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"Stem cell-derived exosomes in the therapy of inflammatory diseases"

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Table of Contents

Abbreviations

AD	Alzheimer's Disease
ADMSC	Adipose-Derived Mesenchymal Stem/Stromal Cells
ADSC	Adipose-Derived Stem Cells
AF4	Asymmetrical flow field-flow fractionation
AFM	Atomic Force microscopy
AIC	Acute Inflammatory Colitis
Aβ	β-amyloid peptide
AβOs	Amyloid β oligomers
BALF	Bronchoalveolar Lavage Fluid
BM-MSCs/BMSCs	Bone Marrow-derived Mesenchymal Stem/Stromal Cells
CAC	Colitis-Associated Cancer
CD	Crohn's Disease
CIA	Collagen-Induced Arthritis
CKD	Chronic Kidney Disease
CM	Conditioned Medium
CNS	Central Nervous System
cryo-EM	cryo- Electron Microscopy
CXCL9	C-X-C motif chemokine 9
DFU	Diabetic Foot Ulcers
DLS	Dynamic Light Scattering
DMBT1	Deleted in Malignant Brain Tumors 1
DN	Diabetic Nephropathy

DPN	Diabetic Peripheral Neuropathy
DPSC	Dental Pulp Stem Cell
DSS	Dextran Sulfate Sodium
EAE	Experimental Autoimmune Encephalomyelitis
EPCs	Endothelial Progenitor Cells
ESCRT	Endosomal Sorting Complex Required for Transport
ESRD	End-Stage Renal Disease
EVs	Extracellular Vesicles
FasL	Fas Ligand
FLS	Fibroblast-Like Synoviocytes
GMP	Good Manufacturing Practice
GMSCs	Gingival Mesenchymal Stem/Stromal Cells
hESCs	human Embryonic Stem Cells
HGF	Hepatocyte Growth Factor
HLSCs	Human Liver Stem-like Cells
HRECs	Human Retinal Endothelial Cells
HSCs	Hemopoietic Stem Cells
HSP70	Heat Shock Protein 70
HSP90	Heat Shock Protein 90
hucMSCs	human umbilical cord Mesenchymal Stem/Stromal Cells
IBD	Inflammatory Bowel Disease
ICM	Inner Cell Mass
IFN-γ	Interferon gamma

IL-10	Interleukin 10
IL-18	Interleukin 18
IL-1β	Interleukin 1 beta
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-7	Interleukin 7
ILV	Intraluminal Vesicles
iMSC	Induced Pluripotent Stem Cell-derived Mesenchymal/Stromal Cells
iNOS	Inducible NO Synthase
IO	Intraocular
iPSCs	Induced Pluripotent Stem Cells
IV	Intravenous
MISEV2018	Minimal Information for Studies of Extracellular Vesicles
MMP	Matrix Metalloproteinase
MPO	Myeloperoxidase
MS	Multiple sclerosis
MSC-CM	Mesenchymal Stem/Stromal Cell Conditioned Medium
MSC-Exos	Mesenchymal Stem/Stromal Cell derived Exosomes
MSCs	Mesenchymal Stem/Stromal Cells
MVB	Multi-Vesicular Bodies
MVE	Multi-Vesicular Endosome
NS	Nervous System
NSCs	Neural Stem Cells

NTA	Nanoparticle Tracking Analysis
OA	Osteoarthritis
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
RA	Rheumatoid Arthritis
ROS	Reactive Oxygen Species
RVG	Rabies Viral Glycoprotein
SC	Subconjunctival
SC-Exo	Stem-Cell derived Exosomes
SEC	Size Exclusion Chromatography
SEM	Scanning Electron Microscopy
SMMSC	Synovial Membrane derived Mesenchymal Stem/Stromal Cells
TE	Trophectoderm
TEM	Transmission Electron Microscopy
TGF-β1	Transforming Growth Factor-β1
Th1	T helper 1 cell
Th17	T helper 17 cell
TLR4	Toll-Like Receptor 4
TMSC	Tonsil-derived Mesenchymal Stem/Stromal Cells
TNBS	Trinitrobenzenesulfonic Acid
TNF-a	Tumor Necrosis Factor alpha
Treg	Regulatory T-cell

TRPS	Tunable Resistive Pulse Sensing
UC	Ulcerative Colitis
U-MSCs	Umbilical cord-derived Mesenchymal Stem/Stromal Cells
VEGF	Vascular Endothelial Growth Factor

Abstract

Exosomes, a subtype of extracellular microvesicles with a diameter of 30–150 nm, are nanovesicles secreted by all types of cells, both eukaryotic and prokaryotic. For many years, scientists believed that exosomes were a disposal mechanism of the cell, however, over the last decades, exosomes received unprecedented scientific attention when it was discovered that they carry genetic material and they act as mediators of cellular communication. The exosomal cargo includes proteins, ribonucleic acids, such as mRNAs and microRNAs, and lipids. By transferring bioactive molecules, exosomes can modulate inflammation, immune response, tumor invasion and metastasis, tissue regeneration and several other biological processes. The research regarding their potential role in the diagnosis, progression and therapy of many diseases is growing exponentially over the last years. Exosomes secreted by stem cells hold great potential in the treatment of various diseases due to the unique properties that are derived from their parental cells. They provide new perspectives for the development of a cell-free therapeutic approach, which is an important pursue due to the reported side effects of stem cell-based therapy, such as immune rejection, mal-differentiation, tumor formation and lung entrapment. Stem cell-derived exosomes have the capacity to repair damaged tissues, promote angiogenesis, regulate immune responses and other physiological or pathological processes, and thus, they are ideal candidates for the treatment of chronic inflammatory and autoimmune conditions. Inflammation is emerging as a major predisposal factor in the pathogenesis of a plethora of diseases. Hence, the therapeutic potential of stem cell-derived exosomes, as well as the mechanism underlying it, is being thoroughly investigated through in vitro and in vivo animal studies. Stem-cell derived exosomes exhibit promising results in the treatment of chronic inflammatory diseases of the gastrointestinal tract, like inflammatory bowel disease and periodontitis, neurodegenerative disorders, such as Alzheimer's disease and multiple sclerosis, and various autoimmune diseases, such as arthritis and diabetes. This report describes the progress regarding the effects of stem cell-derived exosomes in the treatment of many inflammatory diseases and the mechanism behind their action.

1 Introduction

1.1 Extracellular Vesicles/ Exosomes

Extracellular vesicles (EVs) are particles that are naturally released from the cell and delimited by a lipid bilayer, containing cytosol from the secreting cells, but not containing a functional nucleus, indicating the lack of ability to replicate [1]. It is known that all kinds of cells release EVs into the extracellular space, both eukaryotic and prokaryotic. There have been early reports of these secreted particles from various sources. Nonetheless, the importance of the discovery was not recognized at the time, and it was not understood that their functions were part of a shared cell biological property [2]. Specifically, EVs were observed as procoagulant platelet-derived particles in normal plasma, originally reported in 1946 by Chargaff and West, and referred to as ‘‘platelet dust’’ by Wolf in 1967 [2]. Around the same time, in 1969, Anderson described them as “matrix vesicles”, that were surrounded by a membrane and located in the matrix of cartilage. They were thought to be associated with calcification [3]. For years, EVs were detected in many biological fluids, and it was observed that they were secreted by different kinds of mammalian cells. However, it was initially believed that those vesicles were secreted from the cells through a mechanism that involved the outward budding of the plasma membrane. In 1983, it was demonstrated that vesicles are also released by multi-vesicular bodies (MVBs) fusing with the cell membrane, resulting in the release of intraluminal vesicles (ILV) into the extracellular space [4-6]. The term “exosome” was proposed by Johnstone et al. in 1987 to describe the released vesicles of endosomal origin [7]. However, for decades, exosomes were considered “garbage bags”, and they were thought to be the disposal of unwanted cellular components. In 2007, a breakthrough took place when it was discovered that exosomes, carrying mRNA and micRNA, were involved into the communication between cells, through the transfer of genetic material [8]. Today, EVs/exosomes have immerged as mediators of cell-to-cell communication and are receiving unprecedented scientific attention, whilst the research on them is growing exponentially.

1.1.1 Classification and nomenclature

EVs are extremely diverse in terms of their cargo, density and morphology. Because of this diversity, and the many subpopulations that exist, EVs have been categorized in different ways throughout the years. Today, it is commonly accepted that EVs are classified into three subcategories based on their biogenesis: exosomes, microvesicles or ectosomes, and apoptotic bodies [9]. Apoptotic bodies are shed from the plasma membrane of a dying cell during apoptosis, being the result of programmed cell death. They are the population with the largest size and the largest heterogeneity in size, ranging between 200 nm to 5 μm. [9] Microvesicles/ectosomes are considered to be formed from the plasma membrane of viable cells and are 100 to 800 nm in size [9]. Exosomes have the smallest size among the three categories of EVs, ranging between 30 nm- 150 nm, while their origin is endosomal. To release exosomes, several cellular steps need to be completed; formation of ILVs into MVBs, transport of MVBs to the plasma membrane and fusion of MVBs with the plasma membrane [10]. As knowledge concerning EVs is growing, it is observed that the diversity of EVs is larger than anticipated [11, 12]. This fact, as well as the overlap of size, morphology and density in various subpopulations of EVs, and the difficulty in isolating pure preparations using currently available techniques, have created the need for a different type of classification and nomenclature.

Therefore, a universal means of classification has recently been proposed [13]. In 2018, the International Society of Extracellular Vesicles proposed Minimal Information for Studies of Extracellular Vesicles (MISEV2018), updating the guidelines of 2014 (MISEV2014), based on the evolution of the collective knowledge in those four years [14].

“Since consensus has not yet emerged on specific markers of EV subtypes, such as endosome-origin “exosomes” and plasma membrane-derived “ectosomes” (microparticles/microvesicles) [14,15,16], assigning an EV to a particular biogenesis pathway remains extraordinarily difficult unless, e.g. the EV is caught in the act of release by living imaging techniques [14]. Therefore, unless authors can establish specific markers of subcellular origin that are reliable within their experimental system(s), authors are urged to consider use of operational terms for EV subtypes that refer to a) physical characteristics of EVs, such as size (“small EVs” (sEVs) and “medium/large EVs” (m/lEVs), with ranges

described, for instance, respectively, < 100 nm or < 200 nm [small], or > 200 nm [large and/or medium], or density (low, middle, high, with each range defined); b) biochemical composition (CD63+/CD81+- EVs, Annexin A5-stained EVs, etc.) or c) description of conditions or cell of origin (podocyte EVs, hypoxic EVs, large oncosomes, apoptotic bodies), in the place of terms such as exosome and microvesicle that are historically burdened by both manifold, contradictory definitions and inaccurate expectations of unique biogenesis [14]."

In conclusion, the definition of a generic means of classification is important in order for the scientific community to be able to exchange information, and discussions on these issues are on-going.

1.1.2 Biogenesis of exosomes

The identified proteins of exosomes are found in the cytocol, at the plasma membrane or the membrane of endocytic compartments. Furthermore, a lot of these proteins are involved in the endocytic pathway [17,18]. These are the reasons why it is currently believed that the origin of exosomes is endosomal. Exosomes are believed to be created by the endocytic invagination of the PM, and during this event they capture molecules and material from the cytoplasm of the cell [19]. There are two membrane inversions that are taking place during the formation of exosomes, and thus the membrane consistency of exosomes is similar to the plasma membrane of the producing cell. The first inversion happens with the endocytic internalization, while the second occurs when the ILVs are budding off into the lumen of the matured endosome [19]. The cargo in the multivesicular endosome (MVE) that is meant to be degraded by lysosomes, is undergoing ubiquitination. It is not completely understood how this MVE is rerouted for exosome release. It is believed that the endosomal sorting complex required for transport (ESCRT) mechanism is involved in this process. Particularly, Thery et al. suggested in a study that ALIX and TSG100 are involved in the release of exosomes in dendritic cells. ESCRT-I and ESCRT-II are thought to be mediators of the intraluminal membrane budding, and ESCRT-III in the formation of intraluminal vesicles [20]. A study by Trajkovic et al. is referring to another model of

biogenesis that involves a ceramide system into the formation of exosomes [21]. The Rab proteins, a family of small GTP binding proteins are considered to be mediating the intracellular trafficking [22]. Those proteins may regulate the fusion of the MVE with the plasma membrane, as well as SNARE proteins. Exosome release could also be affected by the calcium levels of the cell.

1.1.3 Molecular composition and physical characteristics of exosomes

Exosomes are too small to be observed under optical microscopy. Under TEM, they appear to have a cup- shaped geometry, but it is believed that this shape is an artefact due to the drying process. Their cargo can be specific and related to the parental cell, and the conditions of the secretion may affect it e.g. oxidative stress. Generally, they contain nucleic acids, proteins and lipids. It is not fully understood which mechanism is involved into the selection of cargo.

• Proteins

Proteomic analysis techniques have given us a detailed insight into the exosomal cargo. Early studies have indicated that exosomes contain proteins both dependent and independent of the cell type. On their surface and lumen there are specific marker proteins. Some of them are the tetraspanins (CD9, CD63 and CD81), and the heat-shock proteins (HSP70, HSP90). Furthermore, they contain molecules that are involved in the biogenesis of multivesicular endosomes, such as TSG101 and ALIX, and some proteins of the cytoplasm, such as annexins, Rab proteins, and actin [25]. These are molecules that may play an important role in various diseases. For example, heat-shock proteins are proven to have a cardio-protective molecule. The type of cell that releases the exosome, affects its proteome, and because of this characteristic it is feasible to identify the origin of the

exosome, based on the specific cellular markers that are expressed. For example, CD31 and CD62P are found in exosomes released by platelet cells [25]. There is a database named "ExoCarta" that contains useful information regarding the proteins, RNA and lipids that have been identified in exosomes [26].

• Lipids

The lipid composition of exosomes has not been studied extensively. Nevertheless, it has been shown that they contain numerous lipid species, such as sphingomyelin, cholesterol, phosphatidylserine, and saturated fatty acids [23,24].

• mRNA and microRNA (miRNA)

In 2007, Jan Lötvall *et al.* discovered that cells are exchanging genetic information through exosome-mediated mRNAs and miRNAs [27]. It was a breakthrough discovery in exosome research, and they also proved through in vitro experiments that mRNAs were not only present, but functional too. It was observed that mouse exosomal RNA, when transferred to human mast cells, could provoke the creation of new proteins in the donee cell [28]. This pivotal discovery demonstrated a new way of cell-to-cell communication. This mechanism appears to have similarities with viruses, where exosome-mediated transfer of RNAs, mRNAs and proteins is present. The mRNA and miRNA cargo, like protein cargo, is characteristic to the parental cell, and some conditions like cellular stress can affect the RNA content [29]. Consequently, exosomes could be an indicator of the physiological conditions of the cell they originated from. Various studies have demonstrated the capacity of exosomes to communicate with other cells via miRNA transfer [28,29]. Nevertheless, the range of the amount of plasma miRNA that is present in exosomes is under debate. There are studies suggesting that exosomes carry most of the plasma miRNA [31], and others that plasma miRNAs are mostly argonaute-bound [32].

1.1.4 Characterization of exosomes

There are multiple techniques that can be used for the characterization of exosomes and they are useful tools to describe their physical characteristics such as size, density, morphology and protein cargo. Their morphology can be determined by transmission electron microscopy (TEM), scanning electron microscopy (SEM), cryo- electron microscopy (cryo-EM) and atomic force microscopy (AFM) [33]. TEM and SEM can provide high-resolution imaging, and they can be combined with immunogold staining to locate specific proteins of the surface such as CD63 [34]. SEM can provide detailed information about the geometry of the surface [35,36]. An advantage of the cryo-EM technique is that the samples do not undergo extensive processing before the application of the analysis, in contrary to TEM and SEM techniques, and thus the vesicles can retain their physical state. AFM also enables visualization without the need of extensive preparation, and it provides information regarding the morphology of the surface, the adhesion properties and the density of exosomes. Other methods that can be used to determine the size distribution and quantification of exosomes in a suspension are nanoparticle tracking analysis (NTA), dynamic light scattering (DLS), and tunable resistive pulse sensing (TRPS). A method to define the density of exosomes is gradient centrifugation using sucrose or iodixanol. Western blot and flow cytometry can also be applied to detect selected exosomal proteins that act as markers, i.e. endosomal proteins, transmembrane proteins, cytosolic proteins or proteins specific for other compartments of the cell. A new flow cytometry technique has recently been developed and it allows detection of single vesicles [37].

1.1.5 Isolation of exosomes

There are many techniques that can be used for the isolation of exosomes. Some of them are differential ultracentrifugation, Size Exclusion Chromatography (SEC), filtration, immunoaffinity capture and precipitation [38,39]. The selection of the appropriate technique depends on the characteristics of the sample (e.g. cell culture or biological fluid, quantity of the material), the goal and type of the study. The most broadly used method

for isolation of exosomes is differential ultracentrifugation, with or without purification [40]. It is known that the size and the density of a molecule affect its sedimentation properties, and thus, different centrifugation rotation speeds can produce different preparations. In particular, a low speed is applied at the beginning to dissociate dead cells and cellular waste. Subsequently, a medium speed is selected in order to remove large vesicles. By using high centrifugation speed, small vesicles are finally isolated [41]. Different centrifugation stages are resulting in the isolation of different subpopulations of EVs, such as large vesicles (e.g. apoptotic bodies), middle-sized vesicles (e.g. plasma membrane-derived microvesicles), and exosomes. As it has been mentioned before, there is an overlap of vesicle populations in terms of their size and density, and this fact is a limiting factor to the sufficiency of this method. Furthermore, other molecules could contaminate the population, such as protein aggregates. This limitation could be overcome by using a density gradient, either iodixanol gradients or sucrose gradients [43]. The filtration and SEC techniques are also isolating particles based on their size. Moreover, by using the SEC technique it is feasible to remove soluble proteins, resulting in a preparation that is less contaminated [44,45]. Another advantage of this procedure is that it maintains the integrity of the vesicles, as it causes less damage and aggregates. An effective way to isolate exosomes is by using polyethylene glycol (PEG) [46]. The aqueous PEG could provoke the aggregation of exosomes, and then those aggregates could be precipitated using low-speed centrifugation. Immunoaffinity capture allows the isolation of exosomes with specific protein markers [43]. There is a great need to develop new isolation techniques, to optimize the existing ones or to combine them in order to overcome the drawbacks of the described methods. In this direction, there are innovative techniques that have been applied on exosome isolation, such as ion-exchange chromatography, microfluidics and acoustic isolation, asymmetrical flow field-flow fractionation (AF4), and immunoisolation.

1.1.6 Function of Exosomes

The mechanism of exosome biological function is not yet completely understood. Exosomes are released from a variety of cell types and it is known that they affect biological

processes to a great extent. It is believed that exosomes participate in mechanisms such as inflammation, immunosuppression and regulation and tumor progression.

• Inflammation and exosomes

The role of exosomes in inflammation is complicated. They could act as proinflammatory mediators or as immunosuppressive agents, dependent on the cell that they originate from or the conditions involved. Exosomes are known to be associated with pro-inflammatory proteins (HSP70, TGF-β) [47,48]. In a study, pro-inflammatory exosomes were found in bronchoalveolar lavage fluid (BALF) of patients with sarcoidosis [49]. In another study by Zhang et. al, exosomes were extracted from synovial fluid of rheumatoid arthritic patients, and they were shown to contain pro-inflammatory TNF-α, which delays the T cell activation-induced death, and aggravates their condition [50].

• Immune regulation by exosomes

The discovery that exosomes could possibly mediate immune responses has drawn great scientific attention to them. The condition of the parental cells appears to affect their immunological action. For example, exosomes that are released from mature dendritic cells that have received immunosuppressive treatments, induce immune tolerance [51,52]. Fas ligand (FasL) mediate, a T cell killing molecule, is found in exosomes from a variety of cell types. It is reported that exosomes released by the trophoblast during the early stages of pregnancy induce pregnancy tolerance through Fas mediated apoptosis of T cells [53,54]. Tumor cells secrete exosomes that carry the FasL molecule and it has been shown through in vitro experiments that it kills Jurkat T cells. This secretion seems to help tumor cells to suppress the immune system [55,56].

1.2 Stem Cells

1.2.1 Definitions and properties of stem cells

Stem cells are cells that have the capacity to self-renew and to differentiate into various specialized cell lines. They proliferate indefinitely and produce more cells of the same kind. Stem cells are different from other types of cells, and all of them, regardless of their source, are characterized by some general properties: they can divide and self-renew for long periods, they are unspecialized and they can give rise to specialized cell types [57]. Stem cells can be categorized based on their potency [167], in three main categories: embryonic stem cells, fetal stem cells, and adult stem cells.

Embryonic stem cells are stem cells derived from the inner mass cells of a human embryo, called blastocyst, and they are pluripotent, meaning they have the capacity to differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm, and hence, they can give rise to more than 200 specialized cell types of the body. Embryos of mammalian organisms contain two kinds of cells: of the inner cell mass (ICM), and of the trophectoderm (TE). The ICM cells will produce all the cell types, tissues and organs of the whole body. The TE cells will contribute to the formation of the placenta. In 1988 scientists developed a method to culture human embryonic stem cells (hESCs) in vitro, through the extraction of ICM cells of preimplantation human embryos. This practice resulted in a lot of controversy due to ethical reasons [161, 162]. In 2006, a pioneer work of some researchers resulted in the reprogramming of human adult stem cells into an embryonic stem cell-like state, known as induced pluripotent stem cells (iPSCs) [163].

Fetal stem cells are cells derived from a fetus and can be isolated from fetal blood, bone marrow, and fetal tissues, such as amniotic fluid, placenta, umbilical cord, among others. Fetal stem cells are more “matured” than embryonic stem cells.

Adult stem cells are found in small numbers in most adult tissues, such as bone marrow or fat. They have a more limited ability to give rise to various cells of the body, compared to embryonic stem cells, and thus they are called “multipotent”. They act as a repair system of the organism and provide replacements for cells in cases of injury, disease and other conditions. They include hematopoietic stem cells, mesenchymal stem cells, neural stem

cells, epithelial stem cells and skin stem cells. They can be found in many organs and tissues of the body. They can remain inactive for long periods of time, until activated by a stimulus, in which case they divide in order to repair and maintain tissues [164].

Stem cells have the capacity to self-renew

Unlike most cell types of the body that don't replicate, stem cells have the ability to replicate many times. The result of this proliferation could be either another stem cell, identical to the parental cell, or a more differentiated type of cell. The mechanism behind this selection is yet unknown, and if discovered, it would provide answers regarding the embryonic development, the aging mechanism, or even the development of specific diseases, such as cancer. It could also lead to efficient methods of culturing stem cells in the laboratory [165]. Pluripotent stem cells tend to differentiate into specific cell types, and it was a great challenge for scientists to create conditions in vitro where they would remain undifferentiated [166].

Stem cells have the capacity to recreate functional tissues

Pluripotent stem cells can give rise to all of the specialized cells in the body, such as blood cells, nerve cells or muscle cells. On the contrary, adult stem cells can differentiate into the specific cell types of the tissue or organ in which they are located. Different kinds of stem cells can differentiate into different number of cell types, a property known as "potency" [167]. Each stage of the differentiating process requires specific signals that trigger it, i.e. biological factors released by other cells, communication with neighboring cells and specific molecules and conditions of the microenvironment [168].

Stem cells are capable of dividing and renewing themselves for long periods

Stem cells can proliferate for long periods of time in the laboratory and by doing so they can yield millions of cells. Those cells can remain unspecialized and capable of long term-self-renewal, or differentiate and produce specialized cell types. The factors and specific conditions that are involved into this process of selection (to differentiate or remain unspecialized) is of great interest. Understanding the mechanism behind the activation of stem cells in order to differentiate and replace dead cells and damaged tissue will provide critical information, and it may allow the culture of large numbers of unspecialized stem cells in vitro for further research [169, 170].

Stem cells are unspecialized

Stem cells do not share the same properties or physical characteristics with neighboring cells and thus they cannot perform specialized functions, such as pump blood (heart muscle cell), or carry molecules of oxygen (red blood cell). Those undifferentiated cells can give rise to specialized cells, a process known as “differentiation” [170].

1.2.2 Stem cell-derived exosomes

Many of the regenerative properties and therapeutic potential of stem cells are attributed to their paracrine factors. It is believed that exosomes released from pluripotent and multipotent stem cells induce angiogenesis and cellular regeneration, prevent apoptosis, and regulate the immune system in chronically damaged tissues in living organisms and in vitro [58]. There are various advantages in using stem cell-derived exosomes in comparison to their parental cells. Exosomes do not produce an immune response, they are capable of crossing biological barriers, and they can be stored while retaining their functions. Moreover, there is no danger of mal-differentiation, a potential side effect of cell engraftment [59], teratoma formation or genetic modification that have been linked with iPSCs-based therapies [60,61]. Mesenchymal stem/stromal cells (MSCs), a type of adult

stem cells found in bone marrow, are generally considered safe for clinical applications, however, it is believed that their therapeutic potential could be attributed to the effects of their secreted factors. Exosomes are released by different types of stem cells, including MSCs, hemopoietic stem cells (HSCs), iPSCs and neural stem cells (NSCs) [62,63]. Furthermore, the interaction between exosomes and target cells is complex: cells that have been damaged and need repair release exosomes that carry miRNAs; stem cells internalize them and start to differentiate into specific cell types. On the other side, stem cells release microvesicles that interact with injured cells and induce their self-repair functions [64]. Stem cell derived exosomes are a promising tool in the treatment of various diseases, including inflammatory and autoimmune diseases.

2 Literature Review

2.1 Inflammation

Inflammation is a protective mechanism of organisms in response to external or internal stimuli, such as microbial infection, injury, long exposure to irritants or autoimmune disorders. This immune response of the host is essential in order to remove harmful stimuli and induce the healing process. There are two types of inflammation: acute and chronic. Acute inflammation is part of the innate immunity, and the first defense mechanism against microbial and other invaders, or trauma. The symptoms that accompany the acute inflammatory response are well known and include redness, swelling, pain and heat [65]. Chronic inflammation occurs when a stimulus persists for an extended period of time, and so the immune system continues to pump out white blood cells and chemical messengers that prolong the process. Those cells end up attacking nearby healthy tissues and organs.

Through the years it has been shown that inflammation is a much more complex system than first thought, and there are various regulatory mechanisms that mediate this process. Inflammation is involved in a great range of physiological or pathological mechanisms. Consequently, research in this field has grown tremendously over the last years, but the extend of its effects on human health is not yet completely unraveled [171].

2.1.1 Inflammatory diseases

Inflammatory diseases include a vast array of clinical disorders that are characterized by inflammation, and in particular, chronic inflammation, which is tightly linked to the pathogenesis of these disorders. For example, obesity can cause inflammation, and chronic inflammation can cause diabetes associated with obesity, due to insulin resistance [66]. In rheumatoid arthritis, the lining of the joint, known as the “synovium”, suffers from chronic inflammation due to the existence of macrophages and lymphocytes. As a result, synovial cells are triggered and they secrete synovial fluid. Consequently, billions of neutrophils invade the synovial fluid, further aggravating the chronic inflammation through enzymic

action of dead neutrophils [67]. Inflammation is a complex condition associated with a great range of diseases, such as neurodegenerative diseases (Parkinson's disease, Alzheimer's disease, multiple sclerosis), diseases of the digestive system (inflammatory bowel disease), or autoimmune diseases (arthritis, type 1 diabetes, psoriasis, cancer) among others.

2.1.2 Inflammation and exosomes

Among other properties, exosomes are thought to play a significant role in inflammation processes, thanks to their ability to carry proteins and miRNAs that act as modulators of inflammatory responses. Exosomes can modulate gene expression and cellular functions in close and distant cells. As it has been mentioned before, the physiological or pathological state of the parental cell can affect the components carried by exosomes, and thus exosomes are ideal candidates for novel biomarkers, as they can be collected with non-invasive techniques (blood, urine, saliva) [172]. They could be used as diagnostic and prognostic tools in various diseases, as therapeutic molecules, or to prevent the development of diseases, considering their immunoprotective and anti-inflammatory effects [173].

2.2 Stem Cell-derived exosomes and inflammatory diseases

The role of stem cells in the treatment of various inflammatory diseases has been studied extensively. Although many studies are showing promising results, there are others that refer to serious side effects occurring from stem cell therapy. In particular, many studies have proven that the use of MSCs can promote tumor growth. B16 melanoma cells are not capable of forming tumors, but this changed when they were co-injected with MSCs [77]. Human breast cancer cells have a synergistic effect with bone marrow-derived human MSCs in a mouse model, and they result in a more rapid development of metastases.

[78]. There are studies indicating that MSCs derived from various tissues such as bone marrow [79], periodontal ligament [80], and adipose tissue [81] could alleviate colitis. Nevertheless, there are concerns raised about stem cells, and specifically about their engraftment after injection to the body. Exosomes derived from MSCs have the regeneration and immune regulatory properties like MSCs, but they are less cytotoxic and bio-hazardous, and not so easily degraded [82]. They are also nonimmunogenic, and thus they present a lower risk of allogenic immune rejection from the host organism. Furthermore, as nano-sized particles, those molecules have the capacity to pass the blood-brain barrier.

Consequently, there is an urgent need for alternative approaches regarding stem cell therapy. Pivoting from cell transplantation techniques to cell free therapeutic approaches has been the subject of thorough investigation. To this direction, paracrine factors of stem cells, and especially exosomes, are showing promising results. This thesis investigates the potential role of stem cell-derived exosomes in the treatment of many inflammatory diseases, as an alternative and promising therapeutic approach. Up to date, there are no full-scale clinical trials using stem-cell derived exosome-based treatments, and hence, the results presented below are obtained through in vitro experiments or in vivo animal-based studies.

2.2.1 Gastrointestinal tract

- *Inflammatory Bowel Disease*

Inflammatory bowel disease (IBD) is a general term used to describe disorders that involve chronic inflammation of the gastrointestinal tract. IBD typically includes ulcerative colitis (UC) and Crohn's disease (CD). Symptoms include diarrhea, rectal bleeding, abdominal pain and distension, anorexia and emesis. The number of IBD patients has dramatically increased over the last decade, and it is currently classified as the second most prevalent (after rheumatoid arthritis) chronic inflammatory disease [174]. The exact cause of IBD remains unknown, nevertheless it is believed that there are a lot of factors that affect the pathogenesis and severity of the disease, such as the diet, the

environment, immunological conditions and genetic background, but to name a few [174]. There is no successful etiological treatment up until today, only symptomatic but unsatisfactory, and new therapeutic approaches are needed.

There is growing interest regarding the effects of stem cell-derived exosomes in the treatment of IBD and the research conducted exhibits promising results. MSCs are adult stem cells located in bone marrow, fat (adipose tissue), and there are also fetal MSCs in the umbilical cord tissue (Wharton's Jelly), or amniotic fluid. Their role is to repair skeletal tissue, such as bone, fat found in bone marrow and cartilage. There is growing evidence suggesting that MSCs hold therapeutic potential in the treatment of IBD and other diseases, however studies have shown that their therapeutic efficacy is mainly attributed to their paracrine factors and not to their own engraftment on the target tissue [68,69].

It has been proved that exosomes secreted by human umbilical cord MSCs cells (hucMSCs) can ameliorate dextran sulfate sodium (DSS)-induced IBD in mice [70]. In particular, it is known that in IBD the chronic inflammation of the gastrointestinal tract results in the infiltration of inflammatory cells, mainly macrophages, and when they are stimulated they release cytokines and enzymes, that further aggravate the injury of the intestine [71]. Both the expression of iNOS and infiltration of macrophages to the injured tissues were lower in IBD mice treated with exosomes [70]. Furthermore, this treatment decreased the expression of proinflammatory cytokines (TNF-a, IL-1β, and IL-6) and increased the expression of anti-inflammatory cytokine IL-10 [70]. Moreover, exosomes from hucMSCs were found to suspend the expression of IL-7 pleiotropic cytokine in the colon mucosa tissues and spleens, and in peritoneal macrophages of IBD mice, indicating the suppressive role of exosomes on the expression of IL-7 cytokine in macrophages [70].

Another study indicated that there is a synergistic effect of melatonin and adipose-derived MSCs (ADMSC)-derived exosomes on amelioration of DSS-induced acute inflammatory colitis (AIC) in rats [72]. Specifically, the combination of exosome and melatonin therapy caused a reduction of circulating inflammatory mediators (such as inflammatory cells and cytokines), improved permeability and injury of the colon, and suppressed the inflammatory reaction and the generation of oxidative stress [72].

Yang *et.al* proved that IFN-γ treatment assists MSCs-derived exosomes to attenuate colitis by increasing the level of miR-125a and miR125-b which bind on Stat3 to repress Th17 cell differentiation [73]. In a recent study, EVs derived from inflammatory cytokines (TNF-α and IFN-γ) pretreated canine stem cells showed therapeutic potential in a murine colitis model by enhancing the regulation of immune cells [74]. It is known that chronic

inflammation of the gastrointestinal tract can result in the development of colitis-associated cancer (CAC) triggered by oncogenic factors [75]. The use of MSCs are showing promising results in the reduction of the protracted inflammation resulting to cancer, by the regulation of Treg cells [76]. However, it is possible that paracrine factors of MSCs were involved in this mechanism.

Another study investigated the role of bone marrow MSCs (BMSCs) derived EVs in an ulcerative colitis model. Their findings suggested that BMSCs EVs promoted M2-like macrophage polarization and relieved inflammatory responses, improving DSS-induced UC [83]. When MSC-Exos were systematically infused during the onset of the disease, it was observed that the severity of acute colitis was reduced, and their administration also ameliorated the severity of chronic colitis [83]. Ubiquitination is a mechanism involved in the regulation of many physiological and pathological processes, and it is also involved in the regulation of inflammation. It has been shown that this process is used by exosomes derived from umbilical cord MSCs to alleviate IBD in mice [84]. It is suspected that by regulating the ubiquitin modification level, hucMSC-ex treatment resulted in the recovery of the integrity of tissue structure [84]. HucMSC-Ex treatment also relieves DSS-induced IBD in a mouse model by inhibiting neddylation through miR-326 [85].

Yang *et. al* showed that EVs derived from bone marrow MSCs can improve colitis in mice through inhibition of inflammation and oxidative stress [86]. Most particularly, in this experiment BMSC-EVs down regulated levels of pro-inflammatory cytokines, inhibited NF-κBp65 signal transduction pathways, modulated anti-oxidant and oxidant balance, and moderated the apoptotic mechanism [86]. Another study suggests similar results. EVs derived from human placental MSCs were tested for the treatment of IBD and the results showed that when they were injected in situ, they alleviated hapten reagent 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis by inhibiting inflammation and oxidative stress [86]. In particular, the injection of EVs regulated the balance of pro-inflammatory and anti-inflammatory cytokines, it suppressed oxidative stress by decreasing the activity of myeloperoxidase (MPO) and ROS, and promoted mucosal healing [86].

An interesting experiment confirmed the theory that the anti-inflammatory properties of stem cells are mainly a result of their secretome. Specifically, tonsil-derived MSCs (TMSC) were injected intraperitoneal in acute and chronic murine colitis models and improved the disease activity index, the length of the colon and the expression levels of proinflammatory cytokines [87]. Nevertheless, during the experiments TMSC weren't observed to the inflammation site of the intestine, and thus further research was

conducted to investigate whether the effects of TMSC were actually the effect of conditioned medium (CM) secreted by those cells (TMSC-MC) [88]. It was observed that TMSC-CM presented equivalent efficacy compared to TMSC, regarding the overall improvement of inflammation. Similar results were obtained in a study with MSCs-derived exosomes in a defined medium, and their anti-inflammatory properties were proved more efficient than those of MSCs [89].

Consequently, there is strong evidence suggesting that a cell free therapeutic approach for IBD patients may be possible in the future, although more data is needed in order to support this suggestion.

- *Periodontitis*

Periodontitis, also called “gum disease” is the result of chronic inflammation of the periodontium due to bacterial infection. It damages the soft tissues and bone that supports the teeth. In periodontitis, the increase of proinflammatory macrophages and the decrease of anti-inflammatory macrophages is a factor that contributes to the pathogenesis of the disease.

Dental pulp stem cell-derived exosomes (DPSC-Exo) are thought to be an efficacious treatment for periodontitis. A study investigated the effectiveness of DPSC-Exo-incorporated chitosan hydrogel (DPSC-Exo/CS) and it was shown that the hydrogel can induce the healing of periodontal epithelium and alveolar bone in a murine model [90]. In particular, DPSC-Exo/CS inhibited the conversion of pro-inflammatory macrophages to an anti-inflammatory phenotype, which could be a result of miR-1246 found in DPSC-Exo [90].

Moreover, MSC-derived exosomes are thought to induce regeneration of periodontal tissue and ameliorate periodontal ligament cell functions [91]. Human MSC exosome-loaded collagen sponge was used to investigate their effect to surgically created periodontal defects in rats [91]. It was observed that in treated rats there was significant regeneration of periodontal tissues (newly formed bone and periodontal ligament), and increased cellular infiltration and proliferation [91].

Wang *et. al* evaluated the role of gingival MSCs (GMSC) exosomes in macrophage polarization under inflammation, and the results have shown that they may induce M1 macrophage transformation into M2 macrophages, causing a reduction of the pro-

inflammatory factors produced by M1 macrophages [92]. Consequently, GMSC reduce inflammation and could hold potential therapeutic effects in the treatment of periodontitis, although their efficacy in this disease has not been studied yet.

2.2.2 Nervous system

Stem cell-derived exosomes are proven to be effective in the treatment of various neurodegenerative disorders, such as Alzheimer's Disease, Parkinson's Disease, Multiple Sclerosis, and other conditions of the nervous system, including stroke and brain injury, among others. They seem to exert their therapeutic potential by promoting neuroprotection, neurogenesis, neuromodulation and angiogenesis. In many studies, it was demonstrated that they can induce the restoration of damaged neurons and prevent cell death and apoptosis. It is also believed that stem cell-derived exosomes reduce oxidative stress, which can aggravate many diseases, as well as microglia-induced neuroinflammation, while their immunoregulative properties are useful in the treatment of neurodegenerative disorders.

- *Alzheimer's Disease*

Alzheimer's disease (AD) is a progressive disorder that causes the degeneration of brain cells and is characterized by cognitive decline. It is the main form of dementia and the pathology of the disease involves the presence of amyloid β plaques and tau-enriched neurofibrillary tangles. In the past decade, a third pathological factor is under investigation: the presence of an immune response of the brain. When brain's macrophages (microglia) and other cells of the immune system are activated, they exacerbate amyloid and tau pathology, which could be involved in the pathogenesis of the disease [93]. There are many studies indicating that inflammation plays a significant role in the pathogenesis of AD. While life expectancy is rising, an increasing number of people are affected by the disease, imposing a great financial burden to health system. Consequently, there is an urgent need for the development of novel therapeutic approaches for AD.

The efficacy of stem cell treatment has been investigated in preclinical studies, giving discord results, which have limited the clinical development of this therapeutic plan. Hence, there is a growing need for robust therapeutic approaches. There is a growing number of studies regarding the role of stem cell-derived exosomes in the treatment of AD, most of them suggesting that they hold great potential, with their donor-derived properties, easy storage, low immunogenicity and low risk of tumor development.

In AD, amyloid-β peptide (Aβ) is accumulated in the brain and mediates the neuroinflammatory process. The activation of brain's macrophages regulates the immune response of the nervous system. Human umbilical cord MSC-derived exosomes (hucMSC-exosomes) are mimicking the therapeutic properties of hucMSCs in various inflammatory diseases. In a study, hucMSC-exosomes were used into AD mouse models, and it was observed that they improved cognitive functions, reduced the deposition of Aβ, and modulated the activation of microglia [94]. Moreover, they regulated inflammatory cytokines in peripheral blood and brains of mice, and their immunoregulating effects were also sustained in vitro [94].

One of many factors that is believed to accelerate the pathogenic process in AD is neuroinflammation. The immunomodulatory properties of MSCs are well known, and they are mainly attributed to the secretion of EVs. There are many studies reporting the MSC-EVs properties in regulating inflammatory responses, in which they were administered intravenously or via intracerebroventricular administration in AD mouse models. However, some interesting studies are referring to the intranasal route of administration. In particular, EVs derived from MSCs that were cytokine-preconditioned were administered intranasally to AD mouse models, and they induced immunomodulatory and neuroprotective effects [95]. MSC-EVs crossed the blood brain barrier and reached the brain, where they moderated the activation of microglia cells and they increased dendritic spine density [95]. When tested in vitro, they polarized primary microglia cells into an anti-inflammatory phenotype, which means that their neuroprotective effects could arise from a positive modulation of the inflammatory status [95]. Another study showed similar results, in which secretome collected from MSC exposed in vitro to AD mouse brain homogenates (MSC-CS) was instranasally administered to mice [96]. It was observed that when administered weekly, MSC-CS induced recovery of memory and reduction of plaques, lowered the density of β-amyloid oligomers, and decreased gliosis and the phagocytic marker CD68 [96]. This noninvasive route of administration along with the anti-inflammatory properties of stem cell-derived exosomes, could lead in easier translational exploitation in AD patients.

A problem regarding the use of MSC-Exo in AD, is that when injected intravenously, they could be tracked in other tissues and not on the targeted regions of the brain. Hence, a study proposed a way to overcome this pitfall. Specifically, central nervous system-specific rabies viral glycoprotein (RVG) was conjugated with MSC-Exo through a DOPE-NHS linker, and the brain of transgenic APP/PS1 mice was targeted [97]. The results demonstrated increased delivery of exosomes to the desired site, an amelioration in cognitive functions, a reduction in plaque deposition and Aβ levels, and moderated levels of inflammatory cytokines [97].

Adipose-derived stem cells (ADSC) could be used in the therapy of neurodegenerative diseases such as AD. ADCS-Exosomes have shown modulating effects in AD in vitro models, by ameliorating the progression of Aβ-induced neuronal death [98]. Also, ADSC-Exo are carrying enzymatically active neprilysin, which is the most important β-amyloid peptide (Aβ)-degrading enzyme in the brain, and this degrading capacity holds therapeutic potential against AD [98].

Neural stem cells also secrete exosomes. Exosomes secreted by neural stem cells under the presence of heat-shock were isolated, and it was observed that they were larger in size and showed higher concentration compared to neural stem cell-derived exosomes that were isolated under physiological conditions [99]. When those cells were administered to an AD mouse model, they improved cognitive and motor function, and their overall neuroprotection properties were higher [99]. It seems that under heat-shock stimulus, the exosomal cargo and morphology is altered, although the mechanism behind those characteristics remains unknown. Another study investigated the role of neural stem cell (NSC)-derived EVs in a mouse model of AD, and it was observed that they improved cognitive deficits, mitochondrial function, synaptic activity and SIRT1 activation, and decreased inflammatory response [100].

MSC-derived EVs are believed to have therapeutic effects in various neurological diseases, such as stroke, multiple sclerosis and hypoxic-ischemic brain injury. Their efficacy in the treatment of AD was evaluated in a study. Specifically, intracerebroventricle injection of MSC-derived EVs was administered to mice, and it was demonstrated that their use significantly reduced Aβ induced iNOS expression, ameliorated cognitive behavior and rescued impairment of CA1 synaptic transmission [101]. Soluble oligomers of the amyloid-β peptide (AβOs) are involved in neuronal oxidative stress and synapse damage, causing neurodegeneration and deterioration of cognitive functions in AD [102]. The neuroprotective potential of MSCs and MSC-derived EVs against the damaging effect of AβOs on nerves of the hippocampus was evaluated [102]. It was shown that both MSCs

and MSC-derived EVs protect neurons against AβO-induced oxidative stress and damage of the synapses through different mechanisms: loss of pre- and postsynaptic markers; internalization and degradation of AβOs; release of EVs containing active catalase; and secretion of IL-6, IL-10 and vascular endothelial growth factor to the medium [102].

Furthermore, bone marrow-derived MSCs (BM-MSCs) have been proven to ameliorate cognitive function in AD murine models by improving astrocytic inflammation and synaptogenesis [103]. When BM-MSCs were injected intracerebroventricularly, they attached to the choroid plexus in the lateral ventricle, and thus, it is hypothesized that BM-MSCs secrete exosomes into the cerebrospinal fluid [103]. Astrocytes internalized miR-146a secreted by BM-MSC-derived exosomes, and it was observed that the level of NF-κB in them was decreased [103]. This improvement of astrocytic function could be the key mechanism behind synaptogenesis and the overall amelioration of cognitive impairment. BM-MSC-derived exosomes were also proven efficient in improving the destructive structural changes in the taste buds, which is also a clinical manifestation of AD [104].

The reduction of Aβ accumulation could be used as a therapeutic mechanism for treating AD. In an interesting study, human umbilical cord mesenchymal stem cells (hucMSCs) were cultured in both 3D graphene scaffold and 2D graphene film that were used as a matrix [105]. Subsequently, the supernatant that included secreted exosomes was collected. It was observed that exosomes obtained from 3D culture were dramatically different in terms of their cargo than those obtained from 2D cultures. 3D-Exo could up-regulate the expression of α-secretase and down-regulate the β-secretase in order to decrease the production of Aβ in AD experimental models [105]. 3D-Exo improved memory and cognitive deficits in AD mice [105].

Moreover, it is reported that exosomes derived from DPSCs can reduce cytotoxicity and apoptosis caused by Aβ peptide [106]. This study showed that the secretome of DPSCs contains higher concentrations of VEGF, Fractalkine, RANTES, MCP-1, and GM-CSF than the secretome of bone marrow and adipose stem cells [106].

- *Multiple sclerosis*

Multiple sclerosis (MS) is a chronic inflammatory, autoimmune and demyelinating disease of the central nervous system (CNS). The neurological disability is the result of neuronal

injury caused by the attack of autoreactive T cells to CNS. There are multiple medications available for the treatment of MS, nevertheless, none of them can stop disease progression or reverse neuronal damage. In preclinical studies stem cells have shown immunomodulatory and immunosuppressive properties and they could be used in the treatment of MS. Nevertheless, there are some challenges in stem cell therapy, such as mal-differentiation, tumor formation and lung entrapment. Exosomes secreted from stem cells don't present these drawbacks and it makes them an attractive alternative for the treatment of neurodegenerative disorders such as MS.

In MS patients, the progression of the disease depends on microglia function and balance in their M1/M2 phenotypes (M1/M2). There is evidence supporting that bone marrow mesenchymal stem cell-derived exosomes (BMSC-Exo) hold therapeutic potential in many autoimmune diseases and they have regenerative effects in damaged tissue. The effects of exosomes on microglia polarization and inflammation in CNS were investigated in an experimental autoimmune encephalomyelitis (EAE) rat model [107]. It was shown that BMSC-Exo can reduce inflammation and demyelination of the CNS in an in vivo rat model, through modulation of inflammatory cytokines and polarization of microglia respectively [107].

Furthermore, MSC-Exo can reduce the inflammatory response of lymphocytes, and increase Treg engraftment, which leads to a significant reduction of clinical symptoms in a murine model of relapsing remitting MS [108]. They also improve motor function, reduce damage in oligodendroglia populations, and increase myelination in the spinal cord of treated EAE murine model, by inducing endogenous oligodendrocyte precursor cells to differentiate into mature myelinating oligodendrocytes [109].

In conclusion, stem-cell derived exosomes is a novel and promising therapeutic alternative in the treatment of degenerative disorders such as Alzheimer's disease, Multiple Sclerosis, compared to cell-based treatment that has not been proven effective. Additional studies in this field are required, and efficacy as well as safety standards should be established in order to translate the promising results into clinical applications.

2.2.3 Autoimmune disorders

- *Arthritis*

Arthritis is characterized by pain and inflammation in a joint. The most common types of arthritis are rheumatoid arthritis (RA) and osteoarthritis (OA). For many years, it was believed that only rheumatoid arthritis is associated with inflammation, when osteoarthritis was considered non-inflammatory. Nevertheless, there are studies indicating that inflammation plays a significant role in the pathogenesis and progression of osteoarthritis as well [111, 112]. RA is considered an autoimmune disorder, when OA is classified as a degenerative disorder that occurs overtime due to breakdown of the cartilage and the bones. The use of stem cell-derived exosomes in the treatment of both conditions is undergoing extensive research, and the results are quite promising.

Stem cell therapy in rheumatic diseases has been thoroughly investigated over the last years, and some studies have progressed to the clinical level. However, there is growing interest considering the effects of stem cell-derived extracellular vesicles in the treatment of OA and RA, due to their sometimes-improved efficacy and low immunogenicity. MSCs and MSC-derived exosomes induce repair and regeneration of tissues, promote chondrogenesis, and they have immunomodulatory effects via regulating immune cells and inflammatory factors. Exosomes appear to have more advantages over their parental cells and thus they are a promising cell-free approach in the treatment of those diseases.

There are many animal model-based studies indicating that MSC-derived exosomes could be efficient in the treatment of OA. Exosomes derived from human embryonic stem cells induced repair of the cartilage by increasing proliferation, reducing apoptosis and modulating the reaction of the immune system in a murine model with osteochondral defects that were created on the trochlear grooves [113]. Exosomes regulated the infiltration of M1 and M2 macrophages, with a concomitant decrease in inflammatory cytokines, while also affecting the chondrocyte proliferation and migration, and matrix synthesis [113]. Zhang et al. also compared the effects of hEMSC- derived exosomes and phosphate buffered saline (PBS) in a rat model with the same defects as the previous study. The appearance of exosome-treated defects was enhanced, which was confirmed with histological evaluation, while cartilage and bone were repaired, compared to PBS-treated defects [114].

In another study, the efficacy of induced pluripotent stem cell-derived MSCs (iMSC-Exos) was compared to the efficacy of synovial membrane derived MSCs (SMMSC-Exos) in a

murine OA model induced by collagenase. The study suggested that while both iMSC-Exos and SMMSC-Exos induced chondrocyte proliferation, iMSC-Exos were more efficient in the attenuation of OA. Specifically, histological evaluation demonstrated that the cartilage in iMSC-Exos group was similar to normal cartilage. This observation was further confirmed by immunohistochemistry analysis, which indicated that both in the iMSC-Exos group and in the control group the expression of collagen II was similar [115]. The effect of bone marrow-derived MSC-Exos (BM-MSC-Exos) was investigated in another collagenase-induced OA model in mice. It was shown that BM-MSC-Exos reduced the apoptosis of chondrocytes, increased the differentiation of macrophages and suppressed the immune system, which resulted in the overall protection of cartilage and bone [116].

As it has been mentioned before, the dimensional environment of exosomal cultures could affect their efficacy and functions. Jarmalavičiūtė et al. demonstrated that exosomes secreted from cells that are cultured in a 3D environment present enhanced biological functions, and are more productive and active than those grown in a typical 2D culture [117]. Another study suggested that exosomes collected from 3D cultures of umbilical cord MSCs (U-MSCs) in a hollow-fiber bioreactor demonstrate enhanced osteochondral regeneration activity [118]. Those exosomes presented improved chondroprotective properties by inducing proliferation, migration and matrix synthesis of chondrocytes and reducing apoptosis [118].

Some studies are focusing on reinforcing exosomes' existing functions. For example, in a meniscus OA rat model, exosomes derived from synovial MSCs that were overexpressing miR-140-5p, were shown to ameliorate proliferation and migration of the chondrocytes and they prevented the development of OA in the knee [119]. Similarly, hEMSCs-derived exosomes can improve regeneration of the cartilage after injection in a meniscus OA murine model [120], while EVs secreted from the same type of cells can inhibit the progression of the disease in a rat model with osteochondral defects [121]. In another study, chondral defects were surgically created in the femoral condyles of nine dogs and the injection of mouse BMSC-derived EVs led to the recovery of injured cartilage and chondral tissue [122]. Furthermore, in a collagenase induced murine arthritis model, the immunomodulatory function of MSC-derived exosomes and microparticles was compared, and it was shown that both of them present anti-inflammatory effects on T and B lymphocytes, however Exos were proven more efficient in suppressing inflammation in vivo [123].

Exosomes derived from human infrapatellar fat MSCs are rich in miR-100-5p, and when they were injected in the articular joint of OA mice, the progression of the disease was

restrained, articular damage was improved and gait was ameliorated, via inhibition of mTOR autophagy pathway [124]. Another study suggested that amniotic fluid stem cell-derived exosomes have therapeutic effects in OA in a rat model, and more specifically, they increase pain tolerance and improve histological score [125]. After three weeks of treatment, exosomes that were rich in TGFβ led to the restoration of cartilage, with good surface regularity and with the characteristic of hyaline cartilage [125]. Moreover, after treatment, a significant increase in markers of resolving macrophages (CD163, arginase 1, and TGFβ) was observed [125].

It is believed that miRNAs are involved in the pathogenesis of rheumatoid arthritis. As mentioned before, exosomes are carriers of genetic information from a cell to another, such as miRNAs, and they have been studied as potential vehicles of therapeutic molecules. MiR-146 and miR-155 are considered regulators of primary immune response. Tavasolian *et.* al investigated the therapeutic effect of miR-146a/miR-155 transduced MSC-derived exosomes on the immune response [126]. Exosomes were collected from MSCs over-expressing miR-146a/miR-155, and splenocytes were isolated from collagen-induced arthritis (CIA) and control mice [126]. Then, the effects of those exosomes on regulatory T-cell (Treg) levels was measured [126]. The results showed that exosomes seem to promote the transfer of miRNAs among cells, making them candidates for the treatment of RA. MSC-derived exosomes that are manipulated with anti-inflammatory miRNA could lead to the increase of Treg cell populations and anti-inflammatory cytokines [126].

A previous study was the first to mention that BM-MSC-derived exosomes can transfer mir-150-5p to the joints. Chen *et.* al demonstrated the therapeutic effect of MSC–derived miR-150-5p exosomes on joint destruction in RA [127]. In this study, exosomes from miR-150-5p–expressing MSCs were isolated, and their effects were examined on fibroblast-like synoviocytes (FLS) from patients with RA both in vitro and in vivo in a CIA mouse model [127]. Analysis of patients with RA and FLS from RA showed that miR-150-5p expression was significantly lower in RA than in OA, whereas vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP) 14
expression and angiogenesis were increased in RA compared with OA [127]. MiR150-5p was transferred to BM-MSCs and subsequently transferred by exosomes to RA-FLS. Exo-150 suppressed the expression of the target genes MMP14 and VEGF; therefore decreased migration and invasion, inhibited angiogenesis in vitro, and alleviated the symptoms of RA in vivo [127].

Recently, it was found that the expression of miR-192-5p was decreased in RA-FLS, and it was shown that miR-192-5p targeted and down-regulated C3 botulinum toxin substrate 2 (RAC2). Specifically, MSC-exosomal miR192-5p was injected in a CIA rat model, reached the synovial tissue via the blood circulation, and decreased the levels of RAC2, reduced the clinical score and inhibited hyperplasia of the synovium and joint degeneration [128]. In addition, MSC-exosome-miR-192-5p down-regulated the levels of proinflammatory cytokines in synovial tissues and serum and decreased the release of NO and inducible NO synthase (iNOS) in the serum of rats [128].

The effect of MSC-derived exosomes loaded with microRNA-320a (miR-320a) on the regulation of RA-FLSs was evaluated [129]. In a CIA mouse model with arthritis and bone damage, in the presence of miR-320a, CXC chemokine ligand 9 (CXCL9) was downregulated and led to the restoration of RA-FLS function, via suppressing activation, migration and invasion of RA-FLSs [129]. Consequently, MSC-derived exosomes participate in the transfer of miR-320a and inhibit the progression of RA [129]. In another in vitro experiment, it was found that when MSC-derived exosomes overexpressing miRNA-124-a were co-incubated with hMSC-124a-EV, they could inhibit cell proliferation, migration and promote apoptosis in FLS cell line [131].

- *Diabetes*

Diabetes is a chronic metabolic disorder affecting the glucose levels in the bloodstream. Hyperglycemia as a result of diabetes could lead to end organ failure. There are two broad categories of diabetes, type 1 and type 2 diabetes. Some predisposing factors of the disease include obesity, diet, genetic and environmental factors. Scientists have been focusing on researching the role of adipose tissue, pancreatic beta cell function and gut microbiome in the pathogenesis of the disease, and many clinical trials are in progress. There is growing interest in the role of inflammation in the pathophysiology of both types of diabetes, and targeting inflammation to prevent or control the disease is an emerging goal. Consequently, future research should focus on finding ways to suppress the inflammatory responses that are involved in T1D and T2D, and exosomes, with their immunomodulatory and regenerative capacity, could play a significant role towards this direction.

Exosomes derived from stem cells are known for their immunomodulatory effects in various autoimmune and inflammatory diseases, and experiments show that they can

also be used in the treatment of diabetes. A study demonstrated that exosomes derived from adipose tissue-derived mesenchymal stem cells (AD-MSCs) have immunomodulatory effects of T-cell inflammatory response, and thus they reduce the symptoms of streptozotocin-induced type-1 diabetes mellitus in a mouse model [132]. Intraperitoneal injections of autologous AD-MSC-derived exosomes were performed, and in the treated group of mice it was observed that interleukin-4 (IL-4), IL-10, and transforming growth factor-β increased, whereas IL-17 and interferon-γ decreased, in concordance with the significant increase in the Treg cell ratio in splenic MNCs, compared with T1DM untreated mice [132]. An increase of islets was also observed, while body weight and glucose level remained stable in the two groups [132]. Consequently, AD-MSC's exosomes treatment led to the amelioration of autoimmune reaction, which makes them candidates for the treatment of type 1 diabetes. In another study, the effects of MSC-derived EVs in two autoimmune diseases were evaluated: type 1 diabetes and uveoretinitis [133]. It was concluded that EVs secreted from MSCs effectively prevent the onset of the diseases, by inhibiting the activation of antigen-presenting cells and suppressing the development of T helper 1 (Th1) and Th17 cells [133].

Stem cell-derived exosomes present promising results in the treatment of type 2 diabetes as well. Specifically, hucMSC-derived exosomes were tested in a rat model of T2DM, induced by a fat diet and streptozotocin [134]. The intravenous injection of exosomes reduced the glucose levels in the blood, partially reversed peripheral insulin resistance and inhibited β-cell destruction, by restoring the phosphorylation (tyrosine site) of the insulin receptor substrate 1 and protein kinase B, increasing glycogen storage in the liver, and promoting expression of glycose transporter 4 in the muscles [134]. Another study evaluated the effects of hUCMSC-derived exosomes on hepatic glucose and lipid metabolism in T2DM in vivo model. It was demonstrated that hUCMSC-derived exosomes improved hepatic glucose and lipid metabolism through the activation of autophagy via the AMPK pathway [135].

There are studies aiming at the restoration and regeneration of β-cells of pancreatic islets, the main cells that are undergoing mass and function diminish in diabetic patients. A recent study presented the effects of menstrual blood mesenchymal stem cell-derived exosomes in the pancreas of diabetic mice [136]. Specifically, exosomes derived from menstrual blood MSCs enhanced the β-cell mass and the insulin production, while the presence of insulin in the pancreatic islets was confirmed through immunohistochemistry analysis [136]. It is believed that the regenerative effects of exosomes in the islets is a result of pancreatic and duodenal homeobox 1 pathway [136].

The current treatment options of diabetes mellitus include life-long administration of insulin and other medication. Despite advances in the therapeutic plan, more invasive solutions like islet transplantation pose important difficulties and thus they are not practical for most patients. However, the use of exosomes could ameliorate the success rate of these operations. An islet transplantation is defined as unsuccessful if there is an immune rejection or poor islet function. As it has been mentioned before, hBMSC-derived exosomes can suppress immune reaction and regulate cell-to-cell communication by delivering RNAs and other molecules between cells. A study proposed their use for promoting islet function and inhibiting immune rejection after transplantation [137]. It was found that these exosomes suppressed the reaction of the immune system by ameliorating the function of regulatory T cells (Treg) and inhibiting the proliferation of peripheral blood mononuclear cells (PBMC) [137]. Another reason for dysfunction of beta cells after transplantation is hypoxia. The role of MSC-derived exosomes on beta cells under hypoxic conditions was investigated in a study. Hypoxia caused significant apoptosis of beta cells, but the presence of MSC-Exo improved their survival. The mechanism behind this action includes the inhibition of p38 MAPK signaling pathway, the alleviation of ER stress and the downregulation of apoptosis-related proteins that are upregulated in hypoxia, by carrying miR-21 [138].

Stem cell-derived exosomes could also be used to prevent or repair damage caused by complications of diabetes mellitus, such as impaired wound healing, peripheral neuropathy, nephropathy, retinopathy and cardiovascular disease. Wound complications are common among diabetic patients, and some of the causes include high blood glycose level, neuropathy, poor circulation, immune system deficiencies, and infection, affecting the function of endothelial cells. A great number of studies suggest that stem cell-derived exosomes can improve diabetic wound healing. It was shown that human urine stem cell-derived exosomes can promote angiogenesis via transferring a pro-angiogenic protein called deleted in malignant brain tumors 1 (DMBT1) [139]. Diabetic foot ulcers (DFU) could result in infection and amputation. The malfunction of endothelial progenitor cells (EPCs) and high glucose-induced ROS aggravate the disease. The therapeutic effects of adipose stem cell-derived exosomes (ADSC-Exos) on stress-mediated senescence of EPCs induced by high glucose were evaluated in a study [140]. The results suggested that ADSC-Exos promoted proliferation and angiopoiesis in EPCs, and overexpression of Nrf2 accentuated this effect [140]. In vivo, ulcerated areas in the feet of diabetic rats were reduced, and test on wound beds confirmed increased levels of growth factors, granulation tissue formation and vascularization, and reduced levels of inflammation and oxidative stress-related proteins [140]. Furthermore, stem cell-derived exosomes could be combined with chitosan to improve their regenerative effects on injured tissue. A

study demonstrated the therapeutic effects of chitosan wound dressings incorporating exosomes derived from synovium MSCs overexpressing microRNA-126 [141], and another study assessed the effects of gingival MSC-derived exosomes combined with a chitosan/silk hydrogel sponge [142], both on skin defects in diabetic rat models. According to the above studies, the combined therapy ameliorated skin wound healing, by promoting re-epithelialization and collagen maturity, activating angiogenesis and neuronal ingrowth [141,142].

Non-healing diabetic wounds require novel strategies such as stem cell therapy or delivery of miRNAs to the wound site; however, miRNA is easily degradable in the injured tissue. To overcome this pitfall, engineered stem cell-derived exosomes could act as delivery systems of molecules, such as miRNAs, to the wounds. A similar delivery system was developed using human adipose stem-cell derived exosomes, and miR-21-5p mimics were loaded in them by electroporation [143]. The engineered exosomes significantly promoted proliferation and migration of keratinocytes via Wnt/β-catenin signaling in vitro, and when tested in vivo, they induced diabetic wound healing [143]. Another technique to enhance the biological functions of stem cell-derived exosomes is to pretreat the parental cells with chemical or biological factors, such as atorvastatin. Bone marrow MSCs were pretreated with atorvastatin, and their secreted exosomes exhibited improved pro-angiogenic ability in diabetic wound healing [144]. Specifically, they ameliorated the function of endothelial cells, via AKT/eNOS pathway, by upregulating the miR-221-3p [144].

Many different types of stem cells are exhibiting promising results in the treatment of diabetic wounds through their paracrine factors, and thus, there is extensive ongoing research regarding the mechanisms behind these properties. For example, exosomes derived from human circulating fibrocytes, a population of mesenchymal progenitor cells, appear to exhibit proangiogenic properties, and specifically, in an in-vivo experiment they induced the migration and proliferation of diabetic keratinocytes, activated diabetic dermal fibroblasts, and accelerated wound closure [145]. Moreover, human endothelial progenitor cell-derived exosomes can improve the function of endothelial cells and promote wound healing [146], and fetal dermal MSC-derived exosomes can induce the proliferation and migration of adult dermal fibroblast cells [147]. Exosomes from menstrual blood-derived MSCs have similar regenerative properties on the skin in an in vivo animal study [148].

Diabetic nephropathy (DN) is the progressive loss of kidney function in diabetic patients. It is one of the main causes of chronic kidney disease (CKD) and end-stage renal disease

(ESRD) globally. Bone marrow-derived MSCs have been proven effective in the improvement of DN, however it is believed that their effects are mainly a result of the action of their paracrine factors. In a study, the effects of renal trophic factors, including exosomes, secreted by MSCs were evaluated [149]. MSCs and MSC-conditioned medium (MSC-CM) were administered to a rat model of T2D induced by high-fat diet, and to a diabetic mouse model of insulin deficiency induced by streptozotocin [149]. Both groups exhibited similar curative effects, and specifically, they inhibited the exacerbation of albuminuria and of proinflammatory cytokine expression and fibrosis in tubular interstitium [149]. Exosomes purified from the conditioned medium reduced the apoptosis of tubular endothelial cells and maintained a tight junction structure [149]. Another study investigated the mechanism behind the protective role of MSC-derived exosomes in chronic renal injury. In this experiment, five groups of in vivo rat models were used, and it was shown through the detection of specific autophagy markers that MSC-derived exosomes restore renal tissues by inducing autophagy via the mTOR signaling pathway [150].

Renal fibrosis is common in diabetic nephropathy. EVs derived from MSCs and human liver stem-like cells (HLSCs) could inhibit the progression and reverse renal fibrosis, as demonstrated in an in vivo experiment. In the groups treated with stem cell-derived EVs, parameters of renal function, such as albumin/creatinine excretion, plasma creatinine and blood urea nitrogen, were ameliorated [151]. Moreover, various pro-fibrotic genes were down-regulated in renal tissues, probably targeted by specific miRNAs that were common in both MSC-EVs and HLSC-EVs. Efforts are also made towards the prevention of the disease in its early stages. For example, urine stem cell-derived exosomes have the potential to prevent kidney injury from diabetes by preventing podocyte apoptosis and inducing vascular regeneration and cell survival, as it has been shown in a T1D murine model [152]. Furthermore, it has been proven that diabetic nephropathy can also be suppressed through the action of stem cell-derived exosomal cargo, such as miRNAs, like miR-26a-5p that is contained in EVs secreted by ADSCs [153]. In diabetic nephropathy, this molecule is present at low levels, while toll-like receptor 4 (TLR4) plasmid levels are high. ADSC-derived EVs, and specifically the miR-26a-5p that they contain, can target TLR4 and protect against diabetic nephropathy [153].

A major complication of diabetes is diabetic peripheral neuropathy (DPN), which can result in morbidity or mortality. High blood glucose can cause damage to the nerves throughout the body. There is no effective treatment, however there are several drugs that can be administered to control the progression of the disease, but nerve injury is irreversible. Stem cell-derived exosomes are promising candidates to improve

neurological outcomes of DPN. MSC-derived exosomes were tested in a mouse model, and their effects were evaluated. It was observed that the treatment with MSC-exosomes decreased the threshold for thermal and mechanical stimuli, and increased nerve conduction velocity [154]. Furthermore, MSC-exosomes improved functional recovery of the nerves by suppressing proinflammatory cytokines, and analysis revealed that miRNAs in the exosomes targeted the TLR4/NF-κB signaling pathway [154].

Diabetic retinopathy is a complication that affects the eye of both types of diabetic patients, and it is caused by damage to the blood vessels of the retina, which could result in blindness. There are no effective drugs to reverse the disease progression, and treatment options are mainly focused in the management of diabetes. The effects of MSC-derived exosomes on retinal inflammation were studied. Exosomes were isolated from hUCMSCs, and a transfer of mir-126 followed. Both MSC-Exos and MSC-Exos overexpressing miR-126 were injected intravitreally into diabetic rats in vivo and they were also cocultured with high glucose-affected human retinal endothelial cells (HRECs) in vitro [155]. The results showed that MSC-Exos could reverse the overexpression of caspase-1, interleukin-1β (IL-1β) and IL-18 that was prevalent in high glucose exposed HRECs and resulted in inflammation [155]. However, MSC-Exos overexpressing miR-126 suppressed inflammation more effectively in diabetic rats, by downregulating the HMGB1 signaling pathway [155]. Another study evaluated the effects of rabbit adipose tissue MSC-derived exosomes in retina regeneration in a rabbit model of streptozotocin induced DM. Exosomes were injected by different routes of administration; intravenously (IV), subconjunctival (SC) and intraocular (IO), and their results in retinal regeneration were evaluated after 12 weeks. It was observed that the retinal tissue expression levels of micRNA-222 were negatively correlated to serum glucose levels, and the increased expression of micRNA-222 was associated with the regenerative effects of MSC-derived exosomes after their injection [156].

Erectile dysfunction is common in men with diabetes, especially as the age progresses. The mechanism behind this complication is complex, and it involves nerve, blood vessel and muscle impairment, due to high blood glucose levels. There are medications that can improve erectile dysfunction, however, taking into consideration that many diabetic patients also suffer from cardiovascular disease, these drugs could have serious side effects, as well as interaction with heart drugs. There are studies suggesting that the transplantation of adipose-derived stem cells can improve diabetic erectile dysfunction. The effects of their paracrine factors, and especially exosomes, in diabetic erectile dysfunction, were further evaluated. In a study, ADSC-derived exosomes (ADSC-Exo) exhibited proangiogenic properties in vitro, promoted the proliferation of endothelial

cells, improved erectile dysfunction in vivo, and inhibited fibrosis of corpus cavernosum [157]. These properties of ADSC-Exo were attributed to the specific proangiogenic and antifibrotic micRNAs that they contained. Another study also investigated the effect of ADSC-Exo on erectile function in a T2DM rat model. Intracavernous injection of ADSC-Exo and ADSCs resulted in an increase in the ratio of intracavernous pressure to mean arterial pressure, inhibited apoptosis of corpus cavernosum endothelial and smooth muscle cells, and thus, led to the promotion of recovery of erectile function [158].

Evidence suggest that there is a strong correlation between cardiovascular disease and diabetes. Diabetic patients are two to four times more likely to die from heart disease than adults without diabetes, and it is considered one of the main major risk factors for cardiovascular disease. Predisposing factors of the development of cardiovascular disease in diabetic patients include hypertension, abnormal cholesterol and high triglycerides, obesity, lack of physical activity, poorly controlled blood glycose levels and smoking. There are studies investigating the possible therapeutic potential of stem cell-derived exosomes on cardiovascular disease as a result of diabetes mellitus. Atherosclerosis is a main complication of DM and it is characterized by endothelial damage and inflammation. Endothelial progenitor cells (EPCs) are prerequisites for blood vessels, they produce many factors to regulate blood vessel function, and repair dysfunctions in endothelial cells [159]. In a study it was found that the most highly expressed miRNAs in exosomes derived from EPCs are associated with atherosclerosis [159]. Hence, scientists investigated the role of these exosomes in the treatment of atherosclerosis, and it was found that they significantly inhibited the production of atherosclerotic plaques and inflammatory factors [159]. Moreover, the exosome treatment improved the endothelium-dependent contractile dysfunction of the thoracic aorta of these mice, in an in vitro assay examination [159]. Another study evaluated the effect of MSC-derived exosomes on diabetes mellitus-induced myocardial injury. A streptozotocin-induced DM rat model was establishes and injections of MSC-derived exosomes were administered for 12 weeks. Subsequently, analysis revealed that the exosome treatment down-regulated the levels of molecules that were up-regulated in the diabetic group, such as the level of LVC, FATPs and FA-β-oxidase, TGF-b1, Smad2 mRNAs, and it was concluded that MSC-derived exosomes improved DM-induced myocardial injury via inhibition of TGF-β1/Smad2 signaling pathway [160].

Methods

Various procedures were taken in order to ensure a high-quality review of the literature on stem cell-derived exosomes and their role in the therapy of inflammatory diseases. A comprehensive search of peer-reviewed journals was conducted based on a wide range of corresponding search terms alone or in combination, including: stem cells, exosomes, inflammation, autoimmune. Relevant key words were added in accordance with the specific inflammatory disease that was researched. The selection of specific inflammatory diseases was conducted based on the amount of the available literature on the databases, and the amount of preclinical in vitro or animal-based studies that were conducted in the area of research. Three main databases were searched, including PubMed, Google Scholar and Scopus. Papers published in English language from January 2010 to October 2020 were considered. Preliminary screening of papers by scrutinizing the titles and abstracts of the articles resulted in the exclusion of papers not related to the subject of the dissertation. Additional studies were selected manually from the reference section of papers that comply with the selection criteria, and these articles were screened to meet the final selection and exclusion criteria.

Results

One hundred and eighty papers were ultimately selected for analysis. After completing the analysis of the literature, it was concluded that stem cell-derived exosomes (SC-Exos) hold great potential in the treatment of multiple inflammatory and autoimmune diseases, such as inflammatory bowel disease, periodontitis, Alzheimer's disease, multiple sclerosis, osteoarthritis, rheumatoid arthritis and diabetes, as well as complications induced by diabetes, including nephropathy, neuropathy, wound healing, retinopathy, erectile dysfunction and cardiovascular disease. Some properties of SC-Exos are related to their parental cells, and others are a result of the molecules that they contain, such as specific mRNAs, miRNAs, and proteins, that they use to interact with cells. Stem cell-derived exosomes act as regulators of various biological processes, including immune response, inflammation and regeneration of tissue. In some experiments, it was shown that their anti-inflammatory properties are a result of regulation of inflammatory mediators such as cytokines, infiltration or polarization of inflammatory cells, mainly macrophages, and reduction of oxidative stress. It is reported that they can also overexpress specific mRNAs, or miRNAs, that act as mediators and can affect biological processes, regulate the immune system, alleviate inflammation and promote regeneration. Moreover, SC-Exos contain anti-inflammatory molecules such as interleukin 10 (IL-10) and transforming growth factor-β1 (TGF-β1) that have immunoprotective effects. Other studies highlight their immunoregulating properties, by enhancing or regulating the function and expression of immune cells, such as T or Treg cells, or by suppressing the activation and maturation of dendritic cells. The specific microenvironment of SC-Exos can affect their properties, and pre-conditioning, for example under hypoxia, could ameliorate their function. Furthermore, regenerative properties, partly bequeathed by their parental cells, make them promising therapeutic agents in the treatment of degenerative disorders, or in tissue damage. It is believed that SC-Exos induce angiogenesis, collagen and bone synthesis, and they promote the expression of several growth factors (HGF, IGF1, NGF, and SDF1). According to some studies, they also inhibit apoptosis and induce proliferation and function of cells that are involved in the regeneration or protection of tissues, whereas induce

apoptosis of inflammatory or degenerative factors. The mechanism underlying tissue regeneration by SC-Exos has also been linked to the activation of specific wound healing signaling pathways, such as Akt, ERK, and STAT3.

In conclusion, accumulating evidence suggest that SC-Exos are promising candidates in the treatment of multiple inflammatory and autoimmune diseases, but the exact processes by which SC-Exos derive their therapeutic effects from is still under thorough investigation, and many mechanisms behind these processes remain largely unknown.

Discussion

Over the last years, an increasing number of scientists support that inflammation is emerging as a major predisposal factor that is involved in the pathogenesis of many diseases that were not considered “inflammatory” during the past, perhaps even all of them [175]. Hence, it is a growing necessity to investigate novel therapeutic approaches in the treatment of inflammatory diseases. Stem cells have been in the center of scientific research for many years, due to their regenerative properties and strong immunomodulatory activity [176]. However, the use of stem cell-based therapies has raised serious concerns in the scientific community, due to severe adverse events, such as immune reaction, mal-differentiation, the ability of cells to move from placement sites and the possibility of lung or liver entrapment, abnormalities in the function of the cells, toxicity and tumor formation [177].

Recent studies indicate that the majority of stem cell therapeutic potential, and especially their immunomodulatory and anti-inflammatory properties, can be largely attributed to their paracrine factors, rather than transdifferentiation. Exosomes secreted from stem cells have emerged as powerful components of their secretome, because they carry most of the properties of stem cells, without the risks that are associated with the use of live cells in therapy [178]. Exosomes are nanovesicles, with a diameter ranging between 30 and 150 nm, and unlike their parental cells, they can cross the blood-brain barrier, which makes them appropriate therapeutic candidates for the treatment of neurodegenerative disorders or conditions of the nervous system [179]. Stem-cell derived exosomes are being thoroughly investigated as a cell-free therapeutic alternative, and there are plenty of in vitro or in vivo animal studies that give promising results for the treatment of inflammatory and autoimmune diseases, such as inflammatory bowel disease [81-89], periodontitis [90-92], Alzheimer’s disease [93-106], multiple sclerosis [107-110], osteoarthritis and rheumatoid arthritis [111-131], and diabetes [131-138], as well as complications induced by diabetes, including wound healing [139-148], nephropathy [149-153], neuropathy [154], retinopathy [155-156], erectile dysfunction [157-158], and cardiovascular disease [159-160].

There have been more than two hundred preclinical studies of exosome-based therapies in various animal models [180] and a few exosome-based clinical trials are registered [181]. Although the results are promising, there are many unanswered questions to be investigated before clinical studies can be conducted in a greater scale. First of all, the nomenclature regarding exosomes is inconsistent, and there is a lack of standardized characterization techniques [182,186]. Secondly, up to date there is no purification method specially designed for the isolation of exosomes from conditioned media that is completely efficient and meets good manufacturing practice (GMP) standards [183,186]. Different culturing and purification methods of exosomes could lead in contaminated mixtures and inconsistencies in the results among studies [184,185,186]. Consequently, standardized operating procedures regarding the culture conditions and purification methods need to be established. To this direction, specific guidelines regarding purity and the levels of acceptable contamination are of great significance. Moreover, the optimal dose for humans for each disease and condition being treated in not known, and information regarding the number of doses, quantity of dose, as well as route of administration, should be investigated further [186].

Another important aspect in the field of exosomes that needs to be addressed is the exact biogenesis mechanism that remains largely unknown, circulation kinetics and biodistribution, and natural therapeutic potential that needs to be evaluated in potency assays [186]. Also, the investigation of the safety profile of exosomes is of great importance, and while various clinical trials are trying to evaluate the safety of exosomes, many issues are still unsolved. Although stem-cell derived exosomes present specific advantages compared to their parental cells, such as increased consistency, improved efficacy, and larger scalability of manufacturing, it is not yet known if these advantages are reproducible for clinical translation [187]. Another unmet scientific need is the necessity to increase the production of stem-cell derived exosomes, which can be achieved by pre-conditioning with specific biological factors or chemicals, 3-D culture, bioreactors and microcarriers, and gene editing [183]. It is even more important to establish a method in order to select suitable donors for the production of stem cell-derived exosomes, as well as specific release criteria regarding the size, expression of markers, cargo, and the functionality, before administration to patients. Moreover, the efficacy of stem cell-derived

exosomes can be ameliorated through preconditioning, for example by adding cytokines or chemicals to the cultured medium, or inducing specific conditions, such as hypoxia. Also, CRISPR/Cas9, a genome editing technology can be applied to improve their efficacy. By a deeper understanding of exosomes' biogenesis, these nanovesicles could be engineered and used as vehicles for targeted delivery in molecular therapy [188,189]. Conventional viral vectors are not as resilient, nonimmunogenic and capable of systemic action as exosomes, which makes them a more suitable alternative. Therapeutic molecules, such as specific proteins and RNAs, could be loaded into engineered exosomes that are expressing ligands in order to attach to target cells [189]. This exciting possibility means that in the future exosomes could act as mediators of targeted therapy in many diseases, such as diabetes and neurodegenerative disorders, but further investigation of the efficacy and safety of this practice should be conducted.

Exosome research is in its early stages, and the road from promising observations to clinical applications is long. Currently, exosomes are not yet used in full-scale clinical trials, and progress will depend on the understanding of the mechanisms underlying their biogenesis and function. Many challenges need to be overcome in order to pave the way for applications of these observations in clinical trials, and for new discoveries and potential treatments for various disorders. When these milestones are reached, this very promising field could revolutionize the therapeutic approach in many diseases, and especially in inflammatory and autoimmune conditions.

Conclusion

Taken together, there is accumulating evidence suggesting that stem-cell derived exosomes have tremendous potential in the treatment of several incurable diseases, and especially inflammatory conditions. Their effects are exerted by complex mechanisms and biological processes, and mainly through the genetic information or bioactive proteins that they contain and can affect the function of targeted cells. Efforts are being made in order to establish specific guidelines and standards for the efficacy and safety of exosomes, in order to accelerate clinical application in the therapy of many diseases.

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