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REVIEW ARTICLE

Perde-Schrepler et al: Exosomes in the therapy of hearing loss

Mesenchymal stem cell- derived exosomes as cell-free therapeutics for sensorineural hearing loss

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ABSTRACT

Sensorineural hearing loss (SNHL) can result from various factors, including ototoxic drugs (such as aminoglycosides and chemotherapeutic agents), prolonged exposure to intense sound, and autoimmune or genetic disorders. In adult mammals, the loss of sensory cells in the cochlea is irreversible due to their lack of regenerative capacity. Current treatment options include hearing aids for mild to moderate hearing loss, which rely on residual hearing, and cochlear implants for severe cases, which provide limited auditory recovery while leading to the loss of any remaining natural hearing. Stem cell therapies, particularly those involving mesenchymal stem cells (MSCs), are being increasingly explored in regenerative medicine. MSCs are multipotent cells capable of differentiating into mesodermal lineage cells and possess immunomodulatory and regenerative properties, making them potential candidates for SNHL treatment. However, their administration carries risks, including unwanted differentiation, immune system activation, and potential tumorigenic effects. Exosomes, extracellular vesicles in the nanometer size range, are secreted by most eukaryotic cells. These vesicles, which have a double lipid membrane and contain genomic and proteomic material, play a crucial role in intercellular communication. Exosomes derived from MSCs exhibit similar biological functions to their parent cells but with significantly lower risks, as they do not trigger immune responses or pose oncological concerns. This paper aims to review current knowledge on the use of MSCs and MSC-derived exosomes for inner ear sensory cell regeneration and explore their potential for clinical applications.

Keywords: Sensorineural hearing loss; SNHL; exosomes: inner ear; mesenchymal stem cells; MSCs.

INTRODUCTION

Sensorineural hearing loss (SNHL) is the most common type of hearing impairment. [1] The WHO estimates that about 6% of the world's population suffers of some degree of hearing loss. [2] It affects the persons' communication and speech, as well as cognition, thus having an important impact on social life, education, employment and economy. Responsible for hearing is the organ of Corti, located in the scala media- an endolymph- filled cavity inside the cochlea. It contains 15000 inner and outer hair cells arranged specifically: a single row of inner hair cells and three rows of outer hair cells (HC) separated by the supporting cells SC). [3] (Figure 1). The hair cells stereocilia and kinocilia, in contact with the tectorial membrane, transmit the vibrations generated by the sound (transformed into action potential) along the cochlear nerve and auditory pathways to the brain. [4] In the course of embryonic development, between embryonic days E13-E15, the sensorial cells of mammalian cochlea lose their regenerative capacity, their destruction being irreversible after this timepoint, [5-8] As a result, hearing loss in adult mammals is permanent.

To date, there are no perfectly efficient treatment methods. [9] The golden standard for the treatment of SNHL is the cochlear implant (CI), electrodes surgically implanted in the patients' cochlea bypassing the damaged hair cells and stimulating the auditory neurons directly. Although CI significantly improves speech perception as well as quality of life, [10], it has several drawbacks, such as trouble hearing in noisy conditions, difficulties in music listening as well as the possibility of additionally damaging the already affected inner ear structures in the course of the surgical procedure. [11,13]

Glucocorticoids are often used for the treatment of several conditions affecting the inner ear, based on their anti-inflammatory effect but with limited efficiency, while long-term corticosteroid use was frequently associated with serious side- effects. [14-16]

Growth factors such as epidermal growth factor (EGF), bone derived neurotrophic factor (BDNF), or insulin growth factor 1 (IGF1) were used with moderately positive outcomes. [23-25]

Gene therapy: the transfection of *Atoh1*- a transcription factor essential for the formation of neural cells and inner ear HC or *OTOF* (otoferlin) gene to cochlear HC of patients with hereditary mutations in the *OTOF* gene causing SNHL showed some promising results, but there are few successful clinical trials due to important adverse effects, the lack of an ideal formulation and delivery mode to the target cells. [17-22]

Mesenchymal stem cells (MSC) are multipotent cells isolated from a multitude of organs and tissues. They can differentiate into several cell types of mesodermal lineage and have important roles in immunomodulation, seeming appropriate for the treatment of the damaged cochlear sensory epithelium by replacing the lost HC or neurons. They are an excellent source of exosomes (MSC-Exo), cell-derived membrane-surrounded vesicles carrying bioactive molecules (peptides, proteins, or RNA) and delivering them to recipient cells thus having biological functions similar to the parental cells but possessing lower risks. [26]

The discovery of efficient treatment in SNHL resulting in complete restoration of the structure and function of the inner ear should be based on the understanding of the molecular mechanisms involved in the process of losing the regenerative capacity. Important efforts in the recent years tried to identify new modalities to avoid neurosensory deafness, either by preventing the damage to the inner ear or by stimulating the regeneration of neurosensory cells. This review aims to summarize and critically analyze the existing literature regarding cell therapy employing mesenchymal stem cells as well as stem cell-derived exosomes as efficient alternatives for the treatment of SNHL. We propose to identify the positive findings and critically discuss the limitations requiring further research in order to advance to clinical use.

MESENCHYMAL STEM CELLS (MSC) IN SNHL

Stem cells: embryonic stem cells (ESC), induced pluripotent stem cells (iPSC), mesenchymal stem cells, were intensely studied in medical research for the regeneration of damaged tissues/ organs. Stem cells have the ability of self-renewal and differentiation into several somatic cell types. They can be maintained undifferentiated *in vitro* for long periods. ESC and iPSC can differentiate into almost all cell types in the organism, but the use of ESC encounters ethical issues. iPSC, generated through genetic reprogramming of adult cells solve the problem of ethical concerns, but both ESC and iPSC have high genetic and epigenetic instability, tumorigenicity and immunogenicity [27]

Mesenchymal stem cells are multipotent stem cells. They were isolated from almost all organs and tissues. MSC have important differentiating capacity, being able to differentiate into cells belonging to the mesodermal lineage: osteoblasts, chondrocytes, adipocytes, endothelial-cells etc., but also non-mesodermal cells, such as neurone-like cells. [28]

MSC have immunomodulatory properties and regenerative properties and are easy to cultivate and manipulate. They proved to be suitable for the treatment of the damaged cochlear sensory

epithelium by replacing the lost HC or neurons in several studies. The ideal situation would be that the transplanted MSC engraft in the inner ear giving rise to the correct and functional cells. Several studies attempted to regenerate the inner ear cells transplanting MSC, using different study designs and they obtained promising results. (Table 1)

In vitro studies. Mouse bone marrow derived MSC (BM-MSC) were differentiated towards HC progenitors with the administration of growth factors: neurotrophin 3 (NT3) and fibroblast growth factor (FGF) for 4-5 days followed by NT3 and brain derived growth factor (BDGF) for 7 days. The treated cells expressed progenitor HC markers: *Oct4, nestin, Otx2,* and *Musashi,* proneural transcription factors *GATA3, NeuroD, Ngn1, Math1, Brn3c,* and *Zic2 but* no mature hair cell genes: *myosin VIIa* and *espin*. Transfection of *Atoh1* led to further differentiation into mature HC (*myosin VIIa* and *espin positive*) and SC (expressing *S100A, $p75^{Trk}$, claudin 14, connexin 26,* and *Notch1).* [29] Embryonic stem cells (ESC) cultured in serum-free medium with N2 supplement differentiated into inner ear HC progenitors expressing *Math1, Brn3.1* and *Jagged-1*, *myosin VIIA, espin, parvalbumin 3* and *α9 acetylcholine receptor,* as well as *$p27^{Kip1}$*. [30]

In vivo studies. Mouse BM-MSC showed great biocompatibility after intratympanic injection to immunocompetent adult mice: no oxidative stress, inflammation or increase of apoptosis occured. [31]

BM-MSC isolated from rats and injected into the lateral semicircular canal of mice with hearing loss induced by 3 nitropropionic acid- a mitochondrial toxin, migrated to and could be visualized at the site of the injury. The recorded auditory brainstem response (ABR) thresholds at 40 kHz improved by 23%. [32] Human umbilical cord MSC transplanted through the subarachnoid cavity of congenitally deaf albino pigs reached the inner ear structures (stria vascularis, the basal membrane and the spiral ganglions) changing ABR waveforms but could be also spotted in the brain, heart, liver, kidney, lung. [33]

Bone marrow stromal cells introduced into the posterior semicircular canal of mice with induced spiral ligament degeneration stimulated the regeneration or maintenance of spiral ligament fibrocytes. It also improved the endocochlear potential with a moderate recovery of ABR threshold shifts via paracrine effects. [34] Following the transplantation of a neural stem cell line (cNSC) into the scala tympani of sound damaged mice and guinea pigs, the stem cells were detected in the cochlea showing markers specific for both neural tissues and inner ear tissues (hair cells, supporting cells). This evolution could be an effect of the cochlear microenvironment up-regulating site- specific proteins initiating the differentiation of these stem cells to neural, glial, HC or SC types. [35] C57BL/6 mice exposed to sound trauma and

treated with human umbilical cord MSC (UC-MSC) showed a significant rescue effect: down-regulation of heat shock protein (HSP) family members and cell death effectors and up-regulation of antiapoptotic genes (bcl-2), genes involved in immune response, cell repair and developmental processes, etc. Histological analysis of the organ of Corti revealed the preservation of the HC in the middle turn of the cochlea in the transplanted animals. [36] Neural-induced human MSC (NI-hMSC) from bone marrow expressing high levels of neural markers (NeuN) were transplanted into the scala tympani of mice with noise induced hearing loss causing a significant increase of spiral ganglion neurons. NI-hMSC were observed in the perilymphatic space, the organ of Corti, along the cochlear nerve fibers and in the spiral ganglion. [37] Adult rats with noise-induced hearing loss received human embryonic stem cell-derived MSCs (ES-MSC) intravenously. They had lower ABR thresholds at 4, 8, and 16 kHz, better preserved spiral ganglion and outer hair cells and lower levels of HSP70 and apoptosis markers. A small number of transplanted ES-MSCs were spotted in the spiral ganglion areas. [38]

Cochlear implantation along with stem cells improved the functionality of the first. [39] CI and BDNF-overexpressing MSC introduced in the same time into guinea pig cochlea reduced spiral ganglions degeneration more efficiently compared to BDNF before the implant. [40] *Clinical studies*. The majority of clinical trials using MSC for hearing loss are phases I, I/II, or II. [41] The administration of a single dose of BM-MSC intravenously to two adult patients with SNHL caused no related toxicities but also no improvement in hearing thresholds. [42] 11 children with acquired hearing loss received a unique dose of UC- MSC intravenously. A reduction of ABR thresholds for 62.5% of patients, improved language development and myelination of white matter on MRI were obtained. [43]

A clinical trial used biohybrid cochlear electrodes coated with autologous bone- marrow derived mononuclear cells in one ear and a standard non-coated implant in the contralateral ear. The results were contradictory: one patient experienced similar speech perception in both ears, one patient had better speech perception with the biohybrid implant while the third experienced reduced speech perception with the biohybrid implant. [44] Although inconclusive, these results represent the first attempts of using stem cells associated with cochlear implants.

Although the above presented studies obtained mostly favorable results such as cochlear cells protection and lowered ABR thresholds, there are serious limitations in comparing their results due to important differences in the study designs- different recipient species, sources of stem cells, delivery site and dosage, differences in the timing of treatments and also in the assessed

endpoints.[45] In order to obtain reliable results, more studies are needed, using standardized methodologies.

Mesenchymal Stem Cells (MSC) therapy seems very attractive for the treatment of many diseases not benefiting of efficient treatment. Although approximately 1515 trials (509 completed) using MSC in different diseases were registered on "www.clinicaltrials.gov (accessed on 21.11.2024)", the results obtained could not justify the introduction of MSC treatments in clinical practice for now. There is still no concluded or ongoing trial of MSC in SNHL yet. [46]

The use of stem cells in the treatment of several diseases remains controversial as it raises several concerns regarding the potential risks: immune rejection, limited cell survival in the new environment, and the risk of malignant transformation. [47,48] The production of a sufficient amount of MSCs for clinical use requires a consistent in vitro expansion, which can lead to spontaneous transformation of the cells and genetic alterations of the cells. [49]

MESENCHYMAL STEM CELLS DERIVED EXOSOMES (MSC-EXO).

MSC were assumed to favor tissue regeneration by migrating to the lesion site, engrafting and differentiating in mature functional cells, but several studies claimed that MSC engraftment is not sufficient to explain the amplitude of the regenerating effect. [50] MSC have alternative ways to stimulate tissue repair by increasing cellular viability, proliferation, differentiation, by extracellular matrix remodeling and by inhibiting apoptosis, fibrosis, inflammation through paracrine signaling via secreted factors: cytokines, chemokines, hormones, extracellular vesicles, etc, forming the so- called "secretome" of MSC. [51] (Figure 2). Even if the transplanted cells could not reach the inner ear, an improvement in hearing and the protection of HC were obtained in a study using hASC (Human Adipose tissue Derived Stem Cells) injected intraperitoneally to BALB/c mice with experimental autoimmune hearing loss. This effect can be attributed to the paracrine effect of hASC: [52]

The composition of the "secretome" is specific for the tissue of origin being a mirror of its physio- pathological state (the "secretome" of adipose derived stem cells is richer compared to the one secreted by BM-MSC derived stem cells). [53,54]

By replacing cell transplantation with the "secretome", the side effects like unwanted differentiation, activation of allogeneic immune response, tumorigenicity etc. could be avoided. [55] Another great advantage of the "secretome" as a biological therapeutic product is that it can be modified to increase some of the desired biological effects, can be obtained in large quantities from commercially available cell lines, provides bioactive factors, etc. [56]

EV came into the researchers' attention in the late 80s. [57] They are cell-derived membrane-surrounded vesicles carrying bioactive molecules and delivering them to recipient cells. EV are classified based on their biogenesis mechanism and size in: exosomes (30-150 nm) - endosome-originated EV generated in three steps: biogenesis, transport, and release; microvesicles (100-1000 nm) - formed by the outward budding and shedding from the plasma membrane and apoptotic bodies (>1000 nm) - generated in the process of apoptosis. [58,59]

The differentiation between exosomes and microvesicles can be challenging due to their overlapping size ranges, thus separating them based solely on size is difficult. Although exosomes and microvesicles are generated through different cellular processes, there are no specific biomarkers that distinguish exosomes and microvesicles and they have similar proteins and RNAs in their composition making their differentiation based on biomarkers and molecular content difficult. [60] The International Society for Extracellular Vesicles recommends classifying EV into small EV (< 200 nm) and medium/large EV (> 200 nm). The terms "Exosomes" and "small extracellular vesicles" are used interchangeably in the literature, most authors preferring the first. In this review we refer to both without distinguishing them.

Structure, composition, functions of exosomes

Exosomes are produced naturally by almost all eucaryotic cells being transported in the biological fluids. [61,62] Cellular stress and activation signals can modulate their formation process. [63] Exosomes are generated from late endosomes, (Figure 3) by the inward budding of the limited multivesicular body (MVB) membrane and the formation of intraluminal vesicles (ILVs) incorporating certain proteins and cytosolic components. Most ILVs are released from the cell by fusion with the plasma membrane, becoming extracellular vesicles: "exosomes". [64] After their release into the intercellular space, exosomes interact with the target cells being uptaken by endocytosis (e.g. phagocytosis and pinocytosis), receptor-ligand interaction or fusion. [65-67] Their uptake through cell-type specific mechanisms requires the recognition of specific cell surface molecules. [67,68] These receptor–ligand interactions could be exploited for targeted exosome delivery by surface modification with specific ligands against target receptors. [68-72]

Exosomes have a genetic and proteomic cargo with important role in intercellular communication. 80% of the proteins found in EV are common for all exosomes: fusion and transport proteins Rab2, Rab7, flotillin and annexin, tetraspanins (CD9, CD63, CD81, CD82), heat shock proteins, cytoskeleton proteins including actin, myosin, tubulin, and proteins involved in the synthesis of multivesicular bodies (Alix, TSG101). [73,74] The detection of

these common proteins, characteristic for all exosomes, can be used to confirm their isolation. [75] Some of the exosomes content is specific for the tissue of origin: receptors, transcription factors, enzymes, extracellular matrix proteins, lipids, nucleic acids (DNA, mRNA, and miRNA), adhesion molecules (CAM), integrins, MHC class I, II presented on B lymphocytes and dendritic cells, transferrin receptors on the surface of reticulocytes. The composition of the bioactive cargo of the exosomes, specific for the cell of origin as well as for their current state allows the identification of new diagnostic/ prognostic biomarkers. [76]

The exosomes are unable to multiply, as they have no nucleus but biologically active RNA particles are abundant. [77] The most studied are microRNAs, however, other types of non-coding and coding RNAs have been identified in next generation sequencing studies: mitochondrial DNA, piwi interacting RNAs, long non-coding RNAs, ribosomal RNAs, small non-coding RNAs, transfer RNAs, circular RNAs. miRNA molecules can regulate gene expression. The presence of other types of ncRNA such as the circular RNAs, also having active regulatory roles in the recipient cells, demonstrate the gene regulating role of exosomes and their implication in normal development or cancer. [78]

Exosomes have a bilayered lipid membrane consisting of cholesterol, sphyngomielin, ceramides, etc., The lipid content of exosomes depends on the cells' origin and includes cholesterol, phospholipids, phosphatidylethanolamines, polyglycerols and diglycerides. Exosomes_have a higher organization of the lipid content and a higher stability against detergents compared to other EVs. [79,80] Exosome membranes have different lipid composition and distribution compared to the cytoplasmic membrane being involved in the preservation of exosomes shape and stability. They prevent lipolytic or proteolytic degradation in the circulation. [81,82] Membrane lipids serve as signaling mediators by interacting with prostaglandin and phospholipase C and D, their lipidic composition -sphingomyelin, phosphatidylcholine and bis(monoacylglycero)phosphate-BMP) helping in distinguishing the different types of EV: exosomes have higher sphingomyelin concentration while BMP is a component found exclusively in the endosomes. [83,84] Exosome lipid dynamics and protein domains (tetraspanin domains) have an important role in keeping the optimal conformation of immune proteins, such as MHC class II. [85]

ExoCarta is a database (http://www.exocarta.org) that involves all the published and unpublished data about exosome content, being a good resource for information regarding exosome characterization. It has collected 9769 proteins, 3408 mRNAs, 2838 miRNAs, and 1116 lipids that have been identified in exosomes from different types of cells and from multiple organisms. [86]

Exosomes transfer their content to recipient cells having an important contribution in the intercellular communication and tissue repair through paracrine signaling Being an endogen vector, exosomes have low immunogenicity escaping the rethyculoendothelial system (RES) and avoiding phagocytosis. They can cross natural barriers such as blood brain barrier (BBB) or blood labyrinth barrier (BLB) making them good candidates for the transport of certain drugs, genetic material (lncRNA, miRNA), or small molecules, delivering them to otherwise inaccessible tissues such as the brain or the inner ear. [87,88].

The mechanisms by which exosomes exert their actions in the target cells is still not entirely understood but some component molecules have been identified as being responsible for specific effects: miRNA- for example miRNA133b in the case of recovery after ischemic stroke or miRNA-22 for the antiapoptotic effects in cardiomyocites in cardiac ischaemia. [89,90]

Exosome isolation methods

In order to obtain exosomes suitable for clinical use in SNHL, the isolation method has to provide exosomes with the highest yield and purity. The differences in MSC sources, culture conditions and EV isolation methods lead to important differences in the yield and quality of the obtained MSC-EV preparations [129- Witwer].Several isolation methods have been described, each having its own advantages and disadvantages. [91-103] (Table 2)

To increase the efficiency of exosome isolation, different methods can be combined, like cell media modifications combined with ultrafiltration and size-exclusion chromatography. [104] Choosing between separation methods can be difficult and the decision should be based on the intended use of exosomes in the downstream applications. [105] A sufficient quantity is needed to be isolated to enable exosomes' processing in the tissues, so it is important to obtain high yield of exosomes with a high degree of purity. [106,107]

Ultracentrifugation (UC) is the most frequent method used for obtaining MSC-Exos in clinical trials as well as tangential flow filtration (TFF). [108]

A study published by Kim et al, 2021compared two isolation methods, UC and TFF, the later obtaining a better yield of exosomes isolated from human UCMSC. [109]

For the large-scale production of EV from MSCs ion exchange chromatography (IEX) and ultrafiltration (UF) were used in a study. They obtained EV populations with important anti-inflammatory activity in macrophages and T cells, more important for the EV obtained by IEX. [110]

Exosome engineering

The great potential of exosomes in various pathologies has been demonstrated, but there are several limitations when it comes to their clinical application. Naturally produced exosomes are not able to specifically target certain cells or tissues. These limitations could be avoided by the modification of the exosomes and the development of designed (engineered) exosomes. MSC- exosomes can be enriched in fractions of the vesicular "secretome" to obtain new therapeutic agents for different diseases, including those of the inner ear. Exosomes can be loaded with a range of molecules and serve as drug delivery vesicles. Engineered exosomes can be fabricated either pre- isolation, manipulating the parental cells or after exosome isolation using chemical or mechanical methods. [111]

Exosome production can be stimulated preconditioning parental cells by hypoxia, [112-114] heat-shock, [115] transfections, use of biomaterials, etc. [116] Adding exogenous drugs to donor cells can preload exosomes in situ. Preloading strategies, however, are often not an option for many types of cargoes, and so purified exosomes need to be loaded *in vitro*. Loading cargoes (proteins, drugs, bioactive molecules or mRNA) directly into exosomes requires bypassing the barrier represented by the exosome membrane. Loading can be achieved by two methods: passive loading (simple incubation of the exosomes with the therapeutic material) and active loading using physical methods: electroporation, sonication, freeze-thaw, ultracentrifugation, density gradient chemical methods such as membrane permeabilization with saponin, transfection [117,118] (Figure 4)

Exosomes loaded with biopharmaceuticals have improved *in vivo* stability and cell targeting efficiency.

There are several limitations of preconditioning and engineering methods: the chemical or physical pretreatment of MSC cannot limit the nonspecific aggregation of the produced exosomes during treatment [87]; pre-treatments with cytokines or chemicals can exert long-term effects on the physiological properties of MSC [81]; the different engineering methods could not deliver the desired exosomes consistently requiring additional modification steps increasing the difficulty of their industrial production; [118,-121] the size of the drug- too large molecules. [122] To overcome these limitations, intense collaboration of researchers, clinicians as well as authorities is required to make possible the obtaining of high quality, reproducible engineered exosomes for their safe application in translational medicine. [123,124]

MSC DERIVED EXOSOMES FOR TISSUE REGENERATION.

MSC represent an excellent source of exosomes producing a large quantity, compared to other cell lines. In pathologic conditions, the paracrine gradient produced at the periphery of the affected organ attract MSC to promote tissue healing. [125] MSC-Exo alike their cells of origin, have important immunomodulatory properties: inhibition of mitogen activated T cells, induction of anti-inflammatory phenotype in naïve dendritic cells and NK cells; inhibition of B cells, they contribute to the maintenance of tissue homeostasis, have an important role in intercellular communication and can restore the normal function of a tissue through active catalytic enzymes. [126-128]

Exosomes composition is specific to the tissue of origin. Baglio et al, 2015 compared the small RNA profile of exosomes released by adipose tissue- derived MSC (ASC) and bone marrow-derived MSC (BM-MSC) using RNAseq analysis. The two types of exosomes contained different tRNA species, which could be relevant for subsequent clinical applications. [129] Comparing the capability to induce de novo adipose cell regeneration of small EV and conditioned medium with equivalent protein concentration, EV performed better, the only advantage of conditioned medium being its availability. [130]

To date, there are 25 ongoing or completed clinical trials employing exosomes, the majority using MSC derived exosomes. [131] Their use as therapeutic agents remains challenging, especially when primary MSC are used as the cellular source of exosomes, due to their heterogeneity. Many factors contribute to this heterogeneity, such as the tissue of origin, the differences in donor profiles, the isolation methods and the culture system. The production process parameters can also affect the exosome products, therefore is a stringent necessity for quality control assays, in order to use them in clinical trials. [132,133] Exosomes released from stem cells potentially exert the same therapeutic and clinical benefits as the cells themselves, being able to recover damaged tissues by stimulating tissue regeneration in myocardial infarction, [134-136] or cisplatin affected renal cells. [137] UC- MSC exosomes had anti-inflammatory effect reducing tumor necrosis factor α and interleukin 1 expression as well as increasing neuronal growth factors. [138]

It is important to keep in mind that the administration of exosomes can also have adverse effects, depending on their origin, underlining the importance of safety testing. [139]

MSC DERIVED EXOSOMES IN SNHL THERAPY

There are few studies using exosomes to treat SNHL but the results are encouraging, the proliferative/ protective factors specific to the MSC exosomes protecting inner ear sensorial

cells from ototoxic injuries and stimulating cellular and tissue regeneration of the inner ear. (Table 3) Human UC-MSC- Exo improved survival and primary neurite growth in rats and reduced HC loss with a partial restoration of hearing, demonstrating neuroprotective and regenerative effects. Gene panel analyses revealed that UC-MSC- Exo modulated the expression levels of many genes promoting tissue remodeling and repair. [140,141] In an experimental study aiming to reduce cochlear implant related inflammation, a subject with CI in one ear received an identical one in the contralateral ear four years later together with intracochlear UC-MSC EV. After 24 months, speech intelligibility improved and the mean impedances in the EV-treated side were significantly higher. [142]

Exosomes enriched in HSP70, produced either by heat-shock preconditioning BM-MSC or by treating exosomes directly reduced cisplatin ototoxicity in cochlear explants, reducing pro-inflammatory cytokines IL-1, IL-6, and TNF-α and increasing anti-inflammatory cytokine IL-10 in mice [143,144]. Hypoxic- preconditioned BM-MSC secreted exosomes, overexpressing HIF-1, limited HC loss and inhibited oxidative stress caused by cisplatin in mice. [145]

Exosomes isolated from inner ear tissues also showed otoprotective effects: exosomes derived from inner ear stem cells prevented gentamicin-induced ototoxicity, [146] while exosomes derived from cochlear spiral ganglion progenitor cells inhibited inflammation and attenuated ischemia-reperfusion injury- induced cochlear damage. [147]

UC-MSC derived exosomes added to HEI-OC1 cell line and to cochlear explants after Neomycin reduced hair cell loss, modulated autophagy, up-regulated endocytic gene expression, promoted cell survival, decreased oxidative stress and apoptosis. In mice deafened by Neomycin, exosomes reduced hearing loss. [148] BM-MSC-EV increased neurite growth and growth cone development and prevented SGN degeneration after Ouabain. In vivo, they rescued ouabain induced hearing loss protecting SGN degeneration. [149]

By analyzing these results, it is obvious that MSC-Exo, no matter of the tissue of origin or recipient species protected the inner ear tissues against ototoxic agents and promoted regeneration. There are no recorded clinical trials using MSC-Exo to date, as no sufficient and reliable data exist in this area. The most important limitation of the existing studies comes from the heterogeneity of study designs: source of MSC, exosome isolation methods, characterization, used doses of exosomes (expressed as microgram proteins or number of particles), application time-schedule, site of application, incubation time, evaluated endpoints, etc.

CONCLUSION

Technology breakthroughs provide new and promising tools for the management of hearing loss. Inner ear hair cell regeneration, although challenging, has proven possible. Exosomes produced by MSC highlight new options in regenerative medicine. For their future use in SNHL treatment, high quality clinical trials are required. Exosomes have numerous advantages compared to MSC- cell therapy: not being able to replicate, they are not tumorigenic and there are no ethical concerns regarding their use. Exosomes lack immunogenicity and their small size allows the crossing of natural barriers making them good candidates for the transport of certain drugs, genetic material or small molecules. They are stable and can be stored long-term. Exosome engineering can provide exosomes with improved contents and surface markers for more precise delivery and better therapeutic responses. The difficulties in their use are represented by batch-to-batch variations due to the status of donor cells as well as the isolation methods, the need for large quantities and the lack of regulations. In order to step forward to clinical trials exosome production needs the optimization and standardization of each manufacturing step in an automatic operation system that would allow large-scale production and quality control. Another important issue is the assessment of safety: the potential off-target effects and the long- term safety. All these concerns could be resolved through the joined efforts of scientists, biotechnological companies and regulatory authorities.

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TABLES AND FIGURES WITH LEGENDS

Table 1. Studies using mesenchymal stem cells for hearing restoration.

Type of MSC	Study model	Delivery site and mode: dose, timing.	Outcome	Reference
***In vitro* models**				
Mouse ESC	Three ES cell lines: R1, YC5/EYFP and ROSA26.	Cell culture media: 10 days serum-free medium with N2 supplement, EGF (20 ng/ml^{-1}) IGF-1 (50 ng/ml^{-1}) and bFGF ($10ng/ml^{-1}$) for 8 days.	Differentiation of the embryonic stem cells into inner ear hair cell progenitors	Li, 2003 [27]
Mouse BM-MSC	Mouse BM-MSC cells	•Cell culture media: NT3 (30ng/ml), FGF (10 ng/ml) 4-5 days followed by NT3 (30ng/ml) and BDNF (10 ng/ml) one week •Atoh1 transfection using lipofectamine	Development of hair cell progenitor gene profiles but not hair cell genes Expression of mature hair cell markers	Jeon, 2007 [26]
Animal models				
Murine	Sound damaged mice	Scala tympani- $1.5x10^6$ cells in perfusion (2.5 µl/ min)	Significant increase of satellite cells and Type I spiral ganglion neurons in	Parker, 2007 [32]

neural stem cell line	and guinea pigs	48 h after noise exposure	the stem cell-injected animals. The neural stem cells differentiated into hair cells, supporting cells and spiral ganglion cells.	
Rat BM-MSC	Mouse with hearing loss induced by a mithocondrial toxin (3-nitropropionic acid- 3NP)	Lateral semicircular canal- $1x10^5$ cells, 3 days after 3NP	MSC observed at the site of injury ABR thresholds at 40kHz were improved by 23%	Kamiya, 2007 [29]
Human Neural-induced BM-MSC (NI-hMSC)	Mice with neomycin induced hearing loss	Scala tympani- $1x10^5$ cells, 7 days after Neomycin.	Significant increase of spiral ganglion neurons (SGN) compared to controls. Transplanted NI-hMSC expressing NeuN in the perilymphatic space, the organ of Corti, along the cochlear nerve fibers and in the spiral ganglion.	Jang, 2015 [34]
Human UC- MSC	Congenital deaf albino pigs	Subarachnoid cavity: $3x10^5$-$1x10^7$ cells	UC-MSC found in the stria vascularis, the basal membrane and the spiral	Ma, 2016 [30]

			ganglions, brain, heart, liver, kidney lung. Changes of ABR waveforms.	
BDNF over-expressing MSC +CI	Guinea pig deafened by kanamycin and furosemid	Intracochlear injection ($2.5x10^5$ cells) or administration as coating of the cochlear implant ($5x10^5$ cells)	The MSC survived for 4 weeks *in vivo*. The alginate-MSC coating of the CI significantly prevented SGN from degeneration; MSC alone had no effect	Scheper, 2019 [37]
Mouse BM-MSC	Immunocompetent adult mouse	Intratympanic; $1x10^5$ cells	No oxidative stress generation, no activation of inflammation and apoptosis	Eshraghi, 2020 [28]
Mouse BM-MSC from EGFP-transgenic mice	Mouse model of cochlear fibrocytes degeneration in the spiral ligament	Posterior semicircular canal ($6x10^5$ cells)	Regeneration or maintenance of spiral ligament (SL) fibrocytes. Improvement of endocochlear potential (EP) Moderate recovery of ABR threshold shifts	Kada, 2020 [31]

Human UC-MSC	C57BL/6 mice exposed to sound trauma	Posterior semicircular canal ($1x10^6$ cells)	Significant rescue effect in the MSC treated animals: down-regulation of heat shock protein and cell death effectors; up-regulation of bcl-2, genes of the immune responses, cell repair and development. Preservation of hair cells in the middle turn of the cochlea.	Warneck e, 2021 [33]
Human ESC-derived MSC (ES-MSC)	Sprague-Dawley rats with noise-induced hearing loss	Intravenous ($5x10^5$ cells)	The ES-MSC treated noise-exposed rats showed lower ABR thresholds at 4, 8 and 16 kHz and better preserved spiral ganglion cells and outer hair cells. Reduction of cell death markers AIF, PAR, PARP, caspase 3 and cleaved caspase 3 in the ES-MSC treated rats. ES-MSCs observed in the spiral ganglion area.	Kim SY, et al, 2022 [35]

			Weaker expression of Sry and STEM121 (evidencing human DNA) in the cochlea compared to the lung	
Human studies				
Biohybrid cochlear electrode: (coated with autologous BM-MSC)	Humans (3 patients)	Intracochlear Dose not reported	Contradictory results: one patient experienced similar speech perception in both ears, one patient had better speech perception with the biohybrid implant;the third patient showed reduced speech perception with the biohybrid implant.	Roemer et al., 2016 [41]
Human autologous bone BM-MSC	Humans (2 patients)	Intravenous ($5x10^7$ cells)	No toxicities related to the treatment but also no improvement in hearing	Lee et al., 2018 [39]
UC- MSC	Children (11 children 6 month to 6 years with	Intravenous (8 to 30 $x10^7$ cells/kg body weight)	Reduction of ABR thresholds for 62.5% of patients. Improved language development and	Baumgartner et al., 2018 [40]

	acquired SNHL)		myelination of white matter on MRI.	

Table 2. Exosome isolation methods

Method	Principle	Advantages	Disadvantages	References
Differential ultracentrifugation	Sequential centrifugation at high centrifugation force separation based on density and size	The gold standard of exosome isolation, suitable for large volume samples, relatively cheap high exosome yield and purity	Laborious and time consuming (more than 4h) requires training and an expensive equipment: an ultracentrifuge.	Thery et al., 2006 [89] Monguio-Tortajada et al., 2019 [90]
Size exclusion chromatography (SEC)	Based on size differences of particles. uses the biofluid as a mobile phase and a porous gel filtration polymer as the stationary phase	High purity, short processing time (0.3 h)	Relatively low yield, can be compensated by large starting volumes.	Stranska et al., 2018. [91]

Ultrafiltration	Based on the differences in size and molecular weight	Easy operation, does not request expensive equipments, high purity. <4h	Loss of exosomes on filter membranes, low yield	Cheruvanky et al., 2007. [88]
Anion exchange chromatography	Based on exosome negative surface charge binding to a positively charged chromatographic matrix	High purity and reproducibility	Need additional concentration of the obtained sample by ultrafiltration	Deregibus et al., 2016. [92]
Immunoaffinity capture	Additional step to increase exosome yield and purity based on the expression of surface proteins. Uses antibodies against specific exosome surface markers (CD9, CD63, and CD81). It can use magnetic beads, nanoparticles	Generates specific exosomes. It can isolate subsets of exosomes	Low yield, expensive, time consuming (4-20h)	Koliha et al., 2016,[93] Boriachek et al., 2019, [94] Sharma et al., 2018, [95]; Ghosh et al., 2014 [96]

	coated with antibodies against the surface proteins, markers from parent cells, or exosome-binding molecules such as heat shock protein.			
Precipitation with PEG based reagents	The low solubility of exosomes in the reagent leads to the formation of exosome aggregates which are then precipitated by low-speed centrifugation.	High yield, simple operation, suitable for large samples Operation time 0.3-12h	Low purity (potential contaminants) and specificity	Weng et al., 2016 [97] Konoshenko et al., 2018, [98] Li et al., 2017 [99]
Tangential flow filtration	The fluidics flow tangential to a filter membrane.	High yield	Moderate purity.	Busatto, et al, 2018 [100]

Table 3. Studies using exosomes isolated from MSC for SNHL

Exosome origin	Recipient species	Delivery site and mode: dose, timing	Outcome	Reference
Human UC-MSC	- BV-2 Microglial cell line activated with lipopolysaccharide (LPS)	-1.2x 10^8 exosomes/ml in the culture medium, 1 hour before LPS	-anti-inflammatory effect- significant reduction of IL-1β gene expression; phosphorylation level of NF-κβ p65 was significantly diminished.	Warnecke, A, et al, 2020 [137]
	-Primary rat SGN cell culture	- UC-MSC-EVs from 1 × 10^6, 2 × 10^6, and 4 × 10^6 cells in the culture medium	improved survival, increased primary neurite growth dose-dependently	
	-One-month old female C57BL/6 mice exposed to noise	-posterior semicircular canal 72h after noise trauma- 1 µl EV (2 × 10^{10} particles/ml)	5 days after delivery- protection of the inner ear cells, partial hearing restoration: reduced ABR thresholds; rescue of the organ of Corti	

Human UC-MSC (Wharton's jelly)	-Mice with intraperitoneal Cisplatin induced hearing loss	-100 μL of UCMSC exosomes (1.2 μg/μL) intraperitoneal injection and 10 μL UCMSC exosomes through the round window niche (RWN).	- significant reduction of ABR threshold of 8 and 12 kHz; rescue of the lost cochlear hair cells; reversed miRNA profile of the cochlear tissue	Tsai, SCS, et al, 2021 [138]
Human UC-MSC	Human subject with bilateral hearing loss (Meniere disease)	Intracochlear, simultaneously with cochlear implant- 1×10^8 particles/μl	-no toxicity -better speech intelligibility -significantly higher mean impedances in the EV-treated side	Warnecke, A, et al, 2021 [139]
Human BM-MSC	Cochlear explants from ICR mice treated with Cisplatin and co-cultured with MSC	- Exosomes isolated from the culture medium of the co-culture of MSC with cochlear explants- 2.48×10^{10} particles/	- enrichment of HSP70 in the secreted exosomes -reduced Cisplatin induced ototoxicity-decreased hair cell death.	Park DJ, et al, 2021 [140]

		mL diluted to 1-, 3- or 5-fold; 24h before Cisplatin		
Heat shock treated mouse BM-MSC	- C57BL/6 mice treated with intraperitoneal Cisplatin	-1.2 μg/μL, 1 μL trans-tympanic 30 minutes after Cisplatin	-exosomes reduced Cisplatin ototoxicity- diminished ABR thresholds; reduced hair cell loss, reduced inflammation, decreased apoptosis	Yang, T, et al, 2022 [141]
Mouse inner ear stem cells	*In vitro*: HEI-OC1 cells exposed to Gentamycin	-culture medium: 0, 0.01, 0.1, and 0.3mg/ml same time as Gentamicin	-improved cell viability -reduced oxidative stress - increased relative miR-182-5p expression and decreased FOXO3	Lai, R, et al, 2020 [143]
Mouse cochlear spiral ganglion progenitor cells	Female C57BL/6 mice ischemia-reperfusion injury (I/R) model of hearing loss	-intracochlear: 0.1 μg, 0.2 μg, 0.5 μg, and 1 μg/1 μl, 1 h before the ischemia-reperfusion injury and every 12 h after the injury	- significantly decreased the threshold shift at 8, 16, 32 kHZ - prevented hair cell damage - anti-inflammatory effect: IL-6, IL-1β, TNF-α and Cox-2, were significantly reduced	Yang, T, et al, 2021 [144]

			- inhibition of hair cells apoptosis	
UC-MSC (Promocell)	-Hei-OC1 cell line treated with Neomycin	-30 µg/ml for 24 h in the cell culture medium, 24h after Neomycin	Exosomes reduced hearing and hair cell loss caused by neomycin; modulated autophagy in hair cells, upregulated endocytic gene expression; promoted cell survival, decreased oxidative stress and apoptosis in hair cells	Liu H, et al, 2024 [145]
	-cochlear explants treated with Neomycin	-30 µg/ml for 24 h in the cell culture medium, 24h after Neomycin		
	-C57BL/6 mice deafened by Neomycin	-round window niche (RWN): 20 µg in 10 µl PBS) 2 days after Neomycin. ABR, immune staining after 2 weeks	-exosomes attenuated hearing loss (lower ABR thresholds) and reduced the loss of Myo 7a-positive hair cells in the middle and basal regions of the cochlear tissues	

Rat BM-MSC	-spiral ganglion culture treated with Ouabain -SD rats deafened by intratimpanic Ouabain	-2 µg/µL in cell culture media 48h after Ouabain -200 µg/ml, together with 20 mM ouabain. 7 days after treatment: ABR, immunostaining	-significant increase of neurite growth and growth cone development. -prevent SGN degeneration -EV rescued ouabain-induced hearing loss rescuing the threshold shifts induced by ouabain; -EV Protected SGN from degeneration -inhibit ouabain-induced apoptosis.	Chen A, et al, 2024 [146]

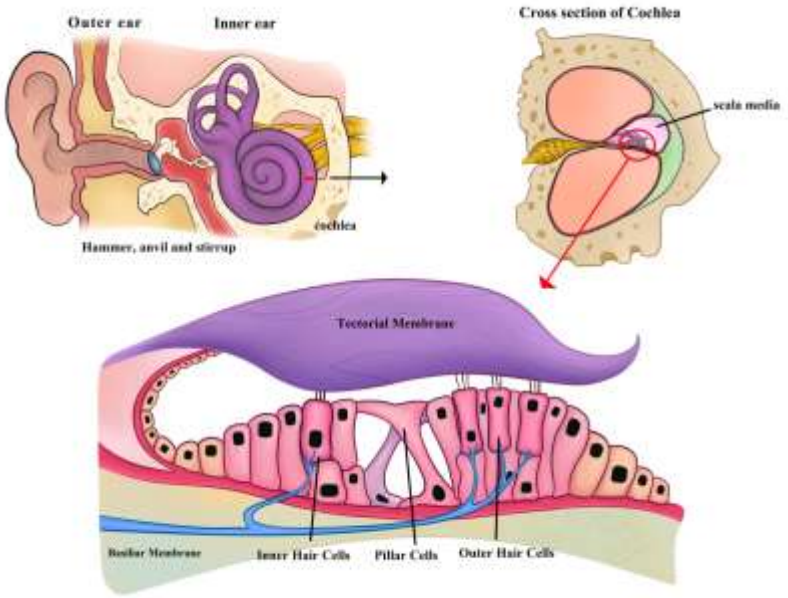

Figure 1. The organ of Corti, located in the scala media- an endolymph- filled cavity inside the cochlea.

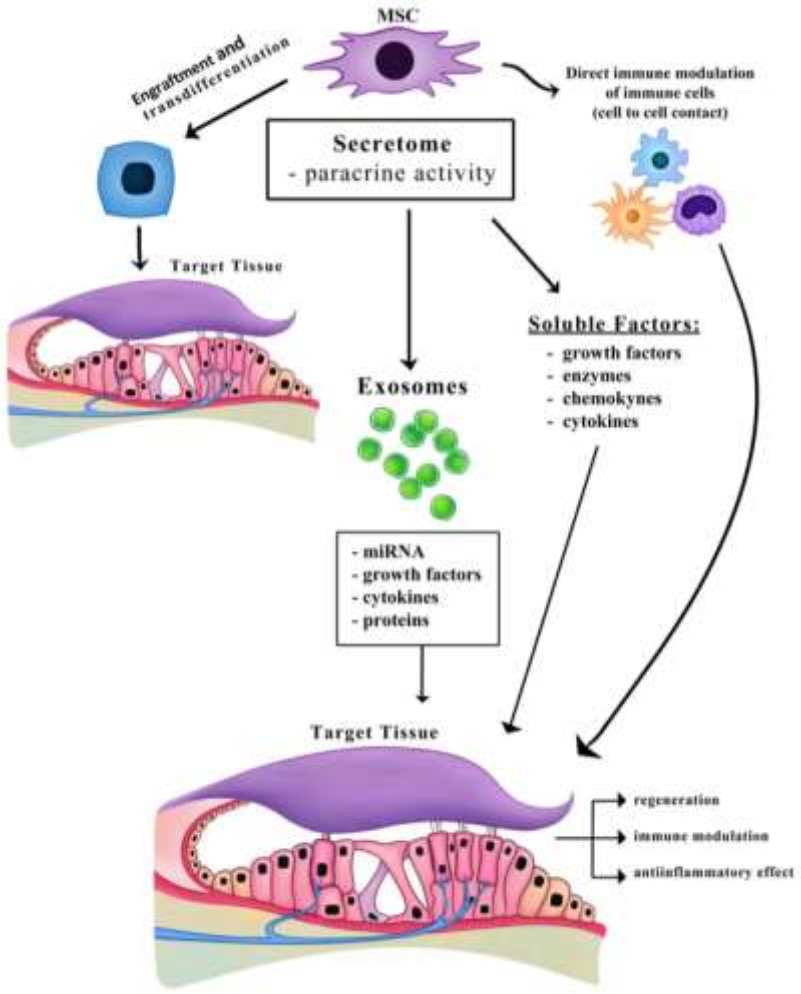

Figure 2. Mesenchymal stem cells and their "secretome"- mechanism of action

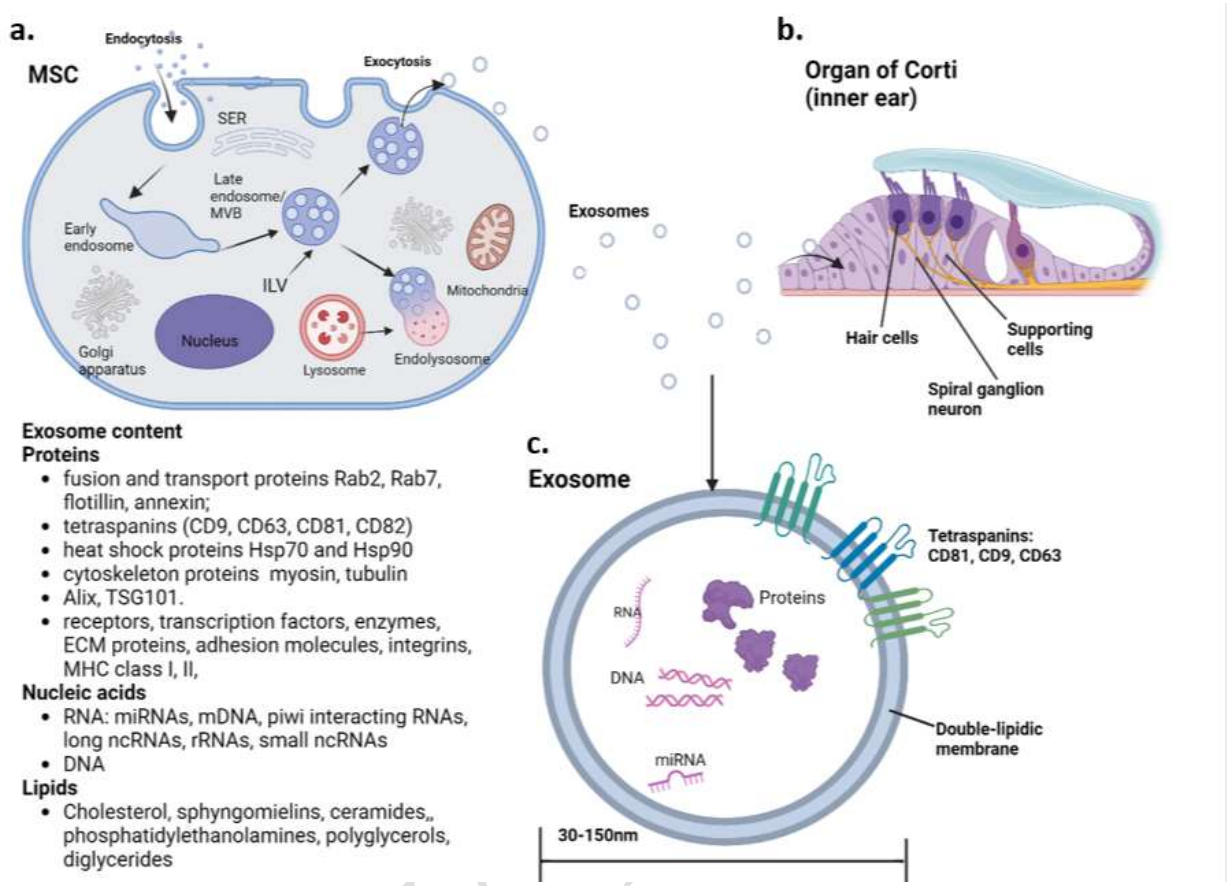

Figure 3. The development of exosomes in the mesenchymal stem cells (MSC) in the endosomal pathway (a). Exosomes' release and uptake by the target cells in the cochlea: hair cells and supporting cells (b). The structure and main constituents of exosomes (proteins, nucleic acids, lipids). (c)

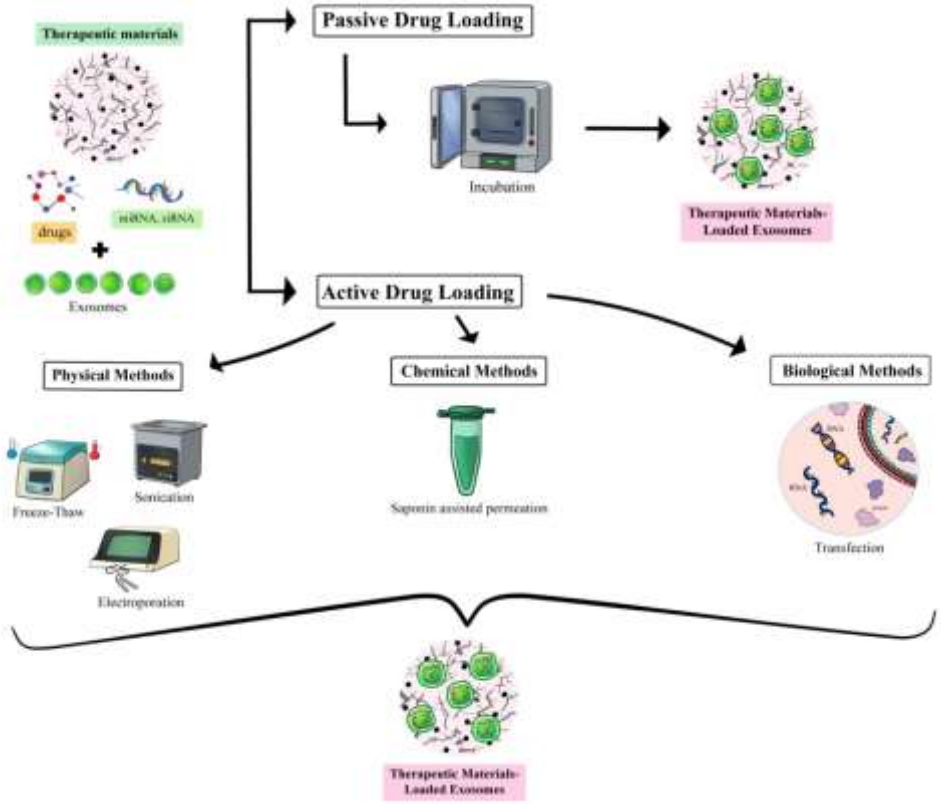

Figure 4. Methods of loading different cargos to target tissues through exosome engineering.