A Systematic Review on Extracellular Vesicles-Enriched Fat Grafting: A Shifting Paradigm

Mohammad Ghiasloo, MD; Laura De Wilde; Kashika Singh, MD; Patrick Tonnard, MD, PhD; Alexis Verpaele, MD, PhD; Olivier De Wever, PhD; and Phillip Blondeel, MD, PhD

Dr Ghiasloo is a Fellow, Department of Plastic and Reconstructive Surgery, Ghent University Hospital, Ghent, Belgium. Ms De Wilde is a medical student, Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium. Dr Singh is a Surgical Resident, Department of General and Visceral Surgery, Evangelical Hospital Bethel Bielefeld, Bielefeld, Germany. Drs Tonnard and Verpaele are co-directors in private practice in Ghent, Belgium. Dr De Wever is an Associate Professor, Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium. Dr Blondeel is a Full Academic Professor, Faculty of Medicine and Health Sciences; and Chairman, Department of Plastic and Reconstructive Surgery, Ghent University Hospital, Ghent, Belgium.

Corresponding Author: Dr Mohammad Ghiasloo, Department of Plastic and Reconstructive Surgery, Ghent University Hospital, Corneel Heymanslaan 10 Route 1281, Ghent B-9000, Belgium.
E-mail: mohammadghiasloo@hotmail.com

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Level of Evidence: 4 (Therapeutic)
Abstract

Background: Recent evidence confirms that mesenchymal stem cells (MSCs) facilitate angiogenesis mainly through paracrine function. Extracellular vesicles (EVs) are regarded as key components of the cell secretome, possessing functional properties of their source cells. Subsequently, MSC-EVs have emerged as a novel cell-free approach to improve fat graft retention rate.

Objectives: To provide a systematic review of all studies reporting the use of MSC-EVs to improve graft retention rate.

Methods: A systematic search was undertaken using the Embase, PubMed and the Cochrane Central Register of Controlled Trials databases. Outcome measures included donor/receptor organism of the fat graft, study model, intervention groups, evaluation intervals, EV research data, in vitro and in vivo results.

Results: Of the total 1717 articles, 62 full-texts were screened. Seven studies reporting on 294 mice were included. Overall, EV treated groups showed higher graft retention rates compared to untreated groups. Notably, retention rate was similar following EV- and MSC-treatment. In addition to reduced inflammation, graft enrichment with EVs resulted in early revascularization and better graft integrity. Interestingly, hypoxic preconditioning of MSCs improved their beneficial paracrine effects and led to a more proangiogenic EV population, as observed by both in vitro and in vivo results.

Conclusions: MSC-EVs appear to offer an interesting cell-free alternative to improve fat graft survival. While their clinical relevance remains to be determined, it is clear that not the cells, but their secretome is essential for graft survival. Thus, a paradigm shift from cell-assisted lipotransfer towards ‘secretome-assisted lipotransfer’ is well on its way.
Adipose tissue (AT) is considered as the ideal soft tissue filler, giving rise to its widespread use in reconstructive and aesthetic surgery. In addition to its biocompatibility, AT is abundant and easy to harvest through liposuction. However, fat grafting is highly unpredictable and is associated with high rates of graft resorption over time. Resorption is mainly caused by local ischemia and delayed revascularization following graft injection. However, liposuction-induced membrane damage, devascularization and ischemic cell injury must be considered as well.

Attempts to improve graft survival have led to the introduction of the cell-assisted lipotransfer (CAL). Although the CAL method was associated with better graft retention compared to conventional fat grafting, this effect was only significant for injected volumes under 100 cc. Furthermore, patients treated with the CAL technique experienced more complications, while undergoing a similar number of procedures compared to patients undergoing conventional fat grafting. While the mechanisms of CAL remain elusive, it is widely accepted that instead of differentiating towards any specific cell linages, the mesenchymal stem cells (MSCs) rather exert a pro-survival secretome, stimulating neovascularization and tissue regeneration. In addition, their paracrine functions appear to be enhanced due to local ischemia and hypoxia. Regardless, cell-based therapies are associated with many limitations, including senescence-induced genetic instability, undesirable long-term side effects and uncontrolled differentiation. Hence, cell-free alternatives appear to be on the rise.

One cell-free alternative are the soluble proteins and growth factors found in the MSC’s secretome. However, concerns have been raised regarding the delivery, dose, cost and safety of such therapies. Another alternative includes the use of MSC-derived extracellular vesicles (EVs); a heterogenous population of nanosized membrane-enclosed vesicles involved in intercellular communication in both physiological and pathological conditions. There are two main EV subtypes: the smaller exosomes (50-150 nm in diameter) originated from endosomes, and the generally larger cell membrane derived microvesicles (50-500 nm in diameter, up to 1 μm). While both populations are overlapping in size and content, it is mostly the smaller exosomes that are believed to be involved in the delicate processes of crosstalk.

Increasingly, MSC-derived EVs (MSC-EVs) have demonstrated therapeutic potential as cell-free alternatives for treatment of various conditions. Several reports have recently identified EVs as a promising alternative to increase fat graft retention rates. The current
study aims to systematically summarize the existing evidence regarding the effects of EV-enriched fat grafting.

METHODS

Protocol
A systematic review was performed according to the guidelines and recommendations of the preferred reporting items for systematic reviews and meta-analysis checklist (PRISMA). Prior to start, a protocol and extraction form were designed and pre-approved by the research team. Institutional review board or ethics committee approval was not required.

Literature search
An electronic search strategy was designed and was refined for the Embase, PubMed and the Cochrane Central Register of Controlled Trials databases. The following search terms and synonyms were used: “(adipose tissue OR lipoaspirate OR microfat OR stromal vascular fraction OR SVF OR stromal vascular fraction cell OR stromal vascular cell OR nanofat OR emulsified fat OR fat emulsification OR fractionated fat OR micro fragmented adipose tissue OR micro fragmented fat tissue OR fragmented fat OR fat fragmentation OR dissociated fat OR adipose cell OR adipose tissue cell OR adipose tissue stromal cell OR adipose stromal cell OR adipose-stromal cell OR adipose derived stromal cell OR adipocyte OR mesenchymal stem cell OR adipose derived stem cell OR adipose derived stem cell OR adipose tissue stem cell OR ADSC OR HADSC) AND (extracellular vesicles OR exosomes OR apoptotic bodies OR microvesicles OR microparticles)”. The search strategy is outlined in Appendix 1. The systematic search of the literature was performed from date of inception until April 12th 2020.

Selection criteria
Studies investigating the results of MSC-EV-enriched fat grafting were included. Abstracts were included when sufficient data was provided. No language restrictions were applied a priori. When necessary, authors of non-English language studies were contacted to provide an English language summary of their findings. Failure to establish contact or to receive results in a timely manner resulted in exclusion. Non-mesenchymal EV sources, in vitro studies, reviews, letters, commentaries, correspondences, case reports, conference abstracts with insufficient information, expert opinions and/or editorials were excluded.

Study selection and data extraction
Studies were selected as follows: after pooling of all search results, titles and abstracts were independently reviewed by MG and LDW. In case of doubt, studies were included for full
Full-text screening was conducted independently by MG and LDW to identify studies fulfilling the aforementioned inclusion criteria. In case of disagreement, the senior authors were consulted and a decision was taken in consensus. A final list of included articles was reviewed prior to data extraction.

Data extraction was performed independently by MG and LDW using a pre-approved form. Disagreements were discussed with senior authors until consensus was reached. The following outcomes were extracted: donor/receptor organism fat graft, study model (type, gender, number of models undergoing preclinical testing), intervention groups (including treatment scheme/dose), evaluation intervals, EV research data (including EV cell source, isolation method and EV characterization methods), in vitro results (including tube formation and migration/scratch assay), and in vivo results (including graft retention rate, graft integrity, neovascularization, adipogenesis, inflammatory profile and extracellular matrix properties).

Assessment of quality of studies

The Risk of bias of the included studies was independently evaluated by MG and LDW using the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) risk of bias assessment tool.

RESULTS

Overall, 2474 articles were retrieved from the aforementioned databases (Figure 1). After deduplication, 1717 articles were screened based on title and abstract. Sixty-one articles were included for full-text screening, of which 55 were excluded. Reasons for exclusion were: other indications (43 studies), conference abstracts (3), no available full-text (2) and other publications types (e.g. review, editorial) (7). Finally, seven studies were included. Four studies evaluated the effects of EVs isolated from hypoxia preconditioned MSCs (hypEVs) of which only three compared the effects of hypEVs to EVs isolated from normoxic preconditioned MSCs (nEVs). One study evaluated different EV doses to a control group (i.e. conventional fat grafting), whereas another one compared the effects of the EVs to their source MSCs. Three studies investigated possible mechanisms of EV-enhanced graft retention. Baseline characteristics are outlined in table 1. Detailed assessment of risk of bias of the individual studies is outlined in table 2.

EV research

EVs were isolated from bone marrow mesenchymal stem cells (BMSCs) and adipose derived stem cells (ADSCs). Hypoxia preconditioning was performed under 1% hypoxia.
5% oxygen. Overall, broad size ranges were observed across all studies, suggesting the isolated EV mixtures to comprise of both exosomes and microvesicles. Compared to nEVs, Han and colleagues reported hypEVs to be larger and more enriched in angiogenic proteins, suggesting hypoxia preconditioning to result in a higher EV cargo capacity. However, this difference in size was not observed by others. Details regarding EV research are outlined in Table 3.

**In vitro**

Overall, EV-enriched human umbilical vein endothelial cells (HUVECs) showed more favorable tube formation and migration capacities compared to non-treated HUVECs. Interestingly, hypEVs were superior to nEVs, further enhancing HUVECs’ migration and tube formation capacities. In addition, EV-enhanced migration and tube formation was dose-dependent; the high-dose hypEV-treated HUVECs increased migration and tube formation by respectively ~1.39- and 1.34-fold compared to the control.

Interestingly, ADSC-EV treatment of peritoneal macrophages increased the proportion of the CD206+ M2 macrophages – the anti-inflammatory ‘repair’ subtype – compared to non-treated macrophages (p = 0.02). Furthermore, the conditioned medium derived from the EV-treated macrophages showed increased levels of catecholamines (p = 0.04). In ADSC adipogenic differentiation assays a higher percentage of adipocytes could be observed in the EV-enriched ADSC group compared to non-enriched MSCs and control. ADSCs treated with the conditioned medium of EV-treated macrophages showed a higher expression of uncoupling protein 1 – marker of beige and brown adipose tissue – and higher oxygen consumption compared to control and EV-treated ADSCs, suggesting the macrophage secretome is responsible for adipose tissue beiging, rather than ADSC-derived EVs.

**In vivo**

**Fat graft retention**

A total of 294 mice underwent fat grafting. Four studies performed supplementary injections post-transplantation. Last evaluation date was at 4, 8, 10 and 12 weeks. Overall, EV-treated grafts had higher retention rates compared to the control (p < 0.05). At 12 weeks, the effects of EVs on fat graft retention were comparable to their source MSCs. However, the EV-group received repeated EV injections at 7 and 14 days post-transplantation, whereas the MSC- and control group received phosphate-buffered saline (PBS) injections. Furthermore, hypEV-treated grafts also had higher retention rates.
compared to the nEV-treated grafts (p < 0.05) \cite{17,21}. Notably, little difference could be observed between the high-dose hypEV-treated compared to the low-dose hypEV-treated group \cite{16}.

**Neovascularization**

CD31-staining showed an increased capillary density in EV-treated grafts compared to the control (p < 0.05) \cite{16-22}. Additionally, hypEV-treatment improved neovascularization significantly compared to the nEV-treatment (p < 0.05) \cite{17,18,21}. The effects of EVs and their source stem cells on neovascularization were at 3 weeks post-transplantation \cite{20}. Expression of proangiogenic factors was increased in the low-dose hypEV-treated grafts compared to the control \cite{16}. Furthermore, hypEV-treatment also elevated the expression levels compared to nEV-treated grafts \cite{18}. Overall proangiogenic expressions were similarly increased in both EV- and MSC-treated grafts. Notably, the expression of hepatocyte growth factor (HGF) was significantly higher in the EV-treated grafts compared with the MSC-treated grafts \cite{20}. Interestingly, Zhu and colleagues observed a distinct difference between graft and host revascularization \cite{21}. While EV-treatment appeared to improve vessel formation within the graft, they appeared to have no influence on the host derived revascularization \cite{21}. This difference in revascularization was ascribed to the use of human lipoaspirate and human MSC-EVs in a murine preclinical model; the co-transplanted human EVs were enriched with human miRNA, whereas the hosting organism was murine, subsequently unresponsive to the human miRNA molecules.

**Integrity**

Independent of dose, MSC- and EV-treated grafts showed less fibrosis and a higher structural integrity compared to the control \cite{16,18,20-22}. Similarly, perilipin-staining showed a higher tissue and adipocyte integrity in EV-treated grafts compared to the control \cite{17,19,20,22}, and hypEVs could further enhance these effects (p < 0.05) \cite{17}. One study observed a smaller mean size of adipose cells in EV-treated grafts compared to the control, after 12 weeks (p < 0.05) \cite{22}.

**Inflammation**

Histological analyses reported less infiltrations of inflammatory cells in EV-treated grafts compared to the control \cite{17,18}. Fat grafts treated with EVs showed increased numbers of M2 macrophages compared to the control in both the peripheral region (after 2 and 4 weeks, both p < 0.01) and the central region (after 2 and 4 weeks, p = 0.01 and p < 0.01, respectively). The proportion of M2 macrophages was also increased after 12 weeks in the EV-treated grafts compared to the control (p = 0.02) \cite{22}.
Expression levels of inflammatory cytokines and chemokines were significantly higher in EV- and MSC-treated grafts compared to non-treated grafts. Interestingly, the EV-treated groups showed higher levels of cytokines and chemokines compared to the MSC-treated groups. However, the repetitive injections of potentially impure EV-mixtures in the EV-group at 7 and 14 days post-transplantation could possibly have affected the week 3 histological evaluation of the inflammatory parameters, and subsequently caused the higher expression levels observed in the EV-group.

**Extracellular matrix**

Higher expression of collagen VI α3 and collagen III α1 could be observed in both EV- and MSC-treated grafts compared to the control at week 10 (all p < 0.05), indicating a more intact and increased adipose extracellular matrix (ECM) structure. The previous suggest that a more complete extracellular matrix structure may contribute to a higher fat graft retention rate.

**Browning of adipose tissue**

As indicated above, EV-enrichment promoted the polarization of M2 macrophages. These macrophages – stimulated by EVs – appear to increase the secretion of catecholamines, in turn inducing browning of AT and increase neovascularization in the peripheral region of EV-treated grafts compared to the control.

**DISCUSSION**

To the best of our knowledge this is the first systematic review describing the effects of EV-enriched fat grafting. Overall, EV treated groups showed higher graft retention rates compared to untreated group's. Notably, retention rate was similar following EV- and MSC-treatment. In addition to reduced inflammation, graft enrichment with MSC-EVs resulted in early revascularization and subsequently better graft integrity. While angiogenesis pathways and their cellular and molecular motors remain elusive, the heterogenous cell populations are believed to contribute differently. In this regard, cells from the endothelial lineage exclusively proliferate and differentiate into endothelial cells, providing building blocks for angiogenesis. The majority of the MSCs from the hematopoietic and mesenchymal lineage exert their function through paracrine signalling. They do so by creating a suitable micro-environment in which the endothelial cells could thrive. Only a small percentage of the MSCs differentiate into pericytes to stabilize the newly formed vessels. Notably, stress-stimuli – including ischaemia, physiological and pathological hypoxia, as well as hypoxic preconditioning – push MSCs towards an
upregulated and adapted secretome. This shift towards a more prosurvival secretome is partially visible in hypEVs and their cargo, as the latter is shown to be adapted to the hypoxic micro-environment present in fat grafts. In fact, in vitro HUVEC migration and tube formation was increased in hypEV-enriched groups compared to nEV groups, suggesting vessel formation to be facilitated by EVs rather than by soluble factors. Presumably, graft enrichment with hypEVs will result in a supraphysiologic upregulation of the proangiogenic pathways as soon as hypEVs and fat graft are mixed, hence facilitating early revascularization within graft.

Several limitations were encountered. First, only seven articles were included. While the evidence is still limited at this point, the timing of this work allows introduction of EV research in general and specifically EV-enriched fat grafting to a broader audience. Furthermore, synthetizing of the current results allows to offer future direction (see infra). Second, not all predetermined data could be extracted, limiting the robustness of the current study and preventing heterogeneity assessment. Subsequently, no meta-analyses could be performed. Finally, the terminology to describe certain EV subpopulations in the included reports was not followed in the current work. Instead, a general term, i.e. EV, was used to report the observed effects. Although most mixtures appeared to mainly contain smaller EVs – often referred to as exosomes – they appeared to contain various EV populations, soluble proteins and cell debris among others. Therefore, one should refrain from coining the term ‘exosome’ until future more fine-tuned – i.e. pure – isolation protocols have been proposed.

Although the field of EV-enriched fat grafting is still in its infancy and has yet to fully develop, the following considerations must be made at this point: 1) Only one study evaluated the effects of BMSC-EV, whereas the other six used ADSC-EVs. Indeed, ADSCs are easier to access, are more abundant – up to 500 times higher in concentration compared to BMSCs, have stronger immunosuppressive properties, secrete several proangiogenic and antiapoptotic cytokines, and display a higher phenotypic variety. However, it is worth investigating how EVs from different MSC sources affect graft retention. This is only logical, as the different MSCs reside in different micro-environments, present different phenotypes and react differently to stress; 2) While enzymatic digestion is considered as the gold standard for stem cell isolation, it is worth investigating whether mechanically isolated stem cells secrete a similar EV population. In this regard, mechanical isolation was observed with a higher EV concentration compared to enzymatic digestion. This is logical, as enzymatic digestion will negatively affect cell secretory functions through
ECM digestion, while simultaneously damaging cell function and viability. In contrast, mechanical isolation is rather gentle, yields more viable cells at a higher concentration, while requiring less than 10% of the starting lipoaspirate volume required for enzymatic digestion. More importantly, mechanical isolation results in a more stress-resistant progenitor cell population within SVF. While it is unclear whether mechanical stress might induce similar changes to the MSC-EV population as hypoxic preconditioning does, it is certainly plausible that EVs derived from mechanically isolated MSCs could result in similar graft retention rates as compared to EVs derived from enzymatically isolated MSCs. Any considerable similarity between both populations will undoubtedly lead to the rise of many novel clinical indications; 3) To date, AT EV isolation remains challenging, mainly due to its viscousity. Hence, fine-tuned isolation procedures are required to allow for the isolation and characterization of specific subpopulations, without contamination of these mixtures with soluble proteins and other contaminants. In turn, this will allow for correct and indisputable evaluation of in vivo effects of specific subpopulations; 4) EVs are carriers of a variety of miRNA and protein molecules among others, and their cargo is continuously fine-tuned depending on the direct micro-environment as well as systemic conditions.

Therefore, extensive EV characterization in accordance to the Minimal information for studies of extracellular vesicles 2018 (MISEV 2018) (e.g. amount, EV markers, size, morphology, cargo, ...) and detailed in vitro testing – to identify key signals through sequencing and reverse proofing among others – are of equal importance to detailed in vivo testing. Future reports solely reporting in vivo results without including extensive in vitro results should therefore be obviated; 5) Application of EVs from hypoxic preconditioned MSCs appears to be an interesting approach in improving ischemic conditions. While the use of hypoxic EVs should be further incentivized, this should be performed under true hypoxic conditions, as characterized by an oxygen concentration between 0.5 – 2%. Regardless, the impact of true hypoxic preconditioning, as well as suboptimal oxygenation of MSCs, their EVs and their subsequent role in fat grafting should be investigated; 6) Similar to MSC-enriched fat grafting, the ideal EV-graft ratio should be pursued. While it is true that Huang and colleagues did not find any difference between their low- (50µg EV/0.2 cc PBS) and high-dose (100µg EV/0.2 cc PBS) group regarding graft retention at 12 weeks, enrichment with varying doses should be further investigated to identify the optimal dose per cc of lipoaspirate, as well as whether there is a dose limit – after which adverse effects are to be expected; 7) As previously mentioned, EV treatment resulted in similar outcomes compared to source MSC. However, the EV group received additional EV injections at day
7 and 14, compared to PBS-injection in the MSC group. While the results are interesting, further research is necessary to identify whether EV-enrichment without repeated injections offers similar results compared to source MSCs. Furthermore, it should be emphasized that repeated injections of any enrichment solution in fat grafting are inefficient and even unrealistic in a clinical setting. Should repeat injections offer indisputable value, then delayed release approaches should be integrated. 8) While it is generally agreed that adipogenesis stabilizes around the 3 month mark, graft stabilization will take up to a year post grafting. It should therefore be encouraged to assess graft retention after a longer period of time (e.g. 4 or 6 months). In the very least, further volumetric assessment (e.g. Magnetic Resonance Imaging, Computer Tomography) should be encouraged; 9) While tempting, it is evident that EVs are not yet primed for full-scale clinical implementation. While diagnostic testing and occasional clinical applications are increasing, our knowledge of EV-specific biomarkers – and potentially their cargo and destination – is still limited. When EV-specific biomarkers are available, cumbersome ultracentrifugation-based techniques can be exchanged by more easily implemented technologies (e.g. ELISA, RNA-based A-PCR). Subsequently, one could potentially develop repeatable EV separation methods with high specificity and purity for extensive preclinical and clinical testing. When considering possible cell competition mechanisms – where ‘ischemic’ graft cells have to compete with ‘healthier’ or even ‘empowered’ CAL cells for survival – as well as increasing cell-therapy limitations, it appears that alternative cell-free approaches are gaining traction. In this regard, ‘cell-free fat extract’ (CEFFE) – the liquid fraction obtained after centrifugation of the mechanically isolated adipose stromal vascular fraction – has shown great potential for a variety of indications such as fat grafting, flap survival and skin regeneration. Combined with our improved understanding of the role of MSCs in angiogenesis and the benefits of EV-enrichment, it is clear that not the cells, but their secretome – i.e. soluble proteins and EVs – is essential for graft survival. While the clinical relevance of EVs has yet to be determined, they have formed the foundation of an emerging research niche regarding the crosstalk of fat cells in fat grafting and revascularization. Despite the many unanswered questions, a paradigm shift from cell-assisted lipotransfer towards a ‘secretome-assisted lipotransfer’ is well on its way. Future studies addressing aforementioned limitations should be encouraged and will undoubtedly improve our understanding of fat grafting.
CONCLUSION
Although the field of EV-enriched fat grafting is still in its infancy, it is apparent that EV-enrichment offers a novel and exciting cell-free approach to improve fat graft survival. In fact, the next step towards a ‘secretome-assisted lipotransfer’ is well on its way. Future in vitro and in vivo studies remain crucial and should be encouraged.

Supplemental Material
This article contains supplemental material located online at www.aestheticsurgeryjournal.com.
REFERENCES


41. Sesé B, Sanmartín JM, Ortega B, Matas-Palau A, Llull R. Nanofat Cell Aggregates: A Nearly Constitutive Stromal Cell Inoculum for Regenerative Site-


Figure Legend

Figure 1. PRISMA flow chart of search strategy results.
### Table 1. Basic Characteristics of the Included Studies

<table>
<thead>
<tr>
<th>Study</th>
<th>Donor organism fat graft</th>
<th>Receptor organism fat graft</th>
<th>Study model (number)</th>
<th>Intervention groups including application scheme/dose</th>
<th>Measurement intervals(s)</th>
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<tr>
<td>Chen B et al. (2019) 20</td>
<td>mouse (inguinal)</td>
<td>mouse (back)</td>
<td>8-week-old female C57/BL6 mice (48)</td>
<td>3 groups: control: 0.200 cc fat + 0.02 cc PBS; MSC: 0.200 cc fat + 0.02 cc PBS; EV: 0.200 cc fat + 40 µg EV + 0.02 cc PBS. EV-groups received supplementary EV injection at day 7 and day 14 (40 µg EV + 0.02 cc PBS), whereas the control and MSC group received 0.02 cc PBS injections.</td>
<td>3 and 10 weeks</td>
</tr>
<tr>
<td>Han Y et al. (2018) 17</td>
<td>human (abdomen)</td>
<td>mouse (back)</td>
<td>6-week-old female BALB/c nude mice (20)</td>
<td>4 groups: control 1: 1 cc fat + 0.1 cc PBS; control 2: 1 cc fat + 0.1 cc PBS; hypEV: 1 cc fat + 50 µg/0.1 cc hypEV; nEV: 1 cc fat + 50 µg/0.1 cc nEV. EV-groups received supplementary EV injections at 1-week intervals</td>
<td>2, 4, 6 and 8 weeks</td>
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<tr>
<td>Han Y et al. (2019) 18</td>
<td>human (abdomen)</td>
<td>mouse (back)</td>
<td>6-week-old female BALB/c nude mice (15)</td>
<td>3 groups: control: 1 cc fat + 0.1 cc PBS; hypEV: 1 cc fat + 50 µg/0.1 cc hypEV; nEV: 1 cc fat + 50 µg/0.1 cc nEV. EV-groups received supplementary EV injections at 1-week intervals</td>
<td>2. 15 and 30 days</td>
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<td>Huang et al. (2017) 16</td>
<td>human (abdomen)</td>
<td>mouse (scalp)</td>
<td>6-week-old female BALB/c nude mice (27)</td>
<td>3 groups: control: 1 cc fat + 0.2 cc PBS; low dose hypEV: 1 cc fat + 50 µg EV/0.2 cc PBS; high dose hypEV: 1 cc fat + 100 µg EV/0.2 cc PBS</td>
<td>12 weeks</td>
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<td>Mou S et al. (2019) 19</td>
<td>human (abdomen)</td>
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<td>6-week-old female BALB/c nude mice (16)</td>
<td>2 groups: control: 0.35 cc fat + 0.02 cc PBS; EV: 0.35 cc fat + 7 µg of EV in 0.02 cc PBS</td>
<td>1 and 3 months</td>
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<tr>
<td>Zhu Y et al. (2020) 21</td>
<td>human (abdomen)</td>
<td>mouse (scalp)</td>
<td>6-week-old female BALB/c nude mice (108)</td>
<td>3 groups: control: 0.300 cc fat + 0.100 cc PBS; nEV: 0.300 cc fat + 0.100 cc nEV (50 µg/cc); hypEV: 0.300 cc fat + 0.100 cc hypEV (50 µg/cc)</td>
<td>3 days, 4 and 12 weeks</td>
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<tr>
<td>Zhu Y et al. (2020) 22</td>
<td>mouse (inguinal)</td>
<td>mouse (scalp)</td>
<td>8-week-old female C57/BL6 mice (60)</td>
<td>2 groups: control: 150 mg fat + 0.200 cc PBS once per week for 12 weeks; EV: 150 mg fat + 0.200 cc EV (100 µg/cc) once per week for 12 weeks</td>
<td>2, 4 and 12 weeks</td>
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</table>

EV, extracellular vesicles; hypEV, extracellular vesicles derived from hypoxic preconditioned mesenchymal stem cells; MSC, mesenchymal stem cells; nEV, extracellular vesicles derived from normoxic conditioned mesenchymal stem cells; PBS, phosphate-buffered solution.
<table>
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<tr>
<th>Study</th>
<th>Sequence generation</th>
<th>Baseline characteristics</th>
<th>Allocation concealment</th>
<th>Random housing</th>
<th>Blinding</th>
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<th>Attribution bias</th>
<th>Reporting bias</th>
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## Table 3. Extracellular Vesicle Isolation and Characterization

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<tr>
<th>Study</th>
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<th>EV isolation method</th>
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<td>Chen Bet al (2019)²</td>
<td>mouse AT; normoxic ADSCs</td>
<td>Low passage (&lt;5 passages) ADSCs were grown to 70 to 80 percent confluence, the culture medium was replaced with basal medium (DMEM supplemented with 2 mM l-glutamine and 100 U penicillin/100 U streptomycin, but no fetal bovine serum), and the cells were cultured for another 48 hours. The conditioned medium was centrifuged to remove cell debris and passed through a 0.22-μm filter. The clarified conditioned medium was then size-fractionated and concentrated 50× by tangential flow filtration using a membrane with a molecular weight cutoff of 100 kDa (Sartorius, Göttingen, Niedersachsen), and then passed through a 100-nm filter.</td>
<td>TEM</td>
<td>123 nm</td>
<td>WB; CD9</td>
</tr>
<tr>
<td>Han Y et al (2018)⁷</td>
<td>human AT; normoxic (20% oxygen) and hypoxic (5% oxygen) cultured ADSCs</td>
<td>ADSCs were cultured under hypoxia (5% oxygen) and normoxia (20% oxygen) in a-MEM containing 10% Exo-free serum for 48 h to collect conditioned medium. Cell debris was discarded by sequential centrifugation at 500 g and 3000 g for 5 min and 15 min, and then passed through a 0.22-μm filter (Millipore, Billerica, Massachusetts). The supernatant was then ultracentrifuged at 110,000 g at 4 °C for 1 h using 45 Ti rotor (Beckman Coulter, Indianapolis, Indiana). The resulting pellets were washed and resuspended in PBS and ultracentrifuged at 100,000 g at 4 °C for 1 h.</td>
<td>TEM</td>
<td>nEV: mean 98 ± 70 nm (mode: 24 nm); hypEV: mean 87 ± 62 nm (mode: 39 nm) (range 20-300)</td>
<td>Microbe ad flow cytometry and WB; CD9, CD63, TSG101</td>
</tr>
<tr>
<td>Han Y et al (2019)⁸</td>
<td>human AT; normoxic (20% oxygen) and hypoxic (5% oxygen) cultured ADSCs</td>
<td>Culture medium was collected and centrifuged at 1500 g for 5 min and then for an additional 5 min at 3000 g to remove cell debris. Then the supernatant was filtered through a 0.22-μm pore membrane filter (Millipore, Billerica, Massachusetts) to remove large membrane vesicles, followed by ultra-centrifugation at 100,000 g at 4 °C for 90 min (Beckman Coulter, Indianapolis, Indiana). The pelat was resuspended in 1 mL sterile/filtered PBS, followed by centrifugation at 100,000 g for 60 min and resuspended in 100 μL PBS.</td>
<td>TEM</td>
<td>nEV: mean 75 ± 61 nm (mode: 22, 58, 97 nm); hypEV: mean 130 ± 65 nm (mode: 76, 94 nm)</td>
<td>FACS and WB; CD9, CD63, TSG101, CD-34, CD-90</td>
</tr>
<tr>
<td>Huang et al (2017)⁶</td>
<td>rat BM; hypoxic (5% oxygen) cultured BMSCs</td>
<td>Passage 4 BMSCs were used. Once the cells reached 70% confluence, they were cultured in serum-free media under hypoxic conditions (94% N2, 5% CO2 and 1% O2) for 48 h. The culture supernatants were collected and centrifuged at 2,000 g for 20 min at 4 °C to remove any large particles of debris. After an additional centrifugation at 100,000 g for 1 h at 4 °C, the pellet was washed twice in PBS.</td>
<td>SEM</td>
<td>mean 228.4±28.8 nm (range 40-300 nm)</td>
<td>CD90, CD29, CD81, and CD63</td>
</tr>
<tr>
<td>Mou S et al (2017)⁶</td>
<td>human AT; normoxic cultured ADSCs</td>
<td>ADSCs from passages 4 to 6 were used. Once cells reached 70% to 80% confluence, they were washed three times with phosphate-buffered saline, and then serum-free DMEM was added. After 24 hours of incubation, the culture supernatants were collected and centrifuged at 700 g for 15 minutes to remove cells and then at 2000 g</td>
<td>TEM</td>
<td>no mean (range 100-1000 nm)</td>
<td>WB; CD9, CD81, β-actin</td>
</tr>
</tbody>
</table>
for 20 minutes to remove cell debris, followed by further centrifugation at 16,000 g at 4°C for 1 hour.

<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Human AT</th>
<th>Medium</th>
<th>Centrifugation</th>
<th>TEM</th>
<th>EV Size</th>
<th>WB</th>
<th>Additional Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zhu et al. (2020)</td>
<td>Normoxic (20% oxygen)</td>
<td>Serum-free DMEM/F12</td>
<td>500, 3000, and 10,000 g for 5, 15, and 60 minutes</td>
<td>TEM</td>
<td>90% of the EVs were between 91.28 and 396.1 nm in size, with a peak size of 220.2 nm</td>
<td>WB</td>
<td>ADSC, adipose-derived stem cells; α-MEM, alpha medium essential medium; AT, adipose tissue; BM, bone marrow; BMSC, bone marrow stem cells; CD, cluster of differentiation; DMEM, dulbecco’s modified eagle’s medium; Exo, exosome; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered solution; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TSG, tumor susceptibility gene; WB, western blot.</td>
</tr>
<tr>
<td>Zhu et al. (2020)</td>
<td>Hypoxic (5% oxygen)</td>
<td>Serum-free DMEM/F12</td>
<td>500, 3000, and 10,000 g for 5, 15, and 60 minutes</td>
<td>TEM</td>
<td>HypEV: mean 180.7 ± 14.9 nm; LIN28B transfected EV: mean 174.5 ± 17.8 nm</td>
<td>WB</td>
<td>ADSC, adipose-derived stem cells; α-MEM, alpha medium essential medium; AT, adipose tissue; BM, bone marrow; BMSC, bone marrow stem cells; CD, cluster of differentiation; DMEM, dulbecco’s modified eagle’s medium; Exo, exosome; FACS, fluorescence-activated cell sorting; PBS, phosphate-buffered solution; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TSG, tumor susceptibility gene; WB, western blot.</td>
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