REVIEW

Mesenchymal Stem Cell-Derived Small Extracellular Vesicles: A Novel Approach for Kidney Disease Treatment

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Abstract: Globally, kidney disease has become a serious health challenge, with approximately 10% of adults suffering with the disease, and increasing incidence and mortality rates every year. Small extracellular vesicles (sEVs) are 30 nm–100 nm sized nanovesicles released by cells into the extracellular matrix (ECM), which serve as mediators of intercellular communication. Depending on the cell origin, sEVs have different roles which depend on internal cargoes including, nucleic acids, proteins, and lipids. Mesenchymal stem cell (MSCs) exert anti-inflammatory, anti-aging, and wound healing functions mainly via sEVs in a stable and safe manner. MSC-derived sEVs (MSC-sEVs) exert roles in several kidney diseases by transporting renoprotective cargoes to reduce oxidative stress, inhibit renal cell apoptosis, suppress inflammation, and mediate anti-fibrosis mechanisms. Additionally, because MSC-sEVs efficiently target damaged kidneys, they have the potential to become the next generation cell-free therapies for kidney disease. Herein, we review recent research data on how MSC-sEVs could be used to treat kidney disease.

Keywords: small extracellular vesicles, mesenchymal stem cells, acute kidney injury, chronic kidney disease, renal cell carcinoma

Introduction

Globally, kidney disease affects approximately 10% of the world's population, which equates approximately to more than 800 million people. Alarmingly, disease morbidity and mortality rates are increasing annually.[1] Kidney diseases include acute kidney injury (AKI), chronic kidney disease (CKD), kidney stones, and renal cell carcinoma (RCC). When compared with the liver and other organs, the kidneys are less capable of self-repair, and currently the most common treatment methods for kidney disease are conservative in nature so as to slow down rather than reverse disease progression.

Mesenchymal Stem Cells (MSCs) are derived from the mesoderm and are a class of pluripotent stem cells widely used in clinical practice, with the potential for multidirectional cell differentiation and self-replenishment.[2] MSCs are isolated from a variety of tissues, such as umbilical cords, endometrial polyps, muscle, dental pulp, bone marrow, and adipose tissue,[3] and under certain conditions, they differentiate into different mesenchymal spectrum cell types, such as osteoblasts, adipocytes, and chondrocytes.[4] MSCs localize to injury sites, secrete anti-inflammatory and growth factors via autocrine, paracrine, and endocrine mechanisms to accelerate wound healing and mediate damaged myocardium repair.[5] They also suppressed lung inflammation and fibrosis in COVID-19 patients by secreting cytokines.[6] Additionally, in the last decade, MSCs were instrumental in treating some musculoskeletal, neurodegenerative, and metabolic disorders.[7] However, as some stem cell therapies are disadvantageous in terms of immune rejection, decreased proliferation and differentiation potential after transmission, and mutations in stem cell genetic material,[8] researchers focused on MSC-small extracellular vesicles (MSC-sEVs) for the following reasons: they have the same functions as MSCs, they are more stable,[9] and they do not induce serious immune rejection reactions. sEVs are lipid bilayer vesicles, they effectively protect bioactive cargoes from external degradation, they do not generate ethical issues, and they have

significant potential for renal disease treatment. In recent years, these qualities have been recognized, therefore in this review, we summarized recent MSC-sEVs research advances in kidney disease treatments.

sEVs

As early as 1971, sEVs microscopic ultrastructures in *Ochromonas Danica* were discovered by Aaronson et al.[10] Almost all human cells secrete sEVs; they are found in bodily fluids such as blood, urine, ascites, and amniotic fluid, and differ in structure and function depending on the tissue of origin.[11] sEVs are composed of lipid bilayers that contain active molecules, including nucleic acids, lipids, proteins, and metabolites, which are related to different cell activities. sEVs act as carriers to transport these active molecules between cells,[12] to cross natural barriers such as the blood-brain barrier,[13] and regulate cell physiological functions while being relevant to numerous diseases.[14]

Structure and Biogenesis

sEVs ensure cell-to-cell or cell-to-extracellular matrix (ECM) connections by transporting proteins, nucleic acids, and lipids.[15] Appearing like typical cup-like structures under transmission electron microscopy, sEVs measure 30 nm–100 nm in diameter, have a density of 1.10 g/mL–1.21 g/mL, and consist of lipid bilayers wrapped around an internal bioactive cargoes.[16] The sEVs surface is rich in lipids (cholesterol, sphingomyelin, and ceramide), fusion-related proteins (annexin I, annexin II, and annexin IV), transmembrane 4 superfamily (CD9, CD63, and CD81), heat shock proteins (HSP) (HSP60, HSP70, HSP90, HSPA5, and CCT2), and some cell-specific proteins.[17]

Precise sEVs biogenesis mechanisms are unclear. Thus far, it is hypothesized that endosomal sorting complex required for transport (ESCRT)-dependent and ESCRT-independent pathways are required for sEVs formation, of which the ESCRT-dependent pathway is a classical pathway.[18] ESCRT consists of approximately 20 proteins that bind to auxilin (Vps4, VTA1, and ALIX) to form ESCRT-0 (Hrs), ESCRT-I (TSG101, Vps28, and Vps37), ESCRT-II (Vps22, Vps36, Vps25, and ESCRT-III (Alix and Vps2) complexes.[19] ESCRT-0 recognizes ubiquitinated proteins in endosomal membranes and interacts with clathrin to enrich their numbers; ESCRT-I and ESCRT-II act together to induce endosomal membrane loading specificity to bud inward; and ESCRT-III shears the bud neck to induce vesicle separation.[20] The ESCRT-dependent sEVs formation pathway consists of two steps; firstly, cells undergo endocytosis and endocytosed cell membranes form multiple vesicles which fuse to form early endosomes (EEs).[21] EEs gradually acidify and mature into late endosomes (LEs) which contain intraluminal vesicles (ILVs), while LEs further form multivesicular bodies (MVBs) rich in ILVs.[16] Then, MVBs are divided into two types according to cholesterol content: MVBs with low cholesterol content are degraded through the lysosomal pathway[22] while high cholesterol content MVBs are transported to and fuse with the cell membrane and release ILVs into the ECM. These ILVs are sEVs.

Biological Functions

By transporting proteins, nucleic acids, and lipids between cells, sEVs are key to most of the life activities, such as maintaining internal environmental homeostasis, immune regulation, angiogenesis, inflammatory responses, and apoptosis.[16] Thus far, sEVs studies have identified two ways in which these entities elicit biological effects:[23] firstly, sEVs directly activate target cell receptors via protein molecules or lipid ligands on the sEVs surface, which activates or inhibits some signaling pathways in target cells. Secondly, sEVs fuse directly with recipient cell plasma membranes or enter cells via endocytosis to release their cargoes, thereby regulating cell function and biological behaviors. By studying sEVs cargoes, we can identify disease development mechanisms and intervene with disease progression. With some diseases and associated development steps, cargo expression in sEVs is altered to some degree.[24] As sEVs research intensifies, growing evidence now suggests that sEVs could function as potential drug carriers and diagnostic markers for several diseases.[25]

sEVs Isolation

To use sEVs in clinical settings, efficient, rapid, and stable extraction methods which maintain sEVs structure, biological function, and integrity are required. Currently, the most commonly used method is ultracentrifugation, which is simple and does not require complex technical support. However, the method is time-consuming, sEVs yields are low, and sEVs quantity and quality metrics are largely influenced by the type of rotor and the angle of settling of the rotor. The main

issue is that precipitates or other vesicles, proteins, or protein/RNA aggregates cannot be easily distinguished from sEVs. Other methods include, sucrose density gradient centrifugation, size-exclusion chromatography, ultrafiltration, micro-fluidics, polymer precipitation, and commercial kits.[26] However, to date, no standardized method has been developed to isolate sEVs, while sEV purity and yield levels from single isolation methods remain largely insufficient to meet downstream experimental requirements (Table 1). To ensure standardized extracellular vesicles (EVs) extraction techniques across clinical research, the International Society for Extracellular Vesicles (ISEV) recommended combined approaches to generate high sEVs purity and yields.[27] Additionally, other extraction factors include but are not limited to; sample sources, EVs subpopulations, and their use in downstream applications. When considering sEVs purity, the ratio of quantitative methods can improve the exosome purity determinations and indicate exosome purity, eg, protein/lipid, protein/particle, and RNA/particle ratios.[27] Although numerous sEVs extraction techniques are available and tend to be standardized and standardized, the establishment of sEVs collection and separation standards, as well as the standardization of product preparation processes, are still issues that need further clarification and resolution.

sEVs Characterization

According to ISEV standards, when Western Blotting (WB) is used to characterize/identify extracted EVs proteins, the evaluation of transmembrane/lipid-bound proteins (CD63, CD81, CD82, HLA, and integrin), cytoplasmic proteins (TSG101/ALIX), and at least one negative protein marker is required to characterize extracted EVs and their purity.[27] For individual sEVs populations, they must be analyzed by at least two methods, such as transmission electron microscopy (TEM) for sEVs morphology and Nanoparticle Tracking Analysis (NTA) to confirm sEVs size.

sEV Storage

sEVs do not store well for long periods; after 2 days at 37°C, sizes decreases by about 60%. Currently, the most common sEVs storage method is freezing at −80°C in phosphate buffer (PBS), in addition to freeze-drying and spray-drying methods.[28]

Table 1 Small Extracellular Vesicles (sEVs) Isolation Techniques: Advantages and Disadvantages

Isolation Technique	Advantages	Disadvantages
Ultracentrifugation	High recovery rates, simplified operation, no chemical contamination.	Costly instrumentation and time-consuming. Low RNA yields, low purity, and may be mixed with other components.
Sucrose density gradient centrifugation	High sEVs purity.	Preparation work is tedious and time-consuming, sEVs levels are low and biological function easily lost.
Size-exclusion chromatography	High recovery rates, high purity, less time consuming, high sensitivity, intact structures and biological function preserved. No chemical contamination. It is currently a better method to extract sEVs.	Expensive instrumentation.
Ultrafiltration	High efficiency, free from chemical contamination, and low extraction costs.	Low elution efficiency, difficulty in removing the effects of proteins, purity, shape, and charge of the sample affecting the separation. Clogging of filter membranes and loss of biological function.
Polymer precipitation	Simple operation, high output, high integrity, no special equipment required, economical.	Cumbersome preparation work, low purity, and susceptibility to chemical contamination.
Microfluidics	Easy to operate, high throughput, automated, less sample volume required for highly pure extracted sEVs.	Costly, low yields, and prone to hole plugging.
Commercial kits	Easy to operate and short times required.	The kits are expensive, without uniform standards, and are not suitable for the extraction of massive samples.
Enzyme-linked immunosorbent assay	High purity, intact isolated sEVs, suitable for the separation of specific sEVs. High specificity.	Antibodies and equipment are costly, low throughput, low isolation efficiency, suitable for cell-free samples only.
Hydrostatic filtration dialysis	Low costs, no chemical pollution, sEVs can be isolated from highly diluted solutions, high throughput, high integrity, and biological functions preserved.	Large sample volumes show decreased efficiency

However, the −80°C storage method in PBS is not optimal as evidenced by Corso et al.[29] The authors evaluated different EVs preservation strategies for up to 2 years, and found that over time, EVs in PBS had much lower recovery rates and that EVs degradation in PBS had already started within minutes of storage. The recommended buffer system, among several tested, was PBS plus human albumin and trehalose (PBS-HAT), which significantly improved short- and long-term EVs sample preservation at −80°C, maintained stability, and significantly improved EVs recovery rates during subsequent EVs studies. Additionally, another study recently analyzed the effects of different preservation strategies and times on EVs. Experiments had three components; firstly, they analyzed the purity and particle size of EVs preserved by different storage methods.; secondly, authors analyzed the effects of both fast and slow freeze-thaw cycles and the number of freeze-thaw cycles on EVs; and thirdly, authors explored EVs membrane fusion after freeze-thawing. This latter study component investigated membrane fusion phenomena after freeze-thawing. Study data were interesting; in the first study, storage at −80°C decreased EVs concentrations over time regardless of the storage state, EVs loading decreased significantly after 6 months, and EVs particle size increased over time at −80°C, which was negatively correlated with particle concentration. The second study showed that both fast and slow freeze-thawing cycles increased EVs particle sizes and decreased particle numbers, and that repeated freeze-thawing cycles increased EVs particle sizes. The third study demonstrated that freeze-thawing processes ruptured EVs films and the subsequent re-micellization of ruptured films into new particles.[30] In summary, EVs extracted from fresh samples are optimal for use, but if long-term storage is required then repeated sample or EVs freeze-thawing must be avoided, and preferably PBS-HAT should be used for storage.

MSC-sEVs

Under TEM, like most other cell-secreted sEVs, MSC-sEVs show a classical cup-like morphology (Figure 1), but they not only express intrinsically expressed sEVs protein markers (CD9, CD63, CD81, TSG101, and ALIX), but also adhesive molecules on the MSC surface (CD90, CD29, CD44, CD73).[31,32] Additionally, MSC-sEVs are rich in cholesterol and sphingomyelin; their fatty acids are predominantly arachidonic acid, docosahexaenoic acid, leukotrienes, phosphatidic acid, and prostaglandin lysophosphatidylcholine.[4] MSC-sEVs also contain high levels of RNA molecules and low mitochondrial DNA levels,[33] and it is these cargoes that allow MSC-sEVs exert their biological effects (Figure 2).

As indicated, MSC-sEVs are safer, more stable, and easier to store than MSCs, and provide a unique cell-free therapeutic approach of great potential. Encouragingly, initial therapeutic MSC-sEVs studies generated promising results for different diseases.[34,35] In the COVID-19 epidemic, MSC-sEVs reduced lymphocytes and decreased inflammatory molecules and neutrophils in COVID-19 patients, thereby reducing cytokine storms in individuals with severe infections.[36] MSC-sEVs not only downregulated immune cells to reduce inflammatory responses, but also rescued damaged immune cells in some diseases. Myelin debris can directly inhibit axonal regeneration and impede neurological repair. After spinal cord injury, macrophage phagocytosis becomes impaired, and local myelin sheaths cannot be cleared, resulting in deteriorating motor functions after injury.[37] Sheng et al[38] reported that sEVs derived from bone marrow (BMSC-sEVs) reached injury sites and were phagocytosed by local macrophages, upregulates the expression of Macrophagereceptor with collagen structure, and restored normal physiological functions to macrophages, thereby removing myelin debris from damaged areas and improving neurological functions.

Thus far, MSC-sEVs have generated impressive results in wound healing and skin regeneration,[39] tumor progression,[40] nerve injury repair,[41] and COVID-19.[42] We next focused on recent MSC-sEVs research in kidney disease.

MSC-sEVs in Kidney Disease
Acute Kidney Injury

AKI etiology is related to ischemia, hypoxia, and reactive oxygen species. Also, because the kidneys require high oxygen consumption for water and electrolyte reabsorption, renal tubules are susceptible to oxidative stress injury.[43] Without intervention, AKI predisposes patients to an increased risk of progressive CKD. MSC-sEVs protect the kidneys in several conditions, including ischemia-reperfusion injury (IRI), drug-induced nephropathy (DIN), and sepsis-induced AKI. The impact of MSC-sEVs on these conditions is discussed.
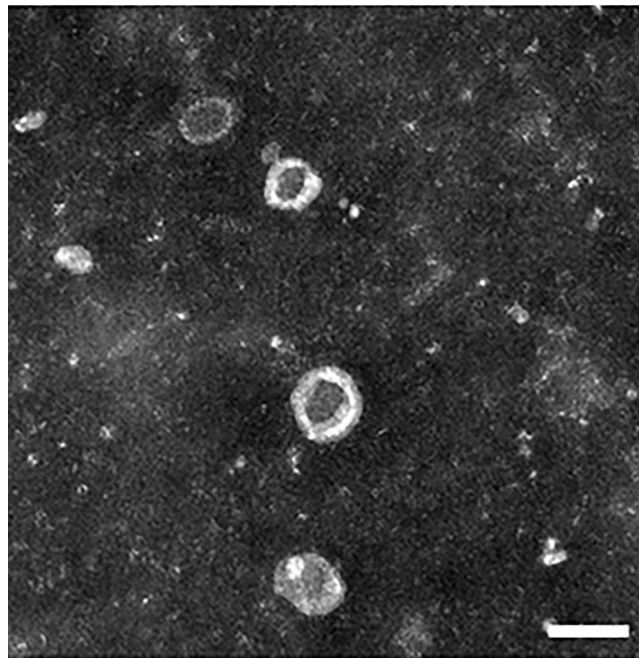
Figure 1 Transmission electron microscope (TEM) hucMSC-sEVs image.
Notes: HucMSC-sEVs show a typical cup-like morphology under TEM (scale bar = 100 nm). Reproduced with permission from He Q, Wang L, Zhao R et al. Mesenchymal stem cell-derived exosomes exert ameliorative effects in type 2 diabetes by improving hepatic glucose and lipid metabolism via enhancing autophagy. *Stem Cell Res Ther.* 2020;11(1):223.[110] Copyright 2020 The Authors. Creative Commons Attribution License.
Abbreviations: hucMSC-sEVs, human umbilical cord Mesenchymal Stem Cell-derived small extracellular vesicles; TEM, transmission electron microscopy.
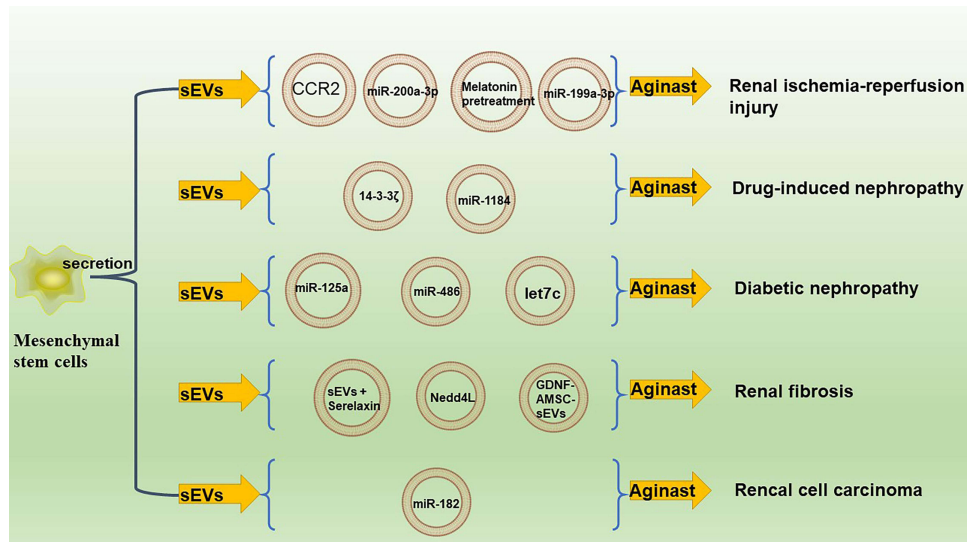

Figure 2 MSC-sEVs cargoes functions in renal disease.
Abbreviations: MSC-sEVs, Mesenchymal Stem Cell-derived small extracellular vesicles; sEVs, Small extracellular vesicles; CCR2, C-C motif chemokine receptor-2; let7c, microRNA-let7c; GDNF-AMSC-sEVs, Glial cell line-derived neurotrophic factor to transfect human adipose mesenchymal stem cells and subsequently isolated sEVs.

Ischemia-Reperfusion Injury

This condition is one of the leading causes of AKI, with approximately 50% of AKI hospitalizations due to IRI.[44] IRI-induced AKI pathophysiology includes inflammation, oxidative stress, renin-angiotensin activation, and mitochondrial dysfunction, while blood reperfusion based on tissue and organ ischemia may lead to IRI.[45]

Early studies reported that adipose-derived MSC (ADMSC) minimized renal injury during renal ischemia-reperfusion injury (RIRI) by reducing oxidative stress and suppressing inflammatory responses.[46] Several studies showed that MSC-sEVs had similar effects in alleviating AKI after IRI, eg, Li et al[47] showed that MSC-sEVs minimized IRI-induced renal structural damage in rats by downregulating inflammatory factors and inhibiting nuclear factor kappa-B (NF-κB) signaling. Zhang et al[48] demonstrated that human umbilical cord MSC-derived sEVs (hucMSC-sEVs) reduced renal pro-inflammatory factors like interleukin-1β(IL-1β), IL-6, tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein-1 (MCP-1) in IRI and reduced oxidative stress to mitigate renal injury. Huang et al[49] established a porcine RIRI model and found that hucMSC-sEVs were beneficial for RIRI as they inhibited inflammatory factor release (Figure 3). Furthermore, by simultaneously administering ADMSC and ADMSC-derived sEVs (ADMSC-sEVs) in renal IRI rats, Lin et al[50] showed that ADMSC and ADMSC-sEVs simultaneous-treatment better protected rat kidneys from acute IRI when compared with animals treated with ADMSC alone. Zhu et al[51] used PKH26-labeled sEVs from human-bone-marrow-derived MSCs (hBMSC-sEVs), injected them into the tail vein of an RIRI mouse model, and found that hBMSC-sEVs accumulate well in the kidney and effectively protected the kidney. Additionally, miR-199a-3p was highly expressed in hBMSC-sEVs and activated phosphatidylinositol 3 kinase (PI3K) /protein kinase B(AKT) and extracellular receptor kinase (ERK) pathways by inhibiting downstream Sema3A expression, thus exerting protective effects toward the kidneys. Several studies reported that MSC-sEVs combinations with other therapies provided better protection for the kidneys. Melatonin (MT) is a hormone secreted by the pineal gland of the brain and improves sleep quality.[52] However, because it has strong antioxidant and anti-inflammatory effects, MT has important protective roles in tissues undergoing IRI.[53] An early 2008 study showed that MSC pretreatment with MT improved MSC survival in vivo,[54] which exerted better renoprotective effect on MSCs. Alzahrani et al[55] compared the renoprotective effects of MT-pretreated MSC-sEVs, MSC alone, and MSC-sEVs without MT pretreatment in kidneys. MT-pretreated MSC-sEVs maximized the protective effects on kidneys by enhancing antioxidant status, inhibiting renal cell apoptosis, suppressing inflammation, and inducing angiogenesis and renal tissue regeneration in IRI tissue. Similar studies reported that combined MT treatments with MSC and MSC-sEVs also minimized RIRI in rats.[56] We previously indicated that sEVs mainly exerted their functions based on their cargoes (especially RNA). Due to sEVs heterogeneity, different sEVs have different cargoes,[57,58] thus, MSC-sEVs can contain protective RNAs (eg, miR-199a-3p[51]) which protect the kidneys by reducing oxidative stress and inhibiting inflammation. Also, MSC pretreatment with MT not only induced MSC proliferation[59] but also upregulated RNAs
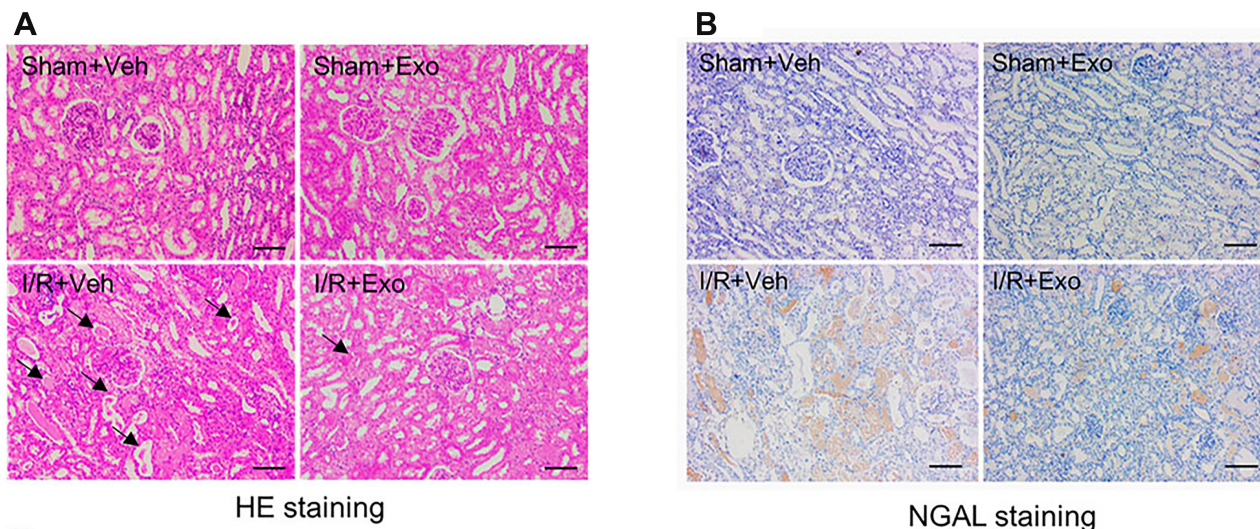

Figure 3 Therapeutic efficacy of MSC-Exo in acute kidney injury.
Notes: Sham+Veh: Sham group treated with vehicle; Sham+Exo: Sham group treated with MSC-Exo; I/R+Veh: Ischemia-reperfusion model treated with vehicle; I/R+Exo: Ischemia-reperfusion model treated with MSC-Exo. (**A**) Kidney tissue hematoxylin and eosin (HE) staining in four groups. Scale bar = 100 μm. Injured tubules with intraluminal casts are indicated by arrows. (**B**) NGAL immunohistochemical staining. Scale bar = 100 μm. MSC-Exo administration caused significant relief in interstitial edema, tubular dilatation, vacuolar degeneration, and necrosis. Immunohistochemistry showed a decrease in NGAL expression after treatment. Reproduced with permission from Huang J, Cao H, Cui B et al. Mesenchymal Stem Cells-Derived Exosomes Ameliorate Ischemia/Reperfusion Induced Acute Kidney Injury in a Porcine Model. *Front Cell Dev Biol*. 2022;10:899869.[49] Copyright © 2022 The Authors. Creative Commons Attribution License.
Abbreviations: MSC-Exo, Mesenchymal Stem Cell-derived exosome; NGAL, Neutrophil gelatinase-associated lipocalin.

with renoprotective effects in MSC-sEVs.[55] Shen et al[60] identified increased CC motif chemokine receptor-2 (CCR2) levels in MSC-sEVs and demonstrated its ability to act as an endogenous CCL2 sponge to deplete ligands, thereby alleviating CCL2-induced macrophage infiltration in mice with IRI-induced AKI. Finally, a recent study reported that 3-dimensional cultured human placental stem cell EVs (hPMSC-EVs) more effectively inhibited apoptosis and inflammatory damage in RIRI when compared with 2-dimensional cultures.[61] Collino et al[62] identified an interesting phenomenon where ADMSCs secreted more EVs under hypoxic conditions, and these ADMSC-EVs were more protective of the kidneys under RIRI when compared with ADMSC-EVs secreted under normoxic conditions. Thus, MSC 3-dimension-culturing and culturing under hypoxic conditions may facilitate MSC-sEVs protective effects.

Drug-Induced Nephropathy

In the body, the kidneys are the main drug metabolism and excretion organs; however, some drugs can seriously damage these organs. DIN is a common cause of AKI, and accounts for up to 60% of community- and hospital-acquired episodes.[63]

Cisplatin (CDDP) is a clinically effective broad-spectrum anticancer drug, and is regulated during renal transport by proximal tubular transport proteins such as OCT2 and MATE1, therefore, CDDP accumulates in proximal renal tubular cells, causes inflammation, injury, and cell death, and leads to nephrotoxicity in 30–40% of CDDP-treated patients.[64] Although studies have highlighted the molecular mechanisms underpinning CDDP-induced AKI (CDDP-AKI),[65] the pathway whereby CDDP regulates cell survival, metabolism, and immune responses is also involved in CDDP cytotoxicity in tumor cells, thus, intervening in this process may attenuate the antitumor effects of CDDP. Therefore, we anticipate that MSC-sEVs better protect renal cells without reducing drug efficacy. Wang et al[66] showed that hucMSc-sEVs reduced TNF-α, IL1-β, and IL6 inflammatory cytokine serum levels in a CDDP-AKI mouse model, thereby suppressing renal injury; similarly, hucMSc-sEVs inhibited CDDP-induced mitochondrial apoptosis and inflammatory cytokine secretion in in vitro renal tubular epithelial cells (TECs), with these effects effectively alleviating CDDP nephrotoxicity by inhibiting mechanistic target of rapamycin(mTOR) phosphorylation and activating autophagy in TECs. Additionally, the 14-3-3ζ protein from hucMSc-sEVs likewise induced autophagy in the CDDP-AKI model. Jia et al[67] found that 14-3-3ζ from hucMSC-sEVs activated autophagy by binding to ATG16L and promoted its localization to the outer surface of phagocytes, thereby protecting the kidney in CDDP-AKI. Zhou et al[68] used in vivo and in vitro approaches to show that hucMSC-sEVs ameliorated CDDP-induced AKI through antioxidant and anti-apoptotic effects and promoted cell proliferation, but did not explain its specific mechanisms. Zhang et al[69] pretreated MSCs with the miR-1184 agomir to generate high miR-1184 expression in pretreated MSC-sEVs, with cells significantly upregulating CDDP-treated HK-2 cell viability, and MSC-sEVs-miR-1184 protective in CDDP-induced AKI via Forkhead Box O4.

Bruno et al conducted several studies using EVs derived from BMSC(BMSC-EVs) in a glycerol-induced AKI mouse model. Their early studies demonstrated that the intravenous administration of human BMSC-EVs had the same efficacy as MSC in restoring renal function and morphology in mice, and that mRNA molecules in BMSC-EVs promoted AKI recovery by activating anti-apoptotic and pro-regenerative programs in TECs.[70] In another study, the authors demonstrated that miRNAs in BMSC-EVs had key roles in recovering glycerol-induced AKI.[71] Interestingly, their recent study reported that in the same model, BMSC-EVs induced renal tubular cell proliferation and attenuated kidney injury (among other functions), which was mainly attributed to mRNA, miRNA, and growth factor cargoes in sEVs.[72] Thus, the anti-apoptotic, regeneration-promoting, and other kidney-protective functions exerted by BMSC-EVs may depend on nucleic acids, proteins, and other cargoes in BMSC-sEVs, but more studies are required to identify which molecules act in which pathways.

In a rat gentamicin-induced AKI model, Reis et al[73] observed that BMSC-sEVs had the same renoprotective role as BMSCs, and that RNAs in BMSC-sEVs mediated AKI recovery via a range of functions, including inhibiting apoptosis, promoting cell proliferation, downregulating pro-inflammatory cytokines, and upregulating anti-inflammatory cytokines. Unfortunately, no studies have identified which RNAs exerted roles in gentamicin-induced AKI, thus mechanisms require further investigation.

Sepsis-Induced Acute Kidney Injury

Gao et al[74] used ADMSC-sEVs tail vein injections to treat a sepsis-AKI mouse model and found that ADMSC-sEVs improved renal function, reduced inflammatory cytokine release, and decreased mortality by activating Sirtuin 1 (SIRT1). hucMSC-sEVs

reduced IRAK-1 target gene expression, which codes for a key protein that positively regulates NF-κB activity, by upregulating miR-146b levels in renal tissue (but not transporting miR-146b), which inhibited NF-κB activity and reduced pro-inflammatory factor levels during sepsis, thus effectively protecting the kidneys from sepsis-induced AKI.[75]

Chronic Kidney Disease

CKD refers to abnormalities in kidney structure and function, is caused by different mechanisms lasting 3 months or longer, and includes many types of kidney disease. Without timely and effective intervention, CKD leads to deterioration and disease progression, or during the disease course, it develops into chronic renal insufficiency, renal failure, and eventually end-stage renal disease (ESRD).[76] One of the most common causes of CKD is diabetic nephropathy (DN); MSC-sEVs have been extensively studied in CKD, especially in DN and renal fibrosis.

Diabetic Nephropathy

DN is a common microvascular complication of diabetes mellitus and a major cause of ESRD. Disease pathogenesis includes abnormal glucose metabolism, renal hyperperfusion hyperfiltration, oxidative stress, immune inflammation, and genetic factors. Currently, the clinical treatment is to slow down DN progression by administering drugs that improve blood pressure or blood glucose levels. Once DN progresses to irreversible stages, alternative treatments such as hemodialysis are inevitable[77]. Li et al[78] reported that mouse umbilical cord MSCs (MUC-MSCs) blocked transforming growth factor-β(TGF-β)-triggered myofibroblast transdifferentiation through paracrine sEVs, inhibited PI3K/Akt and mitogen-activated protein kinase (MAPK) signaling-mediated mesangial cell proliferation, increased matrix metalloproteinase levels in mesangial cells, and reduced renal fibrosis in a DN mouse model. Additionally, hucMSC-sEVs inhibited TGF-β, IL-6, IL-1β, and TNF-α inflammatory cytokine production in HK2 cells when induced by high glucose, thus effectively improving renal function, inhibiting inflammation and fibrosis, and preventing early DN progression.[79] In their DN rat model, Hao et al showed that ADMSC-sEVs significantly reduced blood glucose, serum creatinine, 24-h urine protein, urine albumin creatinine ratio, and kidney weight, and inhibited renal fibrosis and thylakoid hyperplasia when compared with control animals. Moreover, ADMSC-sEVs downregulated IL-6 expression in in vitro DN models, while IL-6 promoted mesangial proliferation. The authors also observed that processes were putatively mediated by miR-125a in ADMSC-sEVs, which bound histone deacetylase 1 (HDAC1) and down-regulated endothelin-1 expression, thereby reducing DN symptoms and kidney injury.[80] Nagaishi et al[81] showed that MSC-sEVs inhibited tubular interstitial fibrosis and glomerular mesangial matrix expansion by reducing p38-MAPK and TGF-β expression, preventing Z0-1 degradation in TECs, and reducing urinary albumin excretion in DN mice. Jin et al[82] indicated that miR-486 in ADMSC-sEVs could be transferred to podocytes, as autophagy activators, to ameliorate podocyte injury by regulating miR-486/Smad1/mTOR signaling to alleviate high glucose-induced cell injury in MPC5 cells and DN mice.

Renal Fibrosis

Renal fibrosis is an irreversible pathological change in end stage chronic kidney disease; its pathogenesis is very complex and the specific mechanisms remain unclear. The main pathological causes may be due to ECM deposition, TEC epithelial-mesenchymal transition (EMT),[83] and vascular sclerosis. In previous research, MSC-sEVs transported miRNA-let7c to damaged TECs and inhibited collagen Iα1, IVα1, α-smooth muscle actin, and TGF-β1 expression by inducing increased let7c expression. Ultimately, this process attenuated renal fibrosis and renal injury.[84] Yang et al[85] showed that miR-186-5p from MSC-sEVs suppressed ECM deposition and EMT by downregulating Smad5, and inhibiting renal fibrosis progression both in vitro and in vivo. BMSC-sEVs reduced M1 and M2 macrophage polarization by activating EP2 receptors, thereby inhibiting pro-inflammatory factor (IL-6) secretion, promoting anti-inflammatory factor (IL-10) release, and ultimately reducing ECM deposits and renal fibrosis (Figure 4).[86] Additionally, combined BMSC-sEVs and serelaxin (antifibrotic drug) effectively treated mice with renal fibrosis while reducing hypertension, renal macrophage levels, T-cell infiltration, and TEC injury.[87] BMSC-sEVs also inhibited TGF-β1-induced EMT in HK-2 cells by transporting Nedd4L, which also improved in vivo renal fibrosis.[88] Jin et al[89] used anti-let-7i-5p to transfect MSCs and observed that MSC-sEVs delivered anti-let-7i-5p to target cells in vitro and attenuated TGF-β1-induced fibrotic responses; in vivo, anti-let-7i-5p exerted anti-fibrotic effects in a renal fibrosis model by activating TSC1/mTOR signaling. Chen et al[90] used glial cell line-derived neurotrophic factor
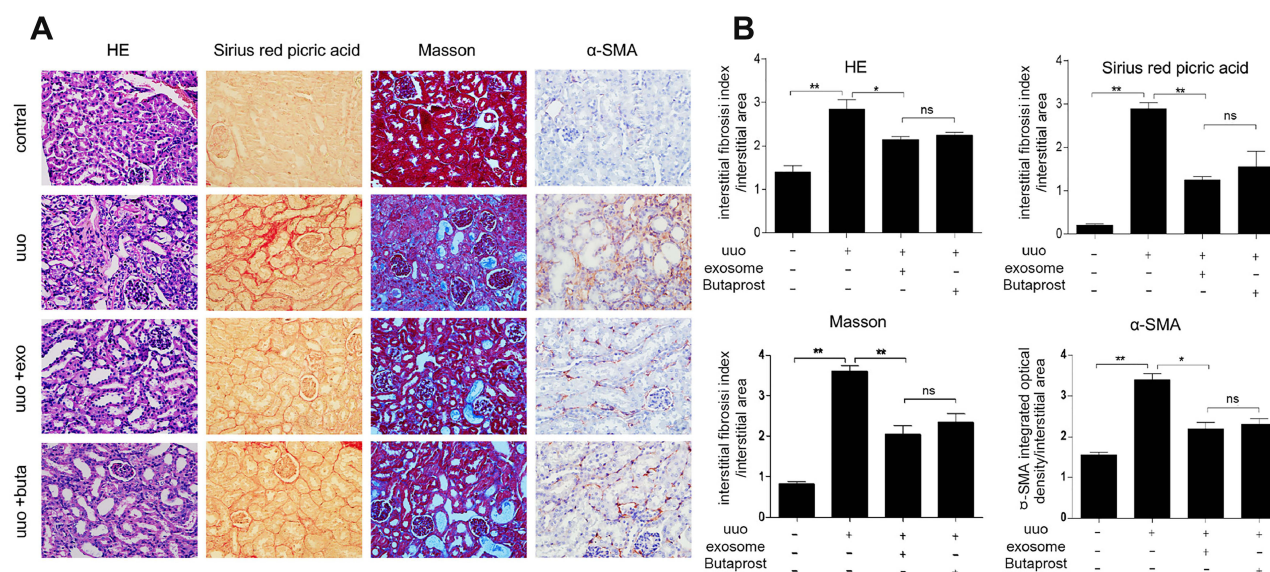
Figure 4 Therapeutic efficacy of BMSC-exo in a renal fibrosis model.

Notes: (**A**) HE, Masson, Sirius red picric acid staining and α-SMA immunohistochemical staining were used to detect renal histopathological changes in the different treatment groups (X400). (**B**) Compared with the control group, the UUO model group showed dilated renal tubules, infiltration of renal interstitial inflammatory cells and increased extracellular matrix deposition. Compared with the UUO group, the BMSC-exo treatment group significantly reduced interstitial matrix deposition and significantly decreased α-SMA expression, with effects similar to those of the EP2 agonist butaprost. *p < 0.05, **p < 0.01. Reproduced with permission from Lu Y, Yang L, Chen X, Liu J, Nie A, Chen X. Bone marrow mesenchymal stem cell-derived exosomes improve renal fibrosis by reducing the polarisation of M1 and M2 macrophages through the activation of EP2 receptors. *IET Nanobiotechnol.* 2022;16(1):14–24.[86] Copyright 2020 The Authors. Creative Commons Attribution License.

Abbreviations: BMSC-exo, exosome derived from bone marrow mesenchymal stem cells; α-SMA, α-smooth muscle actin; UUO, unilateral ureteral occlusion.

(GDNF) to transfect human adipose MSCs and subsequently isolated sEVs (GDNF-AMSC-sEVs), which inhibited apoptosis and stimulated angiogenesis by activating SIRT1 and upregulating phosphorylated endothelial nitric oxide synthase levels, thereby improving renal fibrosis. MSC-sEV pretreatments with different drugs effectively inhibited renal fibrosis and could be used as MSC-sEV applications in the clinic.

Metformin is the drug of choice for treating type 2 diabetes. In addition to anti-hyperglycemic effects, the drug exerts impacts inflammation, autophagy, oxidative damage, apoptosis, and aging, and has protective functions toward the kidneys. After treating CKD MSCs with metformin, Kim et al[91] reported that the paracrine effects of pretreated MSCs were enhanced and inhibited MSC senescence by suppressing SA-β-gal activity, p16 Ink4a expression, and p53 and NF-κB activation, thus effectively reducing CKD inflammation and fibrosis. Furthermore, Liao et al[92] observed that MSCs treated with metformin enhanced EVs production via autophagy-related pathways and showed that elevated ITIH4 levels in EVs, which are critical for regulating cell growth and inflammatory responses, improved disc cell senescence. Although MSC-sEV pretreatment studies with metformin in CKD remain underreported, we hypothesize potential feasibility for treating CKD.

Rencal Cell Carcinoma

RCC is one of the most common urological malignancies,[93] with annual RCC cases at approximately 400,000, and deaths at approximately 175,000. Incidence rates are increasing year on year, with higher rates in men than women.[94] The most common disease subtype is clear cell renal cell carcinomas (ccRCC),[95] which accounts for 70–80% of all RCC cases.[96] To date, several sEVs-related studies have provided new concepts for RCC treatment.[25,97] Li et al[98] used hucMSC-sEVs to treat ccRCC mice via intravenous administration and found not only enhanced in vivo immune responses, but also inhibited in vivo ccRCC growth and metastasis via vascular endothelial growth factor (VEGFA) targeting by miR-182 in hucMSC-sEVs. Another study showed that human liver stem cell-derived EVs upregulated miRNAs with antitumor effects, including miR-145, miR-200b and miR-200c, which slowed down tumor progression in mice by promoting apoptosis and reducing tumor angiogenesis.[99] These studies identified MSC-sEVs superiority in treating RCC; however, MSC-sEVs are a double-edged sword in cancer. Qi et al[100] reported that BMSC-sEVs promoted osteosarcoma and gastric

cancer cell proliferation by activating Hedgehog signaling, while Zhang et al[101] observed that BMSC-sEVs carrying miR-193a-3p, miR-210-3p, and miR-5100 to recipient cells, activated STAT3 signaling to induce EMT and enhance lung cancer cell invasion. Therefore, the main issues are how to safely and stably exploit MSC-sEVs for RCC treatment. As the research on MSC-sEVs progresses, it is believed that the application of MSCs-sEVs in the treatment of RCC can achieve more satisfactory results and be more widely used in the clinic in the future.

Kidney Transplantation

Kidney transplantation is the ideal treatment for ESRD. After more than half a century of development, kidney transplantation has become the most common organ transplantation procedure with high success rates, and has significantly reduced ESRD mortality rates to approximately 29 deaths out of 1000 patients/year.[102] However, some patients may suffer from transplanted kidney failure or progressive renal insufficiency after renal transplantation, which may lead to death, therefore, prognostics for renal transplantation are critical in clinical research. Critically, Wu et al[103] demonstrated that the long non-coding RNA DANCR in BMSC-sEVs promoted Treg cell differentiation by reducing SIRT1 levels in CD4+ T cells, which induced immune tolerance after kidney transplantation.

MSC-sEVs Imaging Studies in Kidney Disease

Despite the potential shown by MSC-sEVs in regenerative medicine, their non-invasive in vivo tracking is highly complex due to variations/changes induced by interactions with recipient cells.[104] Tracking in vivo sEVs distribution, migratory capacity, toxicity, biological effects, communication capacity, and mechanisms may provide a theoretical basis for assessing sEVs levels for treating organs and predicting therapeutic responses.[105]

The first labeling quandary is whether fluorescent labeling should be performed directly or indirectly on MSCs to obtain fluorescently labeled MSC-sEVs. This question was addressed by Grange et al[106] who used the lipophilic fluorescent dye DiD to label MSC-EV to obtain direct EV labeling (DL-EV) and labeled EVs by donor cells pre-treated with the dye (LCD-EV). The authors then administered labeled MSC-EV groups to a glycerol-induced AKI mouse model to assess fluorescence signals using optical imaging. Both labeling methods detected EVs in whole-body images and damaged kidneys, while DL-EV showed higher and brighter fluorescence when compared with LCD-EV whose signals were detectable in dissected kidneys only. These data suggested that LCD-EV exerted higher specificity but lower fluorescence intensity than DL-EVs, therefore, direct EVs labeling appeared to be the better option.

Gupta et al[107] used luciferase NanoLuc and ThermoLuc systems to label MSC-EVs with CD63, and injected cells into mice to determine in vivo MSC-EVs pharmacokinetics. ThermoLuc-labeled MSC-EVs were more suitable for non-invasive EVs follow-up, while NanoLuc was only suitable for in vivo quantification under ex vivo conditions due to poorer substrate distributions, emission wavelength, and toxicity issues, even though it possesses a broader dynamic range. However, fluorescent molecule signals suffer from tissue scattering, poor penetration depth, and low resolution, which can be better facilitated by nuclear and magnetic resonance imaging (MRI)-based methods.

Han et al[108] performed the first MRI tracking study of systemically administered EVs, and improved on previous studies by using electroporation to directly label EVs with superparamagnetic iron oxide. The authors generated magneto-EVs of high purity and sufficient MRI sensitivity by purifying labeled EVs using Ni-NTA columns. Using this method, authors labeled pluripotent stem cell-derived EVs (iPSC-EVs) and injected intravenously them into a mouse AKI model. Unequivocally, iPSC-EVs treatment significantly improved kidney function and increased animal survival. Notably, using MRI signals from labeled EVs, authors observed that iPSC-EVs targeted and rapidly accumulated in damaged kidneys. When compared with previous imaging methods, this method was highly sensitive and allowed for real-time MRI ER tracking in a non-invasive manner, without compromising MSC-EVs therapeutic efficacy. Although MRI facilitated excellent tissue penetration and quantification, it is expensive and time-consuming, and may be difficult to scale-up in future studies, therefore more options are required.

Cao et al[109] labeled isolated MSC-EVs by incubating cells with DPA-SCP and injecting preparations into a RIRI mouse model. DPA-SCP exhibited deeper tissue penetration, lower biological background interference, and higher in vivo biosafety profiles than the conventional dye pkh26, and imaging MSC-EVs by aggregation-induced emission luminogens did not affect the function of EVs membranes and damaged kidneys. Using this method, the authors found

that MSC-EVs were rapidly absorbed by damaged kidneys in RIRI mice to reach TECs and exert nephroprotective effects. Protective effect mechanisms suggested that MSC-EVs transported miRNA-200a-3p to TECs, targeted Keap1-Nrf2 signaling, and targeted and protected TEC mitochondrial functions by reducing mitochondrial fragmentation and normalizing membrane potential in AKI.

These studies demonstrated the following commonalities; firstly, MSC-EVs were rapidly absorbed after entering the body and the highest absorption occurred in the liver; secondly, MSC-EVs had excellent abilities to target damaged kidneys. The above describes some current applications of Imaging study in MSC-EVs for renal diseases. In conclusion, there is an urgent need to develop suitable non-invasive in vivo imaging methods to track MSC-sEVs secretion, transport, and uptake in clinical regenerative medicine (Figure 5).

Conclusion and Future Perspectives

With recent advances in regenerative medicine, MSC-sEVs have the potential to become the next generation of cell-free therapies for kidney disease (Figure 6). EVs have great potential as natural drug delivery systems and EVs bilayer membranes protect cargoes from body clearance thereby prolonging circulating half-lives. From our review, MSC-sEVs have four main application areas. Firstly, MSC-sEVs can transport renoprotective miRNAs to target cells to alleviate AKI by reducing oxidative stress, inhibiting renal cell apoptosis, suppressing inflammation, and inducing renal tissue regeneration. Secondly, MSC-sEVs can slow down DN progression by anti-fibrosis actions, reducing EMT, inhibiting apoptosis, and reducing inflammation. Thirdly, MSC-sEVs have the potential to become anti-tumor vaccines to inhibit RCC progression by enhancing in vivo immune responses. Fourthly, MSC-sEVs appear to improve survival rates in kidney transplanted mice by inducing immune tolerance.

We also reviewed some of the tools currently available for EVs labeling. These technological advances will facilitate a greater understanding of transport trajectories and targeting ability of MSC-sEVs in vivo, with a view to early applications for clinical medicine. Although MSC-sEVs are important for renal disease therapy, many unknowns and challenges remain. Firstly, MSC-sEVs research in kidney disease treatment is still focused on cell and animal studies, and there remains a lack of knowledge on MSC-sEVs duration effects, administered amounts, and therapeutic effects in
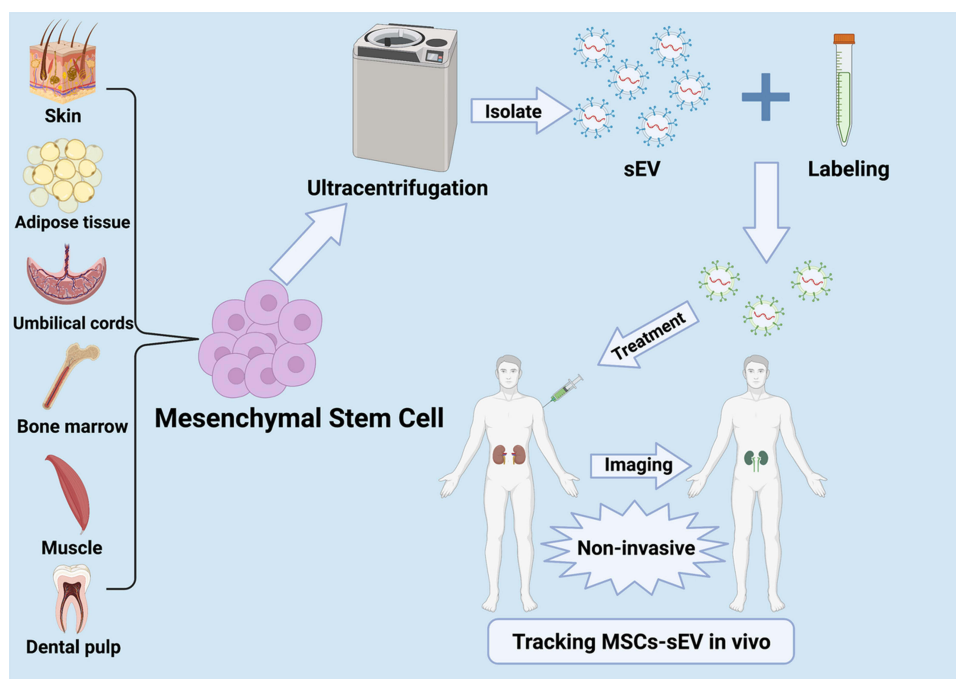

Figure 5 Imaging MSC-sEVs in kidney disease – a flow chart.
Notes: Labeling methods include chemical dye labeling, aptamer labeling, labeling extracellular vesicle surface proteins by genetic engineering, and labeling with radioisotope or magnetic resonance imaging contrast agents.[104] Created with BioRender.com.
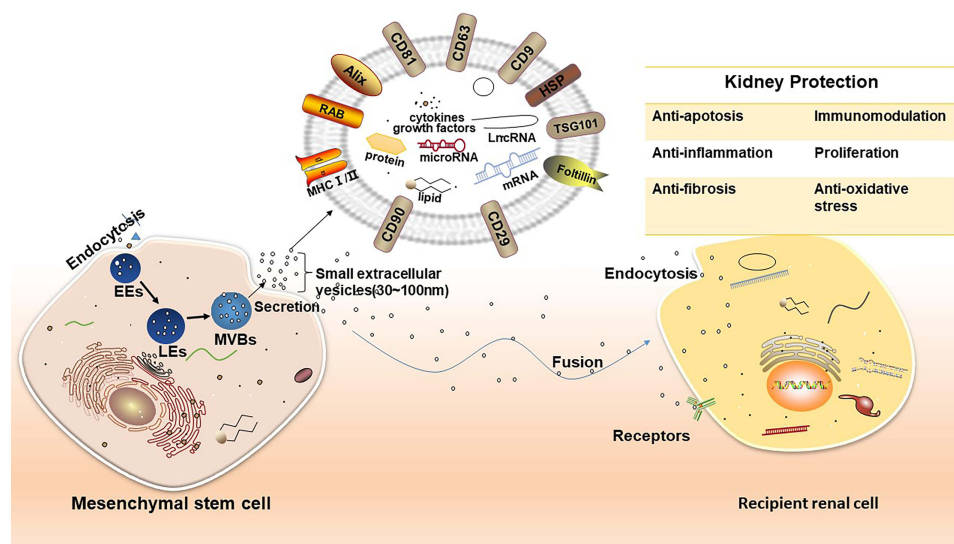**Abbreviation**: sEVs, Small extracellular vesicles.

Figure 6 Mesenchymal stem cells secrete MSC-sEVs to protect the kidney.
Notes: MSCs undergo endocytosis to form EEs, which mature into LEs and eventually form MVBs for transport to the cell membrane to release sEVs outside the cell. MSC-sEVs enter renal cells by endocytosis or bind to receptors, and exert protective effects on the kidney via anti-oxidative stress, anti-apoptosis, anti-inflammation, anti-fibrosis, cell proliferation, and immunomodulation actions.
Abbreviations: EEs, Early endosomes; LEs, Late endosomes; MVBs, Multivesicular bodies; RAB, Ras-associated GTP-binding; Alix, Alg-2 interacting protein x; HSP, Heat shock proteins; TSG101, Tumor susceptibility gene 101; MHC, Major histocompatibility complex; LcRNA, Long non-coding RNA; mRNA, Messenger RNAs.

kidney disease, thus it is unclear if proteins, lipids, mRNAs, and miRNAs in MSC-sEVs may cause other harmful effects in humans. Secondly, due to sEVs heterogeneity, different sEVs sources have different therapeutic effects on kidney disease, so it is important to screen for the most effective sEVs tissue sources. Thirdly, to ensure further research development, more sophisticated isolation and purification techniques are required to generate highly pure and stable sEVs within shorter periods.

Unequivocally, MSC-sEVs have enormous potential for treating renal disease, and its efficiency, safety, pharmacokinetics and specific mechanisms for the treatment of renal diseases will be the focus of the next studies.

Author Contributions
Y.L. collected and analyzed the relevant literature, the writing of the first draft, figure design, and comment revision and feedback. L.W. performed analyses and organized the literature, figure design, and manuscript revision. M.Z. helped write the first draft, graph design, and data collection. Z.C. identified the topic, guided the writing of the manuscript, helped with diagramming, made grammatical corrections to the article and identified the journal for submission. All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure
We have no conflicts of interest to disclose.

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