.

Herein, we focused on the m/lEVs fraction because of the comparable results obtained in the previously cited wok by Las Heras *et al.* [11] in the sEVs and the m/l EVs fractions. Moreover, the more universal accessibility of m/lEVs could fortunately increase the clinical translation of these new biopharmaceuticals. To summarize, we isolated m-lEVs from HF-MSCs to compare their potential neuroprotective and anti-inflammatory effect to AT-MSC–derived m/lEVs. For such aim, HF-m/lEvs and AT-m/lEVs were tested in neurotoxin-treated primary dopaminergic neurons and lipopolysaccharide (LPS)-challenged primary microglia.

Methods

EVs isolation and characterization

Cell culture conditions

HF-MSCs were isolated and characterized from HFs as previously described by Hernaez-Estrada et *al.* [10]. They were further cultured in Dulbecco's modified Eagle's medium (DMEM) (49166029; Gibco, Billings, MT, USA) supplemented with 10% of fetal bovine serum (10270106; Gibco) and 1% (v/v) penicillin/streptomycin (P/S) (15140122; Gibco). AT-MSCs (PCS-500 011; ATCC, Manassas, VA, USA) were cultured following the previous cited conditions. For EVs production, DMEM medium was used with 10% of EV-depleted fetal bovine serum (A2720801; Gibco). HF-MSCs and AT-MSCs were used at passages from 5 to 9 for EVs isolation.

EVs isolation

EVs were isolated and purified from the supernatant of HF-MSCs and AT-MSCs as previously described [11]. At 70–80% of confluence, cells were washed three times with phosphate-buffered saline (PBS) (10010015; Gibco) and the culture medium was substituted with EVs depleted DMEM. After 72 h, the culture medium was collected and new EV-depleted medium was added. After three collections of EV, cells were trypsinized, counted and further re-cultured at 12 000 cell/cm^2 cell density. The collected medium was first centrifuged at 2000 $\times$ *g* for 10 min at 4°C to discard cell debris and then stored frozen at −80°C until the isolation and purification was done. All further centrifugation processes were performed at 4°C. Thawed culture supernatants were centrifuged at 10 000 $\times$ *g* for 30 min to obtain m/lEVs. All pellets were immediately re-suspended in 120 μL of ice-cold PBS and frozen at −80°C until use. As a negative control—named as Blank CTRL in the manuscript—the same exact process with EVs depleted media was performed in flasks without cells to determine the possible carry-over of fluorescence by the culture medium.

Nanoparticle tracking analysis

Particle concentration and size distribution within EV preparations was analyzed using the nanoparticle-tracking analysis by measuring the rate of Brownian motion in a NanoSight LM10 system (Malvern Panalytical, Malvern, UK). For measurement, original EV suspension was diluted from 1:70 to 1:100 with PBS and a volume of 1000 μL was loaded on the camera, and three consecutive video recordings of 40 s each were taken for every sample quantified.

Bead-based multiplex flow cytometry assay

All isolated EVs were subjected to a surface-marker characterization by using a flow cytometry bead-based multiplex analysis (130-108-813; MACSPlex Exosome Kit, human, Miltenyi Biotec, Bergisch Gladbach). Samples were processed according to manufacturer's protocol. The flow cytometry analysis was performed using the MACSQuant Analyzer 10 (Miltenyi Biotec) and results were processed with the MACSQuant Analyzer 10 software (Miltenyi Biotec). The 39 single-bead populations were gated to determine the APC signal intensity on each bead population and the median fluorescence intensity for each capture bead was measured. Values of the corresponding isotype control were subtracted. Only positive markers are shown in the graphics.

Cryo-electron microscopy (cryo-EM)

Freshly glow-discharged 200-mesh grids (R2/1; QUANTIFOIL) were placed inside the chamber of an EM GP2 Automatic Plunge Freezing (Leica Company, Wetzlar, Germany), which was maintained at 8°C temperature and relative humidity close to saturation (90% rH). Then, 4 μL of the sample was dropped onto the grid for 30 seconds. After incubation, most of the liquid on the grid was removed by blotting (blot time was 2 seconds, number of blots was set to 1 and no extra movement was applied) with absorbent standard filter paper (Ø55 mm, Grade 595; Hahnemühle, Dassel, Germany). After the blotting step, the grid was abruptly plunged into a liquid ethane bath, automatically set to −184°C. Once the specimen was frozen, the vitrified grid was removed from the plunger and stored under liquid nitrogen inside a cryo-grid storage box. Cryo-TEM analysis of the samples was performed on a JEM-2200FS/CR (JEOL Europe, Croissy-sur-Seine, France) transmission electron microscope.

EVs functional assays in rat primary cell cultures

Primary cell cultures

All Animal procedures were reviewed and approved by the Local Ethical Committee for Animal Research of the University of the Basque Country (UPV/EHU); (CEEA, ref. M20/2021/345 and M20/2021/347). All of the experiments were performed in accordance with the European Community Council Directive on "The Protection of Animals Used for Scientific Purposes" (2010/63/EU) and with Spanish Law (RD 53/2013) for the care and use of laboratory animals. In summary, dopaminergic neuronal cultures were prepared from Wistar rat embryos at 15, 16 or 17 days of gestation (E15–E17). To obtain primary dopaminergic cell cultures, the protocol was followed as described in this previous publication [15] (supplementary Figure 1A). The obtained dopaminergic cell suspension was seeded at 40 $\times$ 10^3 cells/well density to carry out the neuroprotective assay. In addition, microglial cell cultures were prepared from Wistar rat puppets at day 0 to 2 (P0–P2). The protocol was followed as described before [15] (supplementary Figure 1B) and microglia

cell suspension was employed at 50 × 10^3 cells/well density for cytokine release assay.

Neuroprotective assay

After 7–10 days of maintaining dopaminergic culture, neuroprotective assay was carried out with HF-MSC and AT-MSC derived EVs (HF-m/lEVs and AT-m/lEVs) (supplementary Figure 2A). The media were removed and the EV dose equivalent to a total protein content of 20 μg was re-suspended in PBS (microBCA determined, 23235; Thermo Fisher Scientific, Waltham, MA, USA) and added to the culture 24 h before the incubation with the 6-OHDA neurotoxin. 24 h after, the media was removed and fresh media was added, with a final concentration of 25 μmol/L 6-OHDA neurotoxin and the previously tested concentrations for HF-m/lEVs and AT-m/lEVs, 20 μg. In the case of the Blank Control group, the same volume of PBS corresponding to the non-EVs control group was added to the cell culture. To assess the neuroprotective effect against 6-OHDA neurotoxin, the media were removed 24 h later and cells were fixed with 3.7% paraformaldehyde for 10 min and then washed three times in PBS. DAPI (4′,6-diamidino-2-fenilindol, dihidrocloruro) (D1306; Thermo Fisher Scientific) staining (1:10 000) in PBS for 15 min was used to determine viable cells. For cell viability quantification, fluorescence microscopy images were obtained by means of an inverted microscope (Nikon TMS, Hampton, NH, USA). Three images per well and four wells were used for each group; in total, 12 images were taken per group. Dopaminergic cells were scored as positive if they exhibited defined nuclear counterstaining. The data are expressed as the percentage of the cells group with no treatment but just media change, which was set as 100%.

Pro-inflammatory cytokine release quantification: tumor necrosis factor (TNF)-α and interleukin (IL)-1β

The effect modulating the inflammation of HF-m/lEVs and AT-m/lEVs was carried out in LPS (Ultrapure, tlrl-3pelps; InvivoGen, San Diego, CA, USA) activated microglia primary cells (supplementary Figure 2B). To perform the assay, cells were pretreated for 24 h with 20 μg of protein content of HF-m/lEVs or AT-m/lEVs (microBCA determined). After that treatment, media were removed and cells were incubated for another 24 h with 50 ng/mL LPS and a second dose of EVs (20 μg). After 24 h, the cell media supernatant was collected and stored at −80°C. The levels of TNF-α and IL-1β pro-inflammatory cytokines were analyzed with ELISA assay (900-K73 and 900-K91; PeproTech, Cranbury, NJ, USA). The total amount of cytokine release was normalized according to cell viability measured with CCK-8 assay at the same time point.

Statistical analysis

All results are expressed as mean ± standard deviation. The experiments were n = 2 biological replicates; indeed, each experiment was repeated in two different cultures, two dopaminergic cultures and two microglia cultures. In each experiment of those cultures, at least n = 3 technical replicates were performed with two different batches of EVs. Experimental data were analyzed using the computer program GraphPad Prism (version 6.01; GraphPad Software, San Diego, CA, USA). Two-way analysis of variance followed by Bonferroni's multiple comparison test was used to analyze the data regarding the surface marker evaluated by flow cytometry. One-way analysis of variance followed Turkey's multiple comparison test was used for analyzing all the data regarding the functional assays of EVs, neuroprotective and anti-inflammatory assay. P values <0.05 were considered significant.

Results

EV isolation and characterization

For both cell types, nanoparticle-tracking analysis measurements showed sizes ranging from 100 nm to nearly 400 nm for the m/lEVs in both cell types (Figure 1A). HF-ml/EVs mean size (242.5 ± 2.6 nm) was similar to the values obtained in AT-m/lEVs (255.8 ± 3.4 nm). The size distribution of EVs from cryo-EM images revealed populations approximately 200–300 nm. Moreover, the EV cryo-images revealed a lipid bilayer structure, typical from cellular interior envelope (Figure 1B). EVs were also evaluated by flow cytometry for the expression of 37 surface markers. We analyzed two different batches for each group and found populations positive for CD105, SSE-4, CD9, CD63, CD81, CD29, CD49e, CD11c, CD41b, MCSP, CD56, CD44, CD146, HLA-ABC and CD142 (Figure 1C). As observed, AT-EVs showed statistically significant greater levels for CD105, SSEA-4, CD63, CD29 and CD44 than HF-EVs.

HF-m/lEVs and AT-m/lEVs exhibited a neuroprotective effect

The selected 25 μmol/L dose of 6-OHDA generated approximately 50% of dopaminergic cell death (6-OHDA vs cells, ***$P < 0.001$) (Figure 2A). The incubation of the dopaminergic neuron culture with HF-m/lEVs and AT-m/lEVs before and after 6-OHDA addition resulted in a neuroprotective effect in comparison with the neurotoxin alone.

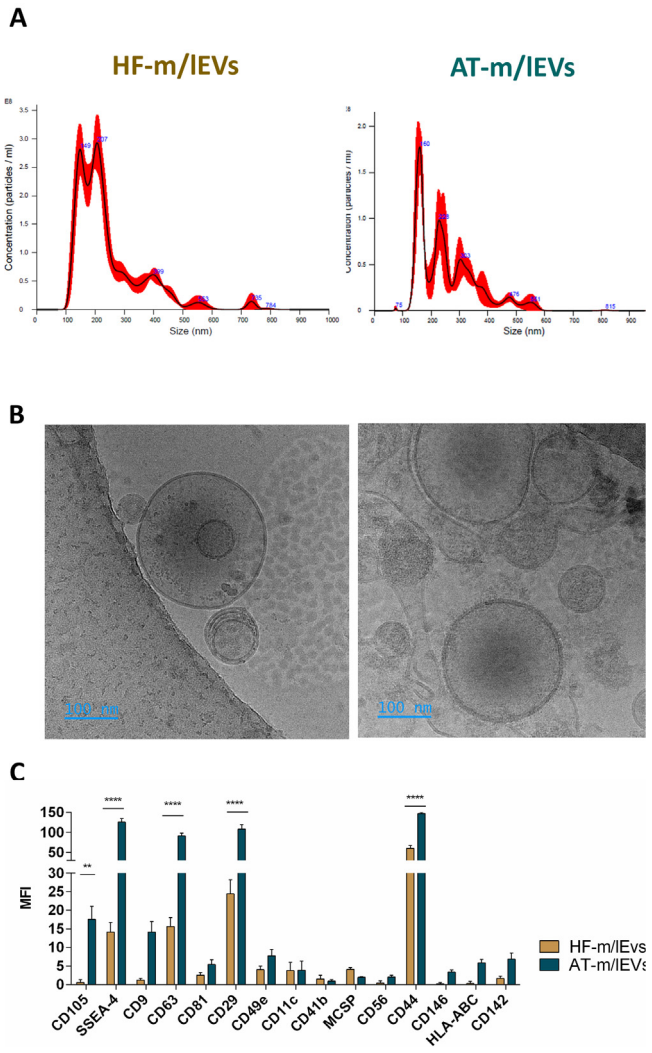

Figure 1. m/lEV characterization. (A) Nanoparticle-tracking analysis size-profile of HF-m/lEVs and AT-m/lEVs. (B) Cryo-EM images of EVs. Scale bars are 100 nm. (C) Multiplex bead-based flow cytometry assay for detection of EV surface markers. (All data are represented with two different batches; n = 3 technical replicates in each batch). Two-way analysis of variance followed by Bonferroni multiple comparison test—comparison between cell type and surface marker ($P > 0.05$)–(****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$). (Color version of figure is available online.)

The values for the remaining living cells in HF-m/lEVs group were almost similar to the cells, which was set as 100%. The obtained values where statistically significant when compared with the 6-OHDA or Blank CTRL group ($^{*}P < 0.05$). Improvement in cell viability was also significant in the AT-m/lEVs group vs 6-OHDA ($^{***}P < 0.001$) and Blank CTRL group ($^{****}P < 0.0001$). In contrast, the treatment with the Blank CTRL group did not increase cell viability, with the remaining living cell values similar to those obtained with the sole neurotoxin incubation. In this regard, no statistical differences were observed between 6-OHDA and the Blank CTRL group. The images taken with fluorescence microscopy showed the difference in living cells between the different groups (Figure 2B).

HF-m/lEVs and AT-m/lEVs decreased cytokine pro-inflammatory release in microglial culture

In order to assess the anti-inflammatory effect of m/l-EVs from two different cell sources—HF-MSC and AT-MSC—microglia cell culture supernatant levels of pro-inflammatory cytokines after LPS stimuli (with or without treatment) were quantified by ELISA technique (Figure 3).

Regarding TNF-α ELISA (Figure 3A), the basal levels of TNF-α statically increased ($^{****}P < 0.0001$) after LPS treatment (50 ng/mL). The incubation with HF-m/lEVs and AT-m/lEVs decreased almost to control levels the values for this cytokine. This anti-inflammatory effect was not seen in the case of Blank CTRL group, highlighting the positive effect of HF-m/lEVs and AT-m/lEVs modulating the inflammatory condition. Moreover, no statistical difference was observed between two groups of treatment of m/lEVs.

Similarly, LPS stimulation also significantly increased IL-1β levels compared with non-stimulated microglia cells (Figure 3B). However, in this case, only treatment with HF-m/lEVs significantly ameliorated LPS-induced IL-1β secretion. However, an obvious reduction in the levels of this cytokine after the treatment with AT-m/lEVs can be observed. In the case of Blank CTRL group, no positive effect was observed decreasing the levels of IL-1β pro-inflammatory cytokine.

Discussion

Although MSC-based therapies have been widely investigated for treating NDs [6], the use of MSC-derived EVs is still in its infancy as a feasible solution for NDs treatment. In this research paper, we aimed to study and compare the effect of EVs derived from HF-MSCs and AT-MSCs. More concretely, in this case we have focused on m/lEVs. In fact, this population of EVs has garnered attention as the result of their ability to deliver healthy and functional mitochondria, allowing the transfer of mitochondrial proteins, lipids and mitochondrial DNA [16], thus leading

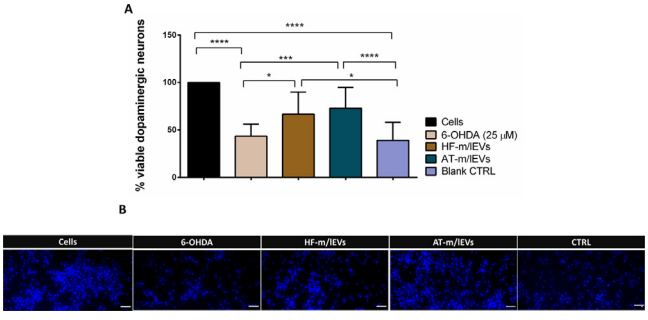

Figure 2. Neuroprotective assay. (A) Graphic representation of the neuroprotective effect of m/l-EVs for the two different cell populations, HF-MSC and AT-MSC. ($^{****}P < 0.0001$ $^{***}P < 0.001$; $^{*}P < 0.05$. One-way analysis of variance, Turkey's multiple comparison test. (B) Representative fluorescence images of the neuroprotective assay with DAPI (4′,6-diamidino-2-fenilindol, dihidrocloruro) staining. The scale bar indicates 50 μmol/L. All data are represented with two different batches of EVs, two different dopaminergic cell cultures and at least n = 3 technical replicates. (Color version of figure is available online.)

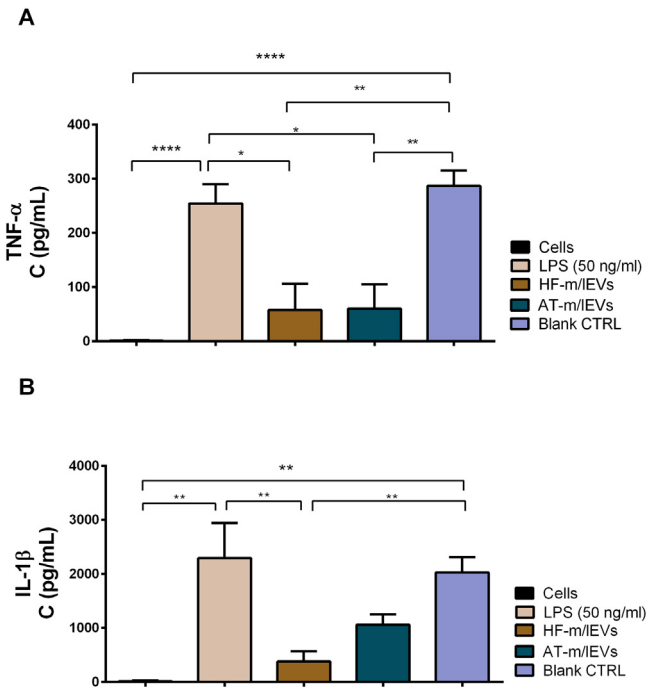

Figure 3. Anti-inflammatory assay. (A) Cytokine levels of TNF-α (pg/mL). ($^{****}P < 0.0001$; $^{**}P < 0.01$; $^{*}P < 0.05$) One-way analysis of variance, Turkey's multiple comparison test. (B) Cytokine levels of IL-1β (pg/mL). ($^{**}P < 0.01$ Cells vs LPS and Blank CTRL; $^{**}P < 0.01$ HF-m/lsEVs vs LPS and Blank CTRL. One-way analysis of variance, Turkey's multiple comparison test. All data are represented with two different batches of EVs, two different microglia cell cultures and at least n = 3 technical replicates. (Color version of figure is available online.)

the repair of potentially damaged cells in plenty of diseases. The m/lEVs may activate the endogenous cellular stress response pathways to allow for repair and metabolic recovery, via revival of mitochondrial function due to the transfer of healthy mitochondria through this EV fraction [17]. Indeed, m/lEV-mediated mitochondrial delivery could be a key step in addressing the unmet clinical challenges for NDs. Moreover, as showed in the previous work conducted by Las Heras *et al.* [11], the regenerative potential of m/lEVs was comparable with that of sEVs for both HF-MSCs and AT-MSCs. According to these data, we performed experiments with only the m/lEVs fraction, to assess, as a first approach, the potential therapeutic effect of this emerging biotherapeutical for NDs.

The obtained values for m/lEVs size were similar to the previous data [11], with values ranging from 70 to 700 nm (Figure 1A,B). In addition, according to surface marker analysis (Figure 1C), EVs were confirmed to be enriched in a collection of proteins, including integrins and tetraspanins (CD9, CD63, CD81) [18] for both batches—more expressed in AT-ml/EVs than in HF-ml/EVs—as well as MSC typical markers (CD105) [19] or several adhesion markers (CD29, CD44 and CD49e) [20], among others.

In this research paper, dopaminergic neuron cell cultures were employed as an *in vitro* model of PD to study the therapeutic effect of HF-ml/EVs and AT-ml/EVs. In this regard, the addition of 6-OHDA neurotoxin—one of the most widely used neurotoxins to model PD [21]—led to a decrease of 50% in neuron cell viability. The treatment with HF-m/lEVs and AT-m/lEVs induced neuroprotection with values of remaining living cells up to 80% compared with the cells without the neurotoxin (Figure 2). These data are in line with the effect of EVs derived from human umbilical cord-MSC (hUC-MSC) [22], where the intravenous administration of hUC-MSC-EVs to a 6-OHDA animal model of the disease improved motor performance increasing brain dopamine levels. In the case of EVs harvested from AT-MSC, the positive effect was only obtained after the modification in cell culture conditions with hypoxia [23]. The same was the case for EVs derived from dental pulp stem cells, in which cell culture condition was modified to stimulate their therapeutic effect *in vitro* [24]. In this case, no

modifications on cell culture media were required to elicit the therapeutic effect of HF-m/lEVs or AT-m/lEVs.

One of the most exciting observations of the past 20 years is that glia plays a central role in NDs, contributing not only to PD pathogenesis and progression but also to the subsequent neuroinflammation [25]. Indeed, neuroinflammation and associated "reactive" microglia have long been recognized as key elements of PD [26]. Hence, the ability of m/lEVs to modulate microglia was studied in the present article. As seen in Figure 3, AT-m/lEVs and, more concretely HF-m/lEVs, decreased two well-known pro-inflammatory cytokines, TNF-α and IL-1β. The potential of EVs to modulate microglia has previously been described with hUC-EVs in status epilepticus [27] or in an AD animal model [28]. In contrast, the EVs derived from bone marrow–MSC have also demonstrated their ability to modulate microglia activation in a hypoxia–ischemia-injured animal model [29] or a 3xTg model of AD [30]. Regarding AT-EVs, the recently published paper by Ma *et al.* [31] showed a decrease on microglial reactive cells levels in APP/PS1 model of AD. In this case, the employed dose for AT-EVs was the same as the one we use in the present research article. Moreover, 20 μg of EVs was also the dose employed in the results reported by Garcia-Contreras *et al.* [32]. Thus, we can conclude that the reported results in this work, with the same dose of EVs, are comparable with those previously reported with MSC source or different *in vitro* or *in vivo* model.

In summary, we showed that m-lEVs derived from HF-MSCs appear to exhibit a neuroprotective and anti-inflammatory potential comparable with that shown by EVs derived from AT-MSC in primary cell cultures of neurotoxin-treated dopaminergic neurons and LPS-challenged microglia. The effect of AT-EVs on NDs has been studied as explained before, with the first research studies conducted by Katsuda et *al.* [13], as well as with the more recently published results in APP/PS1 mice model of AD [31]. However, none of the previously cited research papers focused on the m/l-EVs fraction. Moreover, as far as we know, the effect of HF-MSC or HF-EVs has not been tested before this research article as a therapeutic option for NDs. The positive results presented in this research article for HF-EVs could have a promising effect on the clinical translation of MSC-based therapies. In fact, the less harmful, painful and invasive technique of obtaining HF-MSCs could be beneficial for patients, thus increasing the chances of obtaining a greater number of MSCs for cell therapies or MSC derived EVs-based therapies purposes. Although still-intense research needs to be performed, altogether, we demonstrated that m/lEVs derived from HF-MSC and AT-MSC showed equal potential as new biopharmaceuticals for NDs treatment, opening the field for new MSC sources for EVs-based therapies for ND treatment.

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Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the study: SH, MI, ESV, RHM. Acquisition of data: SH. Analysis and interpretation of data: SH, MI, ESV, RHM. Drafting or revising the manuscript: SH, MI, ESV, RHM. All authors have approved the final article.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2023.04.001.

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