MASTER THESIS

Mouse bone marrow mesenchymal stem cell conditioned media and its derived exosomes in the treatment of Sjögren Syndrome-like disease in NOD mice

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Introduction

Materials & Methods

Results

LIST OF ABBREVIATIONS*

SS	Sjögren's Syndrome
MSC	Mesenchymal Stem Cell
CM	Conditioned Media
Exo	Exosomes
NOD	Non-Obese Diabetic
SG	Salivary Gland
PG	Parotid Gland
SMG	Submandibular Gland
SLG	Sublingual Gland
BM	Bone Marrow
SFR	Salivary Flow Rate
IFN	Interferon
TNF	Tumor Necrosis Factor
DPSC	Dental Pulp Stem Cell
EV	Extracellular Vesicle
MVB	Multivesicular Body
ILV	Intraluminal Vesicle
hUC	Human Umbilical Cord
CNS	Central Nervous System
LG	Labial Gland
OE-MSC	Olfactory Ecto-MSC
UMSC	Umbilical MSC
SHED	Stem Cells from Human Exfoliated Deciduous Teeth
αDMEM	alpha Dulbecco's Modified Eagle's Medium
IV	Intravenous Injection
NTA	Nanoparticle Tracking Analysis
TEM	Transmission Electron Microscopy
DLS	Dynamic Light Scattering
LAMP	Lysosome-Associated Membrane Protein

* Includes only abbreviations that appear 3 times or more in-text.

MCGILL UNIVERSITY

ABSTRACT

FACULTY OF DENTAL MEDICINE AND ORAL HEALTH SCIENCES

Master of Science

Mouse bone marrow mesenchymal stem cell conditioned media and its derived exosome in the treatment of Sjogren's-like disease in NOD mice

By: Crystal To Tam Mai

Introduction: Sjögren's syndrome (SS) is a chronic systemic autoimmune disease that is pathologically characterized by lymphocytic infiltration, autoantibody deposition, and chronic inflammation of the exocrine glands, primarily affecting the salivary and lacrimal glands. Despite its prevalence as one of the most common autoimmune diseases, a cure remains elusive due to the complex etiopathogenesis and insufficient research. Mesenchymal stem cells (MSCs) have shown promise in SS testing. However, their clinical application presents certain risks and limitations, notably the low efficiency of MSCs homing to target tissues and the possibility of remaining MSCs becoming trapped in other organs or potentially leading to the formation of tumour.

Objectives: This study utilizes conditioned media (CM) from mouse bone marrow (BM) MSCs (MSC-CM) and its derived exosomes (MSC-CM-Exo) in a cell- and biologic-based therapy approach for treating SS-like disease in non-obese diabetic (NOD) mice. The first objective of this study is to determine the efficacy of MSC-CM and MSC-CM-Exo treatment in NOD mice with SS-like disease. The second objective will be to evaluate the regeneration ability of MSC-CM and MSC-CM-Exo treatments on salivary gland (SG) structure and function in NOD mice with SS-like disease.

Methods: We used mouse BM to create the MSC-CM. Subsequently, exosomes were isolated from the prepared MSC-CM. Eight-week-old NOD mice with SS-like disease received one of the following treatments: normal saline (control), MSC-CM, or MSC-CM-Exo. Starting from week

11 of age, blood sugar was monitored weekly to detect diabetes. At week 16 of age, the mice were sacrificed followed by SG regeneration studies.

Results: The results demonstrated that both MSC-CM and MSC-CM-Exo treatments effectively preserved the exocrine function of the SGs when compared to the control group. This preservation was achieved by regulating the local inflammatory microenvironment and reducing inflammatory infiltration.

Conclusion: The potential of MSC-CM and MSC-CM-Exo as treatments for SS offers new horizons for safer and more convenient combined biologic- and cell-based therapy options.

UNIVERSITÉ MCGILL

RÉSUMÉ

FACULTÉ DE MÉDECINE DENTAIRE ET DES SCIENCES DE LA SANTÉ ORALE

Maîtrise en Sciences

Milieu conditionné à partir de cellules souches mésenchymateuses de moelle osseuse de souris et leur exosome dérivé dans le traitement de la maladie de type Sjögren chez les souris NOD

De: Crystal To Tam Mai

Introduction: Le syndrome de Sjögren (SS) est une maladie auto-immune systémique chronique caractérisée par une infiltration lymphocytaire, un dépôt anticorps dirigé contre le soi, et une inflammation chronique des glandes exocrines, affectant principalement les glandes salivaires et lacrymales. Malgré sa prévalence comme l'une des maladies auto-immunes les plus courantes, un remède reste hors de portée en raison de l'étiopathogénie complexe et de la recherche insuffisante. Les cellules souches mésenchymateuses (MSC) se sont révélées prometteuses dans les tests SS ; cependant, leur application clinique présente certains risques et limites, notamment la faible efficacité des MSC se déplaçant vers les tissus cibles et la possibilité que les MSC restants soient piégés dans d'autres organes ou conduisent potentiellement à la formation de tumeur(s).

Objectifs: Cette étude utilise des milieux conditionnés de MSC de moelle osseuse de souris (MSC-CM) et ses exosomes dérivés (MSC-CM-Exo) dans une approche basée sur la thérapie cellulaire et biologique pour le traitement de la maladie de type SS chez les souris NOD. Le premier objectif de cette étude est de déterminer l'efficacité du traitement MSC-CM et MSC-CM-Exo chez des souris NOD atteintes d'une maladie de type SS. Le deuxième objectif sera

d'évaluer la capacité de régénération des traitements MSC-CM et MSC-CM-Exo sur la structure et la fonction des glandes salivaires chez des souris NOD atteintes d'une maladie de type SS.

Méthodes: Nous avons utilisé de la moelle osseuse de souris (Ms-BM) pour créer le MSC-CM. Par la suite, les exosomes ont été isolés et purifiés à partir du MSC-CM préparé. Des souris NOD âgées de huit semaines atteintes d'une maladie de type SS ont reçu l'un des traitements suivants : aucun traitement (contrôle), MSC-CM ou MSC-CM-Exo. À partir de 11 semaines de vie, la glycémie a été surveillée chaque semaine pour détecter le diabète. À la semaine 16 de l'âge, les souris ont été sacrifiées, puis des études de régénération des glandes salivaires ont été performées, impliquant étude du débit salivaire régulier, et l'histopathologie de la glande salivaire traitée, préalablement disséquée.
Résultats: Les résultats ont montré que les traitements MSC-CM et MSC-CM-Exo préservaient la fonction exocrine des glandes salivaires (SG) par rapport au groupe contrôle en régulant le microenvironnement inflammatoire local et en réduisant l'infiltration inflammatoire, respectivement.

Conclusion: Le potentiel du MSC-CM et du MSC-CM-Exo en tant que traitements du SS offre de nouveaux horizons pour des options thérapeutiques combinées biologiques et cellulaires plus sûres et plus convenables.

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PREFACE AND CONTRIBUTIONS OF AUTHORS

Crystal Mai was responsible for cell culture, preparation of the MSC-CM and MSC-CM-Exo treatments, molecular biology, the overall design, execution, and data analysis of all experiments. Crystal Mai took charge of designing and planning the intravenous injections for NOD mice, collecting blood for blood glucose monitoring, and measuring salivary flow rate. Dr. You Nan Liu provided valuable assistance in each of these procedures. In addition, Dr. You Nan Liu was responsible for performing euthanasia of all mice via cardiac puncture.

Dr. Janaki Iyer assisted with the housing of the NOD mice, preparation of the MSC-CM and MSC-CM-Exo treatments, and collection of blood glucose from mice for blood glucose monitoring.

Dr. Xinyun Su had previously prepared the mouse bone marrow mesenchymal stem cells and Dr. You Nan Liu assisted with the purchasing the material needed for experiment. Dr. Valentin Nelea performed the DLS experiment, Oscar Boyadjian perform the NTA experiment, and Ms. Jeannie Mui performed the TEM experiment.

All chapters are written by Crystal Mai and edited by Dr. Simon D. Tran and Yuijhun Ng.

1. Introduction

1.1 Sjögren's Syndrome

Sjögren's syndrome (SS) is a chronic systemic autoimmune disease that is pathologically characterized by lymphocytic infiltration, autoantibody deposition, and chronic inflammation of the exocrine glands, primarily the salivary and lacrimal glands [1]. Although the pathogenesis of SS is unknown, it is thought to be multifactorial and caused by a combination of genetic predisposition, hormonal imbalances, dysregulated immune systems, and viral infections. It appears to be a multistep cascade of interconnected events, as well as abnormal cellular and humoral immune system behaviour [2]. T-lymphocytes account for the majority of the glandular infiltration in SS, with B-lymphocytes predominating in the more advanced stages. T-cells differentiate into T helper 1 (Th1) and Th2 cells, which produce cytokines that stimulate the cellular immune response and the humoral immune response, respectively. Hence, Th1 and Th17 cells initiate the pathogenesis of SS, and as the disease progresses, Th2 and follicular helper T (Tfh) cells start to predominate. B-cells are also involved in cytokine, autoantibody production, and antigen presentation. Regulatory B-cells increase the number of regulatory T (Treg) cells while decreasing pro-inflammatory cytokines. Once the infiltrate reaches the acinar epithelium, it will eventually cause glandular dysfunction, which will result in xerostomia (dry mouth), keratoconjunctivitis sicca (dry eyes), as well as enlargement of the major SGs in SS patients [3]. Moreover, SS patients produce a wide spectrum of serum autoantibodies, including anti-La and anti-Ro, validating an autoimmune origin for this disorder [1].

SS occurs in two forms: primary and secondary. Primary SS (pSS) is when the disease presents alone and secondary SS is when the disease is associated with other types of rheumatic diseases, such as systemic lupus erythematosus, rheumatoid arthritis (RA), or systemic sclerosis [3]. Primary SS is an idiopathic inflammatory exocrinopathy that is characterized by both organ-specific autoimmunity and extra-glandular systemic symptoms like fatigue, arthritis, interstitial nephritis, vasculitis, pulmonary involvement, and neuropathy [1]. Primary SS has a population prevalence of approximately 0.5% and predominantly affects female with a female to male ratio of 9:1 [2]. Secondary SS, on the other hand, is more common with approximately 60% of SS patients acquiring this form [3].

The primary approach to treating SS is mainly focused on alleviating symptoms and addressing complications of the disease in its early stages. The current therapeutic goal is to minimize the damage caused by chronic xerostomia and keratoconjunctivitis sicca. One important receptor involved in regulating tear production and saliva secretion is the muscarine M3 receptor, which is present in the acinar cells of the salivary and lacrimal glands. As many SS patients still retain some functioning acinar cells, the administration of muscarinic agonists like pilocarpine hydrochloride and cevimeline hydrochloride can provide therapeutic benefits for xerostomia and keratoconjunctivitis sicca. Furthermore, corticosteroids and targeted therapies specific to the affected organs or systems can be utilized to treat systemic manifestations of the disease [3].

Despite substantial molecular, histological, and clinical research, the pathophysiology and underlying cause of SS remain unclear [1]. On account of this, there is presently no cure for SS as well as no treatment available to reduce the glandular lymphocytic infiltration that contributes to the progression of exocrine gland dysfunction in SS. This disorder can appear in a wide range of clinical manifestations due to its systemic nature, which adds to the uncertainty and delay in diagnosis. In addition, the symptoms of xerostomia and keratoconjunctivitis sicca are widespread and relatively subjective. In a study of 618 patients, 15% of patients with pSS and 26% of those with secondary SS did not report any symptoms of xerostomia [4]. A more thorough approach to diagnosing this disorder is encouraged by increasing the understanding of SS and its diverse manifestations [3].

1.2 Salivary Glands

The human SGs play a vital role in maintaining the homeostasis of the oral cavity, serving as fundamental organs. These glands are responsible for the production and secretion of saliva, a fluid with multiple indispensable functions. Saliva provides crucial mucosal lubrication, contains antibacterial compounds, supplies salivary electrolytes, and encompasses a variety of enzymes that protect the oral mucosa and teeth. As a result, ensuring the proper functioning of salivary secretion becomes paramount for optimal oral health and has been reported to have a direct relationship to overall oral function and quality of life [4]. The autonomous nervous system primarily regulates the SFR. Saliva generated through sympathetic stimulation tends to

be more viscous, while saliva produced through parasympathetic stimulation appears waterier in nature [5]. Due to gland loss or degeneration, there can be a partial or complete reduction in saliva production. As our understanding of the morphological and biogenetic aspects of SG development deepens, it opens new avenues for interpreting glandular health and diseases in innovative ways [4].

Human minor SGs are dispersed throughout the oral cavity, comprising an estimated 600-1000 glands. In contrast, the major SGs in humans consist of just three pairs: the parotid, submandibular, and sublingual glands. Although these major glands occupy different locations, they possess similar anatomical features. These shared features encompass acini, which are glandular secretory end pieces, as well as branched secretory ducts that discharge their contents into the oral cavity [4].

Parotid Gland

The parotid glands (PG) are the largest major SGs in humans, consisting exclusively of serous acini. These acini secrete a watery saliva that is rich in amylase [4, 5]. When stimulated, the PGs contribute to 50% of the total volume of saliva produced, and when unstimulated, they account for 25% of the total volume [7]. The primary excretory duct of the PG is known as Stensen's duct, which serves as a conduit for the saliva to enter the oral cavity. This duct traverses through the masseter muscle and the buccinator muscle before reaching the buccal mucosa near the second maxillary molar (Figure 1) [6].

Submandibular Gland

The submandibular glands (SMG) are the second largest pair of major SGs in humans. They are composed of a mixture of mucous and serous acini, with the latter being more prominent. SMG secrete a viscous and mucin-rich saliva that is predominantly composed of glycoproteins sulfated cystatins as well as epidermal and neuronal growth factors, thereby facilitating oral mucosa protection and lubrication [4]. Upon stimulation, the SMGs contribute to approximately 35% of the total volume of saliva produced, and when unstimulated, they account for 60% of the total volume [5]. Its primary excretory duct is referred to as the Wharton’s duct

which allow saliva produced from the SMG to travel to the oral cavity and be released on both side of the lingual frenulum (Figure 1) [4].

Sublingual Gland

The sublingual glands (SLG) are the smallest among the major SG groups in humans. Although the SLG comprises both mucous and serous acini, it predominantly consists of mucous acinar cells. Consequently, the SLG produces saliva that is viscous and rich in mucin [4]. Upon stimulation, the SLG contributes to approximately 7-8% of the total volume of saliva produced, and in the unstimulated state, it accounts for a similar percentage of the total volume [5]. The SLG is connected to the oral cavity through a common duct known as Bartholin's duct, as well as small ducts called ducts of Rivinus [4, 6]. Moreover, Bartholin's duct connects to Wharton's duct at the sublingual caruncle (Figure 1) [4].

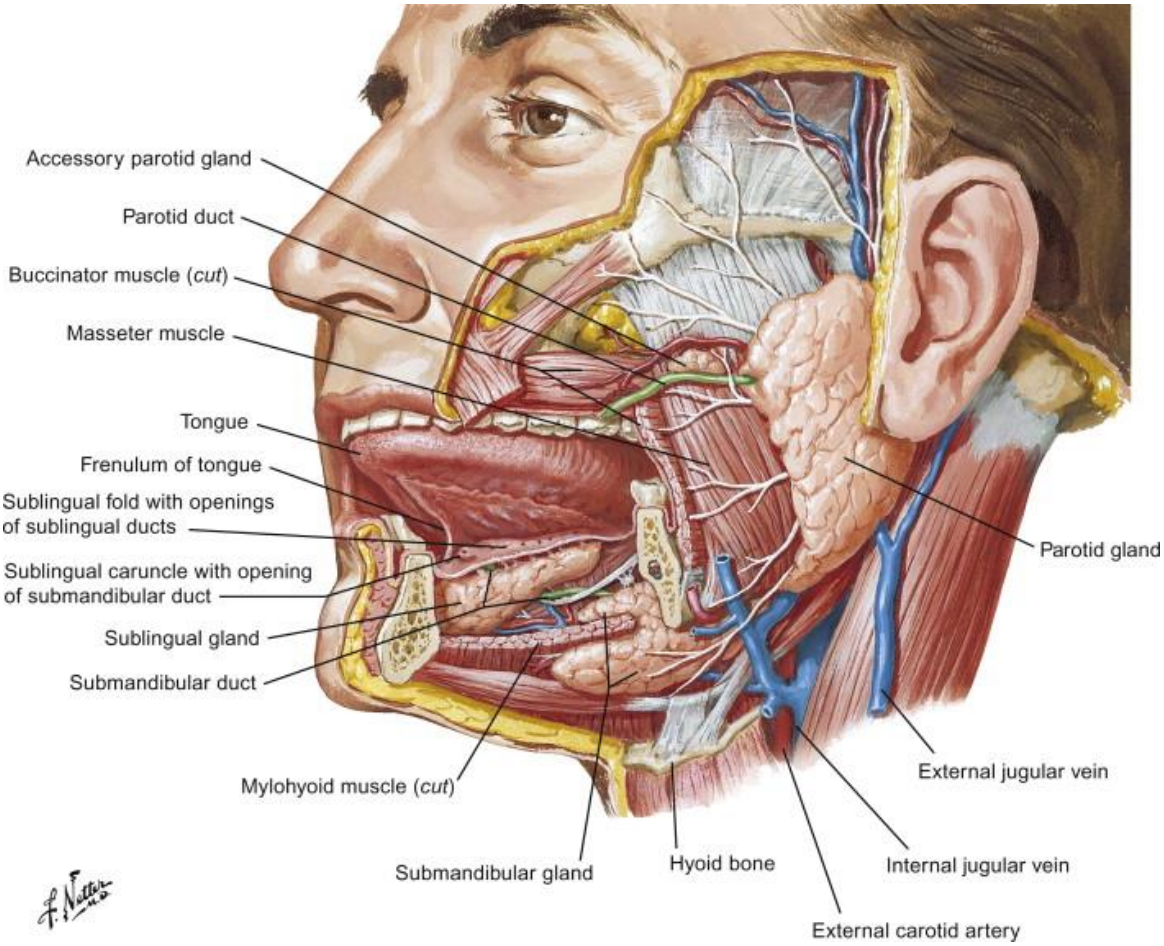


Figure 1. Diagram of major salivary glands and oral cavity components. The PG are located behind the external auditory canal (not shown) of the skull base and mandible on both the right and left side of the head [4]. The SMG are positioned behind the free insertion of the mylohyoid muscle in the submandibular triangle, which is a region under the mandible bounded by the anterior and posterior bellies of the digastric muscle (not shown) [5]. The SLG can be found between the mucosal floor of the oral cavity and the mylohyoid muscle [4] (Image from [6]).

1.3 Saliva

Saliva is a transparent, mildly acidic mucinous-serous secretion made up of 99.5% water, 0.3% proteins, and 0.2% small organic and inorganic substances [7]. The PG, SMG, and SLG collectively contribute to 90% of total saliva production, while the remaining 10% is derived from minor SGs [8]. On an average day, an adult human produce approximately 0.5-2 liters of saliva. However, saliva production notably decreases during nighttime, accounting for only 2-10% of the overall fluid output. Under normal conditions, the basal SFR varies significantly among individuals, ranging from 0.30 to 0.55 mL/min. During activities such as chewing or talking, SFR can surge up to approximately 10 mL/min, whereas during sleep, it diminishes to less than 0.25 mL/min. In response to a strong stimulus like food, SFR can reach levels of 1.5-2.3 mL/min, while pharmacological agents such as pilocarpine can elevate SFR to 5.0 mL/min. Factors including the duration of sleep, meal frequency and composition, as well as emotional stimuli, have been reported to influence the daily volume of saliva [7].

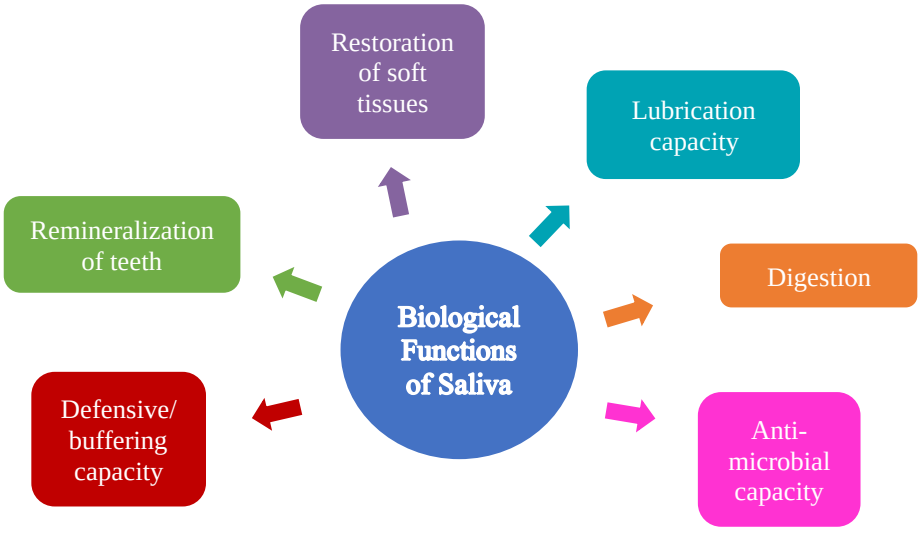


Figure 2. Saliva plays a vital role in maintaining essential biological functions. It possesses bicarbonate, phosphate, and protein buffer systems that contribute to regulating the optimal pH level in the oral cavity, typically ranging from 6.0 to 7.5. One of its notable qualities is being oversaturated with calcium hydroxyapatite, which helps prevent the demineralization of teeth. Salivary epidermal growth factor not only protects the esophageal mucosa but also aids in the restoration of the oral mucosa. Salivary mucins serve to lubricate intraoral structures and establish a protective barrier against microbial invasion. In neonates with pancreatic dysfunction, specific substances present in human saliva, such as α-amylase and lipase, have been associated with the digestion and decomposition of starches as well as the breakdown of triglycerides. Furthermore, human saliva contains antibacterial components like salivary peroxidase, lysozyme, lactoferrin, and histamines. Notably, histatins and lactoferrin also exhibit antifungal properties, expanding the protective capabilities of saliva [8]. *Illustration by Crystal Mai*.

1.4 NOD Mouse Model for Sjögren's Syndrome

The NOD (Non-Obese Diabetic) mouse model holds great significance in SS research due to its capacity to recapitulate several important features of the human condition. Moreover, it has proven to be an invaluable tool for uncovering early disease markers and delving into the underlying biological and immunological dysregulations. Here are some notable resemblances between the NOD mouse model and humans within the context of SS:

Autoimmune Features: SS manifests as an autoimmune disorder characterized by the immune system's misguided attack on the body's own tissues. Notably, NOD mice exhibit autoimmune features, producing autoantibodies that selectively target a range of self-antigens, such as salivary gland protein 1 and carbonic anhydrase 6. These autoantibodies detected in NOD mice closely resemble those observed in human patients with SS [8].

Spontaneous Development of Autoimmune Features: NOD mice exhibit spontaneous development of autoimmune features that closely resemble SS, including lymphocytic infiltration within the salivary and lacrimal glands. The infiltration of lymphocytes, specifically CD4+ T-cells, into these glands is a distinctive feature of SS. This immune cell infiltration plays a pivotal role in causing glandular damage and impaired secretory function observed in human SS patients. Remarkably, NOD mice display a comparable lymphocytic infiltration pattern in

their salivary and lacrimal glands, rendering them an invaluable model for investigating the complex mechanisms and consequences associated with immune cell infiltration in SS [9, 10].

Xerostomia and Keratoconjunctivitis Sicca Phenotypes: Xerostomia and keratoconjunctivitis sicca are prominent clinical manifestations in individuals with SS. Notably, NOD mice exhibit diminished saliva production and tear secretion, resulting in akin manifestations of dryness in the oral cavity and ocular surfaces. This recapitulation of dry mouth and dry eye phenotypes in NOD mice bears resemblance to the observations made in human SS patients, thereby enabling researchers to delve into the fundamental mechanisms and explore potential therapeutic approaches aimed at ameliorating these symptoms [9, 11].

Genetic and Immunological Similarities: The NOD mouse model possesses a genetic background that bears resemblance to human populations predisposed to SS. Moreover, NOD mice display immune system dysregulation, characterized by aberrations in various immune cell types, cytokines, and signaling pathways. These immunological similarities between NOD mice and human SS patients render the NOD mouse model an invaluable resource for investigating the fundamental mechanisms underlying the development of SS [9].

However, it is crucial to acknowledge that no individual mouse model has successfully replicated all elements of SS pathogenesis and clinical characteristics. This is primarily attributed to the multifaceted nature of SS, which involves a combination of susceptibility genes and environmental factors. Nonetheless, mouse models remain valuable instruments for comprehending the mechanisms underlying the disease, validating hypotheses, and fostering the development of prospective therapies prior to their translation into human clinical trials [9].

1.5 Xerostomia and Therapy in Sjögren's Syndrome

The development of xerostomia in SS primarily occurs as a result of inflammation within the SGs, leading to impaired glandular function. The pathogenesis of SS involves the participation of both acinar and ductal cells. The secretory function of acini is impeded by the atrophic destruction of the glands and inflammatory apoptosis. Furthermore, ductal cells contribute to the amplification of the inflammatory cascade by functioning as antigen-presenting

cells (APCs). In addition, the degree of atrophy in the exocrine glands is influenced by various factors, including inflammatory mediators, autoantibodies, and apoptotic signals [8].

As more is understood regarding the pathogenesis of SS, researchers have made significant progress in the development and clinical testing of new therapeutic approaches. These modalities encompass a wide range, including palliative topical treatments, immunomodulatory interventions, and biological therapies [8]. Symptomatic and supportive therapies for xerostomia are among the most common and include behavioural changes in lifestyle, patient education, utilizing topical lubricating agents (i.e. saliva substitutes), pharmacological agents (i.e. pilocarpine, cevimeline, etc.), and oral prophylaxis [12, 13]. In severe cases of the disease, immunomodulatory and immunosuppressive medication have shown to be useful however, the effects are transient and prolonged use has been associated with a wide range of adverse effects. Furthermore, these medications ultimately fail to completely restore proper glandular function. Recent clinical trials have investigated the efficacy of biological therapies that target specific molecules, including cell surface markers, cytokines, receptors, and clusters of differentiation. Unfortunately, these therapies have been linked to serious side effects, such as viral and opportunistic infections, depression, tumour development, as well as cardiovascular and neurological issues. Despite the advancements in scientific research, effective and satisfactory treatment for xerostomia remains elusive. Current studies suggest that regenerative medicine may hold promise in the clinical management of xerostomia caused by head and neck cancer, radiation damage, and autoimmune diseases like SS [13].

1.6 Regenerative Medicine

When referring to the healing of damaged tissue, the term "repair" is defined as the recovery of tissue structure and function following injury and damage. The process of tissue regeneration occurs when new growth entirely restores the damaged tissue back to its normal state [14]. Functional organ restoration, which involves stem cell therapy, tissue engineering, and developmental biogenesis, has lately gained prominence as an effective therapeutic strategy [2]. Regenerative medicine, an interdisciplinary field of experimental science, capitalizes on the body's inherent healing capabilities to regenerate organs and restore them to their optimal

functionality. This is achieved by leveraging the principles of growth factors, scaffolds, and graft cells [11].

1.7 Stem Cell Therapy; Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs), also known as mesenchymal stromal cells, are a type of adult stem cells that possess the remarkable ability to self-renew and differentiate into multiple cell lineages [11]. Recent in-vitro studies have revealed that MSCs may offer therapeutic benefits through cell "enhancement" achieved by secreting anti-inflammatory and trophic factors, which have the potential to restore the physiological environment [12]. Originally discovered in BM, MSCs have also been identified in various tissues, including muscle, adipose tissue, teeth, peripheral blood, placenta, and umbilical cord [13, 14]. Although MSCs may exhibit certain variations depending on their tissue of origin, they must adhere to the three essential criteria outlined by the International Society for Cellular Therapy [15]. Firstly, MSCs must demonstrate initial plastic adherence when cultured in-vitro. Secondly, they should not express certain markers such as CD45, CD34, CD14 or CD11b, CD79 or CD19, and HLA-DR, while expressing surface antigens such as CD73, CD90, and CD105. Thirdly, under specific culture conditions, MSCs must be capable of differentiating into various mesodermal cell types, including chondrocytes, adipocytes, and osteoblasts [16-19]. This plasticity exhibited by MSCs makes them highly advantageous in tissue regeneration [20]. MSCs possess a unique combination of immunomodulatory and regenerative properties, setting them apart as a particularly valuable type of stem cell. Consequently, they have been extensively employed in the treatment of various inflammatory and degenerative disorders [21].

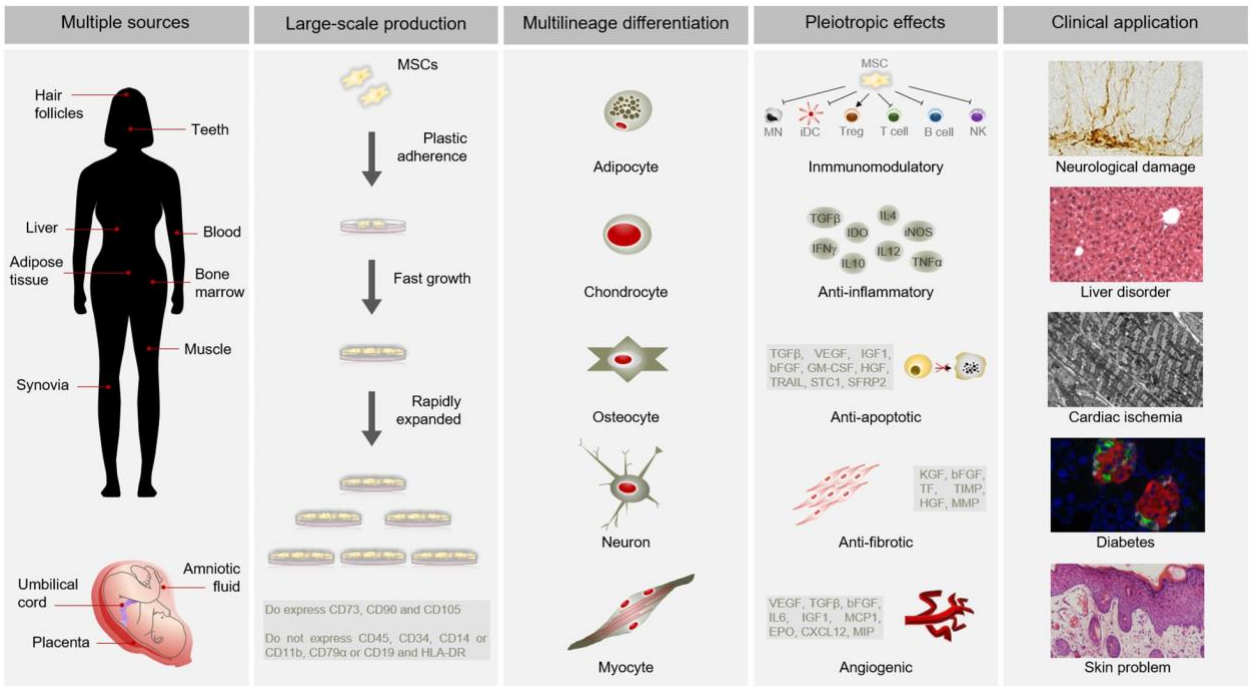


Figure 3. Advantages of MSCs for clinical application. MSCs have emerged as the most extensively used stem cell type for therapeutic application due to their numerous benefits. Among other benefits, MSCs can be isolated from a variety of sources, produced in large quantities, differentiated into a wide range of cell types, and have pleiotropic effects. With all these benefits, MSCs are well suited for therapeutic usage of a broad range of pathological conditions including neurological damage, liver disorder, cardiac ischemia, diabetes, and skin problems [15] (Image from [15]).

Tissue Repair and Regeneration: MSCs are known to secrete a variety of modulatory factors that can regulate angiogenesis, fibrosis, inflammation, cell death, and tissue regeneration. It has been reported that MSCs secrete trophic factors that stimulate cell survival (e.g. IGF-1, SDF-1, HGF), cell proliferation (e.g. HGF, EGF, NGF, TGF-α), as well as tissue angiogenesis (e.g. VEGF). Another characteristic of MSCs are their ability to migrate through the stromal extracellular matrix and peripheral blood to sites experiencing injury via chemoattractant gradients. At these injury sites, MSCs are activated by nearby factors including cytokine milieu, Toll-like receptor ligands, and hypoxia [16]. For instance, C-X-C motif chemokine ligand 12 (CXCL12) is a common triggering factor at sites of injury, and it has been demonstrated that a specific subset of MSCs expresses the C-X-C chemokine receptor type 4 (CXCR4), which

controls cell migration by attaching to its ligand, CXCL12 [15]. This wide range of stimuli induces the production of multiple growth factors, which then works synergistically to accelerate the process of tissue regeneration [25].

Anti-inflammation: In-vitro studies show that MSCs appear to increase inflammation when the immune system is underactive and decrease it when it is overactive. MSCs detect pro-inflammatory signals in the early stages of inflammation and promote inflammation via receptors for IL-1β, interferon (IFN)-γ, Toll-like receptors, and tumour necrosis factor (TNF)-α. They stimulate T-cell activation by secreting chemokines such as C-X-C motif ligand (CXCL)9, CCL5, CXCL10, and macrophage inflammatory protein-1, as well as recruit more lymphocytes. In later stages of inflammation, elevated levels of pro-inflammatory factors (i.e. IL-1β, IFN-γ, and TNF-α) allow MSCs to decrease inflammation and prevent autoimmune reactions by releasing TGF-β, IL-10, IDO, or iNOS. From this, the migration, maturation, and antigen presentation of dendritic cells (DC), as well as the function and proliferation of T-cells and the growth of Treg cells, are all inhibited. Thus, it has been postulated that the levels of IDO or iNOS determines whether MSCs have pro- or anti-inflammatory effects. Therefore, MSCs may suppress inflammation processes in different ways, through the upregulation of anti-inflammatory factors or the downregulation of proinflammatory factors [17].

Immunosuppressivity: Major studies have revealed that MSCs inhibit the proliferation of T- and B-cells, lowers the activation of natural killer cells, and prevent monocytes from differentiating into dendritic cells. Furthermore, MSCs can promote the formation of Treg cells, which are known to have immunosuppressive properties. Although soluble factors are essential for MSCs' immunosuppressive function, cell-to-cell contact also play a role in immune responses. For instance, it has been demonstrated that direct contact between MSCs and proinflammatory macrophages induce the production of tumor necrosis factor-stimulated gene-6 (TSG-6), which in turn enhances immunological tolerance. Thus, MSC-mediated immune response modulations trigger vital inflammatory processes that help to regenerate and repair damaged tissue by encouraging healing, scarring, and fibrosis [15].

In SS patients, there is a growing interest in the use of MSCs as a cell source to preserve the exocrine function of the salivary and lacrimal glands [11]. Khalili et al. previously

demonstrated that treating NOD mice with a mixture of MSCs and complete Freund's adjuvant (CFA) was successful in minimizing inflammatory mediators and lymphocytic infiltration in the SGs [18, 19]. Moreover, Abughanam et al. discovered that MSCs as well as their extract can both be used as therapeutic agents in NOD mice with SS-like disease. This is due to how both the cells and the extract downregulated the expression of tumour necrosis factor (TNF), matrix metallopeptidase 2 (MMP2), caspase 3 (CAP3), and IL-1β, while upregulating the expression of AQP5, EGF, FGF, bone morphogenetic protein 7 (BMP7), lysozyme (LYZ1), and IL-10. They also determined that greater serum levels of epidermal growth factor (EGF) cause glands to proliferate more rapidly and as a result, cornea integrity and epithelial thickness were preserved due to more consistent rates of tear flow [11]. However, there are several complications with MSCs and it has been reported that transplanted MSCs often do not survive long in the host due to loss of cells, harsh micro-environment, and insufficient or no cell adherence [20, 21]. Thus, the transformation of MSCs into CM and the subsequent isolation of exosomes present a promising theoretical approach to address various limitations (Figure 4). Previous studies conducted by our research group and others have demonstrated the therapeutic potential of BM cell extract, colloquially known as "soup," in the context of mitigating irradiation-induced SG damage and myocardial infarction [22-24]. However, to date, no investigations have explored the efficacy of mouse BM MSC-CM and its derived exosomes, specifically in the treatment of SS-like disease in NOD mice or in any other relevant experimental models.

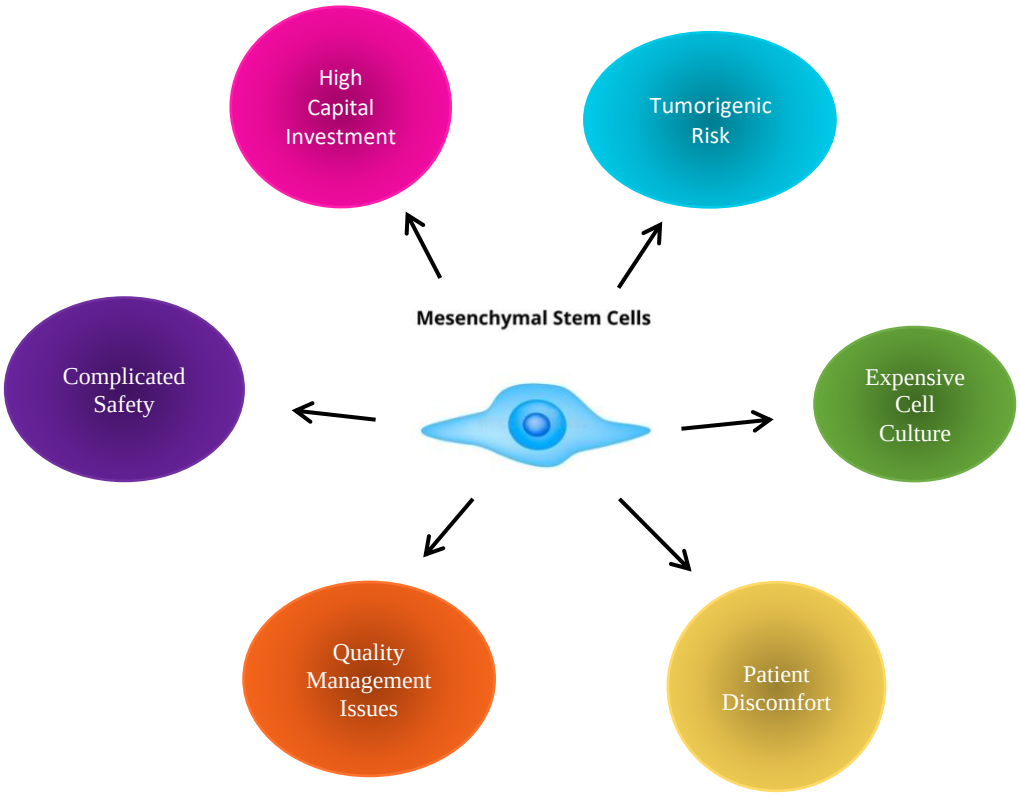


Figure 4. The drawbacks of MSC-based therapies. These drawbacks include complicated safety, high capital investment, expensive cell culture, patient discomfort due to the invasive cell collection procedure, and quality management issues in handling the cells [20]. In addition, MSCs are capable of self-renewal and continuous proliferation, but in-vivo, it is challenging to predict and control these characteristics. Therefore, MSCs may induce the formation of new tumours or accelerate the growth of already existing ones [11]. *Illustration by Crystal Mai*.

1.8 Stem Cells Trophic Factors: Conditioned Media

Recently, studies have revealed that implanted MSCs secrete a wide range of paracrine factors (i.e. growth factors, chemokines, cytokines) with immunomodulatory properties that can accumulate in serum-free CM during cell culture [20]. In response to the inflammatory environment, MSCs has been observed to increase the expression of immunosuppressive factors and through paracrine activity, they stimulate target cells. This leads to the readily production of signalling molecules including TGF-β, IFN-α, IFN-β, CCL9, IL-10, nitric oxide (NO), HGF, VEGF, FGF, PDGF, and membrane bound vesicles (i.e. micro-vesicles and exosomes) [22]. It has been reported that these signalling molecules may be responsible for the therapeutic effects

of MSCs. Due to how these signalling molecules accumulate in serum-free MSC-CM, MSC-CM therapy can theoretically be used as an alternative treatment approach without the aforementioned complications and drawbacks of using MSC as therapy (Figure 4) [22, 23]. Furthermore, numerous studies have shown that cell paracrine factors function similarly to cells, but are more efficiently preserved and have a lower risk of tumour development as well as immunological rejection [23].

Ogata et al. evaluated the therapeutic effects of dental pulp stem cells (DPSC) CM on salivary function in a SS mouse model. Their results showed that DPSC-CM was able to mitigate hyposalivation caused by SS by lowering the expression of inflammatory cytokines, activating Tregs in the spleen through the TGF-β/Smad pathway, modulating the local inflammatory microenvironment, and diminishing apoptosis in the SMGs. In addition, numerous immunosuppressive factors, including TGF-1, HGF, IL-10, and IL-13, were detected in DPSC-CM. One of their most important findings, however, was that DPSC-CM increased the differentiation of T-cell into Treg cells while decreasing Th1 and Th17 cell production in the spleen [23]. The majority of the lymphocytes that infiltrate the SS-affected SGs are CD4+ T-cells [10]. Patients with SS had greater levels of both Th1 and Th2 cytokines in their SGs [24, 25]. The Th17/Treg ratio in SS patients, on the other hand, was found to be higher in healthy controls but lower in patients in the experimental group, indicating a modest degree of imbalance and an aberration in the early stages of T-cell differentiation inside the body [26]. Thus, Ogata et al. came to the conclusion that DPSC-CM may have immunomodulatory effects through regulating the differentiation of T-cells [23].

1.9 Mesenchymal Stem Cells Trophic Factors: Exosomes

Exosomes, also referred to as intraluminal vesicles (ILVs), are tiny membrane microvesicles derived from endosomes [27, 28]. They are surrounded by a single outer membrane and secreted by all types of cells. It has been reported to be detected in saliva, tears, urine, plasma, semen, breast milk, cerebral spinal fluid, amniotic fluid, bronchial fluid, synovial fluid, serum, lymph, gastric acid, and bile [27]. Exosomes have only recently been established as functional vehicles capable of transporting a complex cargo of proteins, lipids, metabolites, and nucleic acids to target cells they encounter. Due to their ability to travel via physiological fluids

such as saliva, blood, urine, and cerebral spinal fluid, exosomes can reach distant recipient cells and potentially reprogram them. They can be taken up by the recipient cells, and the molecules they carry can affect how these recipient cells function and behave. In light of this, exosomes constitute a novel method of intercellular communication and may participate in a variety of cellular processes, including immunological response, signal transduction, antigen presentation, tissue repair, and tissue development [28].

History of Exosome Therapy: In 1983, two scientific papers were published in close succession, one in the *Journal of Cell Biology* (Harding et al., 1983) [29] and the other in *Cell* (Pan & Johnstone, 1983) [30]. These studies revealed that transferrin receptors were found to be associated with small vesicles measuring around 50 nm in diameter during the maturation process of blood reticulocytes. Interestingly, it was discovered that these vesicles were released from the reticulocytes into the extracellular space. A few years later, Rose Johnstone came up with the name "*exosome*" to refer to these specific extracellular vesicles (EVs). During that period, there was limited exploration into the therapeutic possibilities of exosomes [31]. These vesicles were thought to be involved in clearing the cell's cytoplasm of extracellular proteins. Additionally, they were classified as a method for removing unwanted components such as receptors, from the plasma membrane during the maturation of reticulocytes [32].

From the late 1990s to the early 2000s, researchers started to acknowledge the functional roles that exosomes play in a wide range of physiological and pathological processes. Raposo et al. made a significant discovery in 1996 when they demonstrated that EVs derived from immune cells can present antigens. This important discovery underlined the functional roles that EVs could potentially play across a range of biological processes. Subsequently, the discovery that EVs could potentially function physiologically, serve as biomarkers, and possess therapeutic benefits sparked a wave of interest and fascination within the field of EV research [33].

In the decade following the year 2000, researchers became progressively interested by the complexities of EVs. They studied the proteome and lipidome of these vesicles derived from all different cell types. Further research revealed that EVs had the ability to release cytokines, especially EVs originating from immune cells due to their prominent role in the function of the immune system. The growing fascination with EVs derived from tumours, combined with a

deeper understanding of their functionality within the immune system, has emphasized a promising pathway for their application in anti-tumour therapies. Moreover, recent entries in the scientific literature have begun to reveal the in-vivo functions of EVs, demonstrated by their potential to prevent and alleviate disease across multiple animal models [33].

From 2006 to 2008, there were many discoveries that demonstrated how EVs can transport RNA cargo to target cells [33]. In 2006, Ratajczak et al. conducted a study that revealed how microvesicles have the ability to reprogram hematopoietic progenitor cells. They made this discovery by observing how microvesicles can transfer both mRNA and protein cargo to these recipient cells. This finding highlighted the important functions of microvesicles as potential messengers of intercellular communication as well as mediators that play a role in the reprogramming of hematopoietic cells [34]. Furthermore, there was another study conducted by Valadi et al. that reported similar results. In their study, they revealed how exosomes have the capacity to transfer genetic material, particularly mRNA and microRNAs, from donor to recipient cells. Notably, this transfer of genetic material was found to have functional implications as it can alter the gene expression as well as influence the behaviours of these recipient cells. These ground-breaking discoveries really solidify the potential of exosomes as carriers of genetic information and hence, the important role they play in cell-to-cell communication [35].

Another noteworthy discovery was made in 2008 by Skog et al., who discovered that microvesicles derived from glioblastoma cells carry RNA and proteins that can actively promote the growth of tumours. In their study, they observed how these microvesicles derived from glioblastoma cells transfer their cargo to neighbouring cells and consequently altering their behaviour to promote tumour growth. Furthermore, this study also highlighted the potential ability of these microvesicles to serve as biomarkers for glioblastoma, offering a non-invasive approach to detect and monitor the disease. These discoveries continue to add to our understanding of the functional role of microvesicles as well as emphasize their potential for future therapeutic and biological applications [33].

Origin and Size: Exosomes are a subtype of EVs and range in size from 30 to 150 nm in diameter [28]. Exosome vesicles are formed through the process of inward budding of the

limiting membrane of early endosomes, which later mature and develop into late endosomes, which is also referred to as multivesicular bodies (MVBs) [27]. Following invagination of the late endosomal membrane, small ILVs begin to form within the large MVBs (Figure 5) [28]. MVBs will undergo one of two pathways: MVBs can either fuse with lysosomes and as a result, the cargo contained within the ILVs become degraded, or MVBs can fuse with the cell plasma membrane (PM) and release ILVs into the extracellular space as exosomes (Figure 5) [33].

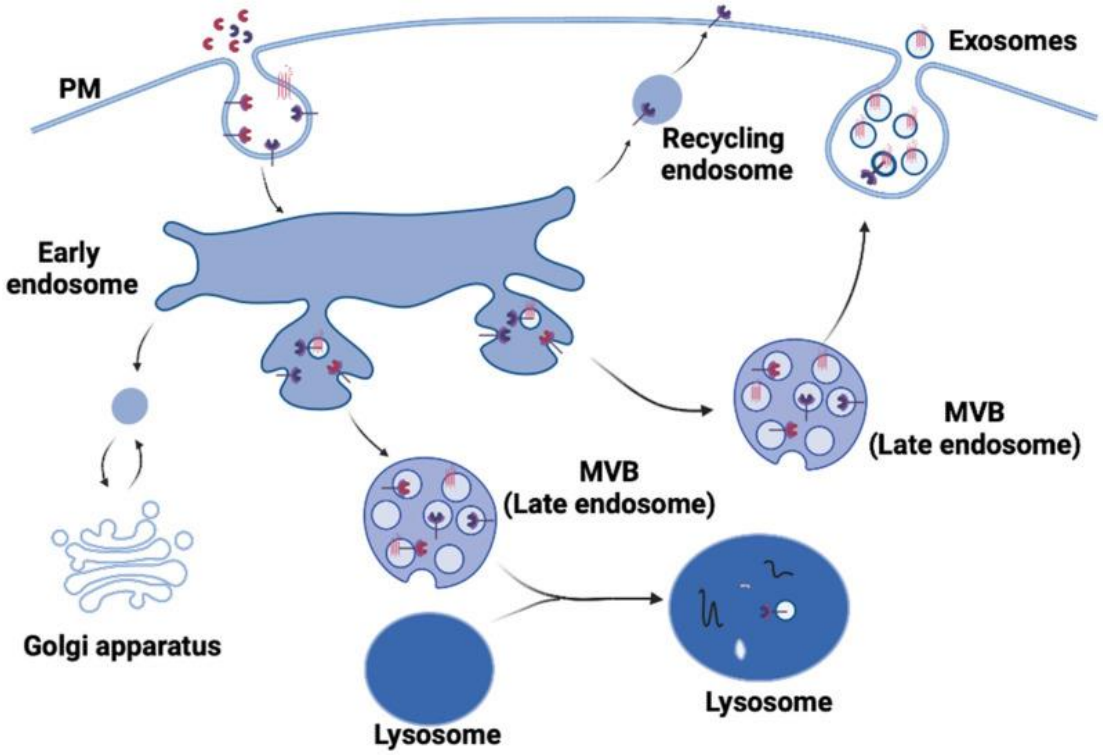


Figure 5. Diagram of the exosome system. Through endocytosis, cargos are sorted and transported to the early endosomes. The early endosomes commit to and undergo the endosomal maturation pathway which causes late endosomes to appear multivesicular (MVB). Exosomes are eventually released once the MVBs fuses with the cell PM. (Image from [33]).

Exosomes have emerged as a promising tool in the field of regenerative medicine for promoting regeneration and repair of tissues [34]. Exosomes can stimulate regeneration through several mechanisms:

Cell-to-cell Communication: Exosomes function as important messengers, carrying specific bioactive molecules (i.e. proteins, lipids, nucleic acids, mRNA, microRNAs, metabolites, growth hormones, etc.) and transferring them from donor to recipient cells. Exosomes may impact the behaviour and function of the recipient cells by transmitting these bioactive molecules which in turn, support tissue regeneration. For example, the delivery of RNAs through the fusion of exosomes with recipient cells contributes to the regulation of various aspects of the cell's behaviour and phenotype. This includes controlling the cell cycle, influencing its susceptibility to apoptosis, facilitating migration, modulating inflammation, and promoting angiogenesis [35]. Different endocytic mechanisms, including clathrin-mediated endocytosis, clathrin-independent endocytosis, caveolin-dependent endocytosis, phagocytosis, and micropinocytosis can be used by exosomes to interact with recipient cells [36, 37]. The chosen route is dependent on the expression of proteins on the surface of exosomes as well as the proteins found within the exosomes [36].

Stem Cell Activation: Exosomes derived from stem cells, such as MSCs, have been reported to activate and differentiate resident stem cells within tissues which in turn, promote tissue repair and regeneration. Exosomes have the ability to bind to specific stem cells and regulate their activation and differentiation through increasing intracellular communication [38].

Anti-inflammatory Effects: Exosomes have anti-inflammatory properties as well as the potential to modify the immune response. Numerous studies have demonstrated their ability to diminish the levels of several pro-inflammatory mediators and cytokines such as NF-κB, IL-1β, IL-6, IL-8, IL-18, COX-2, MMP1, MMP3, MMP9, iNOS, CXCL, IFN-γ, and TNF-α. Suppression of these pro-inflammatory mediators and cytokines leads to a reduction in inflammation [41]. This anti-inflammatory effect contributes to establishing a favourable environment for tissue regeneration [49]. Moreover, owing to their nature as cell-free therapies, exosomes induce lesser immune responses and exhibit lower toxicity in contrast to cell-based therapies [39].

Angiogenesis Promotion: Exosomes possess the remarkable ability to stimulate angiogenesis—a vital biological process involving the formation of new blood vessels from pre-existing ones. This phenomenon holds immense importance in tissue regeneration, as blood vessels are crucial conduits of nutrients and oxygen to regenerating tissue while also aiding in the removal of waste. Within exosomes, a reservoir of pro-angiogenic factors may exist, effectively shuttling them to specific area, thereby contributing to the establishment of new blood vessels. In addition, these microscopic messengers have been observed to participate in fostering angiogenesis throughout the progression of cancer. They achieve this by transporting pro-angiogenic biomolecules such as microRNAs, vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMPs). Beyond this, exosomes contribute to the promotion of angiogenesis by reducing the production of HIF-1, a protein recognized for its role in inhibiting the angiogenic process [42].

Extracellular Matrix Remodeling: Exosomes have the ability to influence the remodelling of the extracellular matrix, which functions as a basic framework for connective tissues, offering cells both mechanical reinforcement and chemical sustenance. They can stimulate the synthesis of new extracellular matrix components, including adhesion molecules, while also regulating the activities of various enzymes involved in tissue remodelling. This process of remodelling assumes pivotal significance in enabling tissue regeneration and repair [42].

The use of exosomes as therapy has gained considerable attention and has been explored in various areas of medicine:

Regenerative Medicine: In 2015, Zhao et al. embarked on a study to explore the potential of using exosomes derived from human umbilical cord MSCs (hUC-MSCs) to address acute myocardial ischemia (AMI). Through the use of a rat model of myocardial ischemia, the researchers delved into the therapeutic effects of these exosomes. The outcomes revealed that the introduction of exosomes derived from hUC-MSCs into ischemic hearts yielded a notable decrease in the mortality of myocardial cells, an augmentation in cardiac function, and a further boost in angiogenesis stimulation. These findings underscore the remarkable regenerative capacity of exosomes derived from hUC-MSCs within the realm of cardiovascular diseases. The

study not only suggests the potential of these exosomes as a regenerative approach for managing AMI-induced injuries but also presents them as a therapeutic strategy to expedite the process of heart repair and recovery [43].

Neurological Disorders: Back in 2011, Alvarez-Erviti et al. embarked on a study that revolved around using specialized exosomes to transport small interfering RNA (siRNA) to the brain of mice. The core objective of the researchers was to explore the feasibility of using exosomes as vehicles to transport therapeutic agents to the brain. To achieve this, they engineered exosomes derived from dendritic cells by incorporating a brain-targeting peptide on their surface and loading them with siRNA molecules tailored to target a specific gene. The outcomes of the study demonstrated the successful transportation of siRNA molecules to the brain via these modified exosomes after being introduced systemically. This process led to the suppression of the targeted gene within brain cells. This investigation not only highlighted the potential of exosomes as a promising avenue for delivering therapeutic compounds, such as siRNA molecules to the brain, but also reveals new prospects for devising treatments for neurological disorders [43].

Cancer Therapy: Recently, Li et al. undertook a study with the objective of creating personalized cancer immunotherapies. Their approach involved using exosomes derived from dendritic cells, loaded with neoantigens. The researchers set out to forge a novel method for inducing a strong immune reaction against cancer cells, using neoantigens that were specific to each patient's tumour. These personalized neoantigens were then loaded into the exosomes derived from dendritic cells. These exosomes were then subjected to testing to determine their potential in inciting a strong immune response against cancer cells. The results of the study demonstrated that exosomes carrying these patient-specific neoantigens effectively stimulated immune cell activity, initiating an immunological response against cancer cells. This study highlighted the possibility for a personalized approach to cancer immunotherapy using exosomes that contain patient-specific neoantigens, opening up new avenues for the advancement of effective therapies for cancer [44].

Immune Disorder: Li et al. conducted a study in 2018 to investigate the therapeutic potential of MSC-derived exosomes in reducing inflammation and demyelination in the CNS of rats with experimental autoimmune encephalomyelitis (EAE), an immunological disorder. The study focused on the effects of MSC-derived exosomes on the polarization of microglia, which are immune cells present in the CNS. The findings showed that MSC-derived exosomes successfully decreased inflammation and demyelination in the CNS of EAE rats and that this beneficial effect was attributed to the exosomes' ability to modulate microglia polarization toward an anti-inflammatory state. These findings emphasize MSC-derived exosomes' intriguing therapeutic potential in modulating immunological responses and assisting tissue repair in immune diseases affecting the CNS, suggesting their potential use in the treatment of such conditions [44].

Cardiovascular Diseases: Exosomes derived from cardiac progenitor cells (CPCs) have been investigated in 2019 for their capacity to prevent apoptosis induced by coxsackievirus B3 (CVB3) in the context of viral myocarditis. Li et al. sought to understand the mechanisms by which CPC-derived exosomes impact CVB3 proliferation and alter signalling pathways including mechanistic target of rapamycin (mTOR). The researchers discovered that CPC-derived exosomes efficiently reduced CVB3-induced apoptosis through inhibiting CVB3 proliferation and regulating the mTOR signalling pathways. These findings highlighted the remarkable therapeutic potential of CPC-derived exosomes in preventing cardiac cell death caused by CVB3 infection, giving vital insights into their prospective utility in the treatment of viral myocarditis and other cardiovascular diseases [39].

1.10 Mechanisms of Action Underlying Exosome Therapy in Sjögren's Syndrome:

The mechanism underlying the role of exosomes in SS is currently the subject of active investigation and extensive exploration. Promising research have demonstrated that exosomes derived from various cell sources, including MSCs or SG epithelial cells (SGECs), exhibit potential therapeutic effects in SS [40].

In a pivotal study conducted in 2005, Kapsogeorgou et al. made a significant observation that both SGEC lines of SS and non-SS patients secreted exosomal vesicles to a similar degree. These SGEC-derived exosomes contained substantial amounts of cytoskeletal proteins specific to epithelial cells, as well as autoantigens such as anti-Ro/SSA, anti-La/SSB, and Sm ribonucleoproteins. While the secretion of these exosomes was not exclusive to SS-derived cells, this ground-breaking discovery marked the first identification of SS-specific autoantigens within exosomes. These findings suggest that exosomes may contribute to the presentation of intracellular autoantigens to autoreactive lymphocytes, potentially playing a role in the development of SS [41]. Additionally, Cortes-Troncoso et al. reported that the transfer of exosomes derived from T-cells carrying miR-142-3p into SGECs could influence intracellular Ca2+ signaling and decrease cyclic adenosine monophosphate (cAMP) production. Such disruptions in signaling pathways have the potential to induce dysfunction in glandular cells, potentially contributing to the development of SS [42].

Currently, the research focus on exosomes in SS predominantly revolves around the analysis of tears and saliva, as these biofluids offer a convenient and non-invasive means of sample collection. The ease and safety of obtaining these fluid samples have facilitated extensive investigations into the exosomal content and potential biomarkers associated with SS. However, considering the multi-organ involvement in SS, it is imperative to extend the investigation of exosomes to other tissues and organs to attain a comprehensive understanding of their involvement in the disease pathology. Such explorations would contribute to unraveling the complex role of exosomes in SS and provide valuable insights for developing effective diagnostic and therapeutic strategies [40].

Accurate diagnosis plays a pivotal role in the management of SS due to several factors of significance. SS presents as a multifaceted autoimmune disease, often sharing symptoms with other disorders like RA, which complicates arriving at a conclusive diagnosis. Currently, the diagnostic process predominantly hinges on subjective evaluations and the detection of specific autoantibodies such as anti-Ro/SSA and anti-La/SSB. However, these criteria may not adequately detect early-stage or atypical cases, highlighting the crucial need for more sensitive and specific diagnostic tools capable of early detection and differentiation from similar conditions. Consequently, the development of advanced diagnostic techniques becomes

paramount in enhancing the accuracy and efficiency of diagnosing SS. This, in turn, enables enhanced patient care and more effective management [46].

In 2017, Aqrawi et al. undertook a study that focused on the isolation of EVs, including exosomes and microvesicles, from the saliva and tear fluids of individuals with SS. Their objective was to identify potential biomarkers within these EVs using advanced liquid chromatography-mass spectrometry (LC-MS) [49]. Likewise, Michael et al. led the way in isolating exosomal microRNAs (miRNAs) from the parotid saliva of SS patients. Their ground-breaking study suggested that the composition of miRNAs present in salivary exosomes might serve as diagnostic markers for various SG diseases, including SS [50]. In alignment with these discoveries, Alevizos et al. validated the reliability of SG miRNA expression profiles in differentiating SS patients from healthy individuals. These findings underscore the promising potential of miRNAs as markers for identifying inflammation or dysfunction in SGs associated with SS [51].

These findings add to our understanding of how exosomes contribute to the evolution of SS, underlining the promise of exosomes and miRNAs as potential indicators for both detecting and predicting the advancement of this autoimmune disorder. Nevertheless, additional research is imperative to gain a more comprehensive understanding of the fundamental mechanisms through which exosomes function in the context of SS and to validate their effectiveness as components for diagnostics and potential treatment strategies [46].

1.11 Previous Studies Utilizing Exosomes as Therapeutic Agents in Sjögren's Syndrome:

The management of SS is a complex endeavor that requires a comprehensive, multifaceted approach encompassing various strategies. These include the use of saliva substitutes and artificial tears, alongside drug prescriptions like corticosteroids, nonsteroidal anti-inflammatory drugs (NSAIDs), and hydroxychloroquine. In addition, although immunosuppressive drugs like methotrexate, mycophenolate mofetil, and biological agents have shown to be effective, their prolonged use requires careful consideration owing to their potential adverse effects. This concern has sparked significant interest in the exploration of innovative therapeutic approaches for SS, aiming to enhance efficacy and patient satisfaction. Among these

emerging strategies, exosomes have gained considerable attention as highly promising candidates, motivating the need for more extensive research [46]. As previously mentioned, exosomes play a crucial role in promoting tissue regeneration and repair, attributed to their unique ability to execute cell-to-cell communication, remodel the extracellular matrix, activate and differentiate stem cells, promote angiogenesis, and exhibit anti-inflammatory properties [36-42, 49]. Moreover, exosomes have demonstrated encouraging results across a spectrum of medical domains including regenerative medicine, neurological disorders, immune disorders, cancer treatment, and cardiovascular diseases [43-45, 50, 51].

Extensive research has been dedicated to exploring the potential application of exosomes in the context of SS, driven by the growing interest in their use as a treatment strategy. Exosomes have been studied as a potential therapeutic intervention for SS in several notable studies [46].

In 2021, Li et al. conducted a study using a mouse model of SS to delve into the potential therapeutic effects of exosomes derived from labial gland MSCs (LG-MSCs). The findings of the study presented compelling evidence of the effectiveness of LG-MSCs and their exosomes in mitigating SS symptoms. This positive outcome was attributed to their capacity for regulating the delicate equilibrium between Treg and Th17 cells. This remarkable modulation of immune cell populations played a critical role in reducing inflammation and enhancing SG function [52].

During that very year, Rui et al. initiated a research project using mice with experimental SS (ESS). Their aim was to explore the therapeutic potential offered by exosomes derived from olfactory ecto-MSCs (OE-MSC-Exos). The results they obtained indicated a significant alleviation of SS symptoms when OE-MSC-Exos were administered. This positive impact was attributed to the capacity of OE-MSC-Exos to induce modifications in the behaviour of myeloid-derived suppressor cells (MDSCs). The researchers observed a significant enhancement in the suppressive capabilities of MDSCs upon treatment with OE-MSC-Exos, as evidenced by the upregulation of arginase expression and the elevation of reactive oxygen species (ROS) and nitric oxide (NO) levels. Notably, IV administration of OE-MSC-Exos exhibited a substantial impact on attenuating disease progression and restoring MDSC function in ESS mice, thereby underscoring the promising potential of OE-MSC-Exos as a therapeutic intervention for the treatment of SS [43].

In the following year, a group of researchers from the Kagoshima University in Japan sought to identify non-invasive diagnostic markers for SS. They collected mouth rinse samples from SS patients and healthy volunteers to investigate exosome-derived miRNAs as potential biomarkers. Using microarrays and PCR, they identified 12 miRNAs as potential candidates, with 4 showing significantly higher expression in the SS group. Logistic regression analysis highlighted the greater influence of miR-1290 and let-7b-5p in the SS group. Combining these miRNAs produced a diagnostic formula that effectively distinguished SS from healthy controls, achieving a sensitivity of 91.7%, specificity of 83.3%, positive predictive value of 84.6%, and negative predictive value of 90.9%. These findings proposed that increased levels of miR-1290 and let-7b-5p in mouth rinse exosomes could serve as a novel and non-invasive diagnostic marker for SS, representing a significant advancement in diagnosing and screening of the disease [44].

Xing et al. reported in the same year that LG-MSC-derived exosomes contained miRNA-125b, which can relieve SS symptoms in NOD mice. They discovered that miRNA-125b targeted and suppressed PRDM1, a gene involved in plasma cell development and autoantibody generation. In the NOD mice, treatment with LG-MSC-derived exosomes enriched with miRNA-125b reduced plasma cell infiltration, autoantibody generation, and enhanced glandular function. These findings demonstrated that exosomes derived from LG-MSCs can transport miRNA-125b to target and repress plasma cells, hence slowing the development and progression of SS [45].

A team of researchers based in Shanxi, China, conducted a study in 2023, aiming to explore the impact of exosomes derived from umbilical MSCs (UMSCs) on CD4+ T-cells within patients diagnosed with pSS. The outcomes of their investigation revealed significant immunosuppressive effects of UMSC-derived exosomes in individuals with pSS. These effects were characterized by the successful suppression of CD4+ T-cell activation and proliferation, marked by a decrease in activation markers on these T-cells. Furthermore, the exosomes induced the differentiation of CD4+ T-cells into Treg cells, which hold a crucial role in the regulation of immune responses. Notably, the exosomes derived from UMSCs also hindered the production of pro-inflammatory cytokines by CD4+ T-cells, suggesting their potential as agents with anti-inflammatory properties. These findings underscore the potential of UMSC-derived exosomes to modulate CD4+ T-cells' immune responses in individuals with pSS, thereby presenting new and

promising pathways for the regulation of immune responses and the alleviation of inflammatory processes associated with pSS [52].

In the same year, Du et al. investigated the therapeutic potential of exosomes derived from stem cells from human exfoliated deciduous teeth (SHED) in the context of SS-induced hyposalivation. As per the researchers' observations, exosomes derived from SHED exhibited a significant capacity to alleviate hyposalivation in NOD mice. Their investigation revealed that these exosomes triggered an elevation in the expression of ZO-1, a crucial protein responsible for maintaining tight junctions and thus SG function. This effect was attributed to the activation of the Akt/GSK-3/Slug signaling pathway, which consequently led to the heightened expression of ZO-1. Overall, these findings serve as evidence of the potential utility of SHED-derived exosomes as a therapeutic intervention to enhance SG functionality in individuals with SS. This potential is rooted in their ability to regulate the Akt/GSK-3/Slug-mediated pathway responsible for ZO-1 expression [53].

Another study conducted by Hu et al. in 2023 explored the revitalizing impact of exosomes derived from dental pulp stem cells (DPSC-Exos) on the functioning of SG epithelial cells (SGECs) in NOD mice. Their research discovered significant revitalizing effects of DPSC-Exos on SGEC functionality within the NOD mice model. This revitalization was intricately tied to the activation of the G protein-coupled estrogen receptor (GPER), which in turn initiated the cAMP/PKA/CREB signaling pathway. This activation cascade yielded an array of favourable outcomes, including heightened cell proliferation, reduced apoptosis, elevated secretion of proteins linked to saliva production, and an overall enhancement in glandular function. These observations suggest the potential role of DPSC-Exos as a prospective pathway for treating and restoring SG functionality in individuals with SS. This potential hinges on the modulation of the GPER-mediated cAMP/PKA/CREB signaling pathway [54].

While the current body of literature on exosome-based therapies in SS is rather limited, these initial findings establish a starting point for future investigations and offer substantial potential for pioneering innovative therapeutic strategies in addressing this complex autoimmune disorder. These preliminary findings not only lay a solid scientific foundation, but also provide optimism for future advances in exosome-based therapies [46].

Author	Year	Sources of Exosomes	Technique (i.e. protein concentration)	Route of Administration
Li et al. [46]	2021	Human Labial Gland-Derived MSCs (LG-MSCs)	50 μg/mouse	IV into the tail vein on alternate days for two weeks
Rui et al. [43]	2021	C57BL/6 mice Olfactory Ecto-MSCs (OE-MSCs)	100 μg/mouse	IV into the tail vein twice
Xing et al. [45]	2022	Labial Gland-Derived MSCs (LG-MSCs)	50 mg diluted in 200 mL PBS	IV into the tail vein on alternate days for 14 days
Du et al. [47]	2023	Stem Cells Derived from Human Exfoliated Deciduous Teeth (SHED)	50 mg diluted in 25 μL PBS	Subcutaneous injection into the SMGs at multiple points
Hu et al. [48]	2023	Human Dental Pulp Stem Cells (DPSCs)	25 mg/kg body weight of mouse	IV into the tail vein once a week for 10 weeks

Table 1. Summary of previous studies using exosomes as therapeutic agents in Sjögren's syndrome. The studies are presented in chronological order from the earliest to most recent. Exosomes can be derived from a range of sources. These sources include MSCs derived from human LG-MSCs, C57BL/6 OE-MSCs, SHED, and human DPSCs.

1.12 Rationales of the Study

To the best of our knowledge, no previous studies have delved into the potential therapeutic applications of BM MSC-CM and its derived exosomes in the context of treating SS-like disease in NOD mice. Recognizing this gap in knowledge, we embarked on this study with the explicit intention of investigating the efficacy of using mouse BM MSC-CM and its derived exosomes as therapies for treating SS-like disease and to evaluate their influence on the restoration of SG function in NOD mice. In addition, we sought to analyze the extent of lymphocytic infiltration through histopathological examination of serial H&E-stained sections from the SMG and PG, as well as regular monitoring of the blood glucose levels in NOD mice to assess the potential onset of diabetes.

The specific aims of our study are,

I. Assess the blood glucose profiles of the NOD mice across all experimental groups:
    I. Monitor blood glucose levels in NOD mice from 11 to 16 weeks of age, identifying instances of diabetes.

II. Investigate Salivary Flow Rate (SFR) in NOD Mice across all experimental groups:
    I. Quantify the SFR of the mice to assess the functionality of their SGs.
    II. Explore potential correlations between SFR and blood glucose levels, delving into the impact of SS on SG function.

III. Evaluate the severity of lymphocytic infiltration in SGs of the NOD mice in all experimental groups:
    I. Perform histopathological analysis of serial H&E-stained sections of the SMG and PG SGs from the mice.
    II. Utilize the focus score system to assess the severity of lymphocytic infiltration in the SMG and PG SGs of the mice.
    III. Compare the focus scores among mice in distinct experimental groups, aiming to establish potential connections between SS and the level of SG inflammation.

2. Materials & Methods

2.1 Animal Model

All experimental procedures were performed following the guidelines imposed by the Canadian Council on Animal Care. Our protocol (5330) was approved by the University Animal Care Committee (UACC) at the McGill University.

2.2 Recipient

Four-week-old female NOD mice with SS-like disease purchased from Taconic Biosciences (Albany, NY, USA) were randomized into 3 groups. Control: NOD mice treated with normal saline (n = 19), Group 2: NOD mice treated with MSC-CM (n = 10), Group 3: NOD mice treated with MSC-CM- derived exosome (n = 21).

2.3 Blood Glucose Monitoring

From 11 weeks of age onward, fasting blood glucose levels of all mice were monitored once a week using the Accu-Check® (Roche) system, with blood samples taken from the middle third of their tails. Mice with diabetes (blood glucose > 250 mg/dL) received subcutaneous insulin administration and were checked daily thereafter. Mice with borderline glucose levels (150–250 mg/dL) were monitored twice a week. Mice with normal glucose levels were monitored weekly until hyperglycemia was detected, at which point the above protocol was implemented.

Figure 6. Image of blood sample taken for blood glucose monitoring. This NOD mice had a blood glucose level reading of 5.4 mmol/L (98 mg/dL) which indicated the mice does not have diabetes.

Figure 7. Image of insulin used to treat mice with diabetes. The insulin used was *Humulin N* from Eli Lilly and Company. Prior to injection using a 0.5 mL BD™ U-100 insulin syringe with a 28 G x 1/2-inch gauge, the middle third of the tail was sterilized with an alcohol swab.

2.4 Cell Preparation

Mouse BM MSCs were obtained from a liquid nitrogen tank. Prior to this, the MSCs were harvested from 8-week-old male C57BL/6 mice by our lab and cryopreserved in liquid nitrogen until needed for future experiments. The differentiation capacity and the flow cytometry of these cells had formerly been characterized. Subsequently, the MSCs were cultured in a mixture of alpha Dulbecco's Modified Eagle's Medium (αDMEM), low glucose, 10% Fetal Bovine Serum (FBS), and 1% antibiotic-antimycotic, and maintained at 37°C, 5% CO2, and 95% air. Upon reaching 80% confluency and assuming a spindle shape, the cells were passaged at a 1:3 ratio. Passaging was conducted to enrich the MSCs, as non-MSCs and hematopoietic cells tend to adhere strongly to the culture vessels. Once the cells reached passage 8, they were detached and prepared for treatment.

2.5 Preparation of MSC-CM Treatment

After achieving 80% confluence, the BM MSCs were replenished with serum-free αDMEM (Gibco, Rockville, MD, USA) containing 2% antibiotic-antimycotic solution. The cell-cultured CM was collected after 48 hours of incubation (at 37°C, 5% CO2, and 95% air), centrifuged at 2000 x g for 30 minutes to remove cells and debris, and filtered using 0.22 μm pore filters. The MSC-CM was stored at -80°C until experimental use.

2.6 Isolation of MSC Exosomes and Preparation of MSC-CM-Exo Treatment

The Total Exosome Isolation Reagent (Invitrogen™ by Thermo Fisher Scientific) was used for the isolation and recovery of intact exosomes from the prepared MSC-CM. This reagent demonstrates a high efficiency in precipitating less-soluble components, specifically vesicles, from the solution through a mechanism that involves binding water molecules. To achieve this, 0.5 volumes of the Total Exosome Isolation reagent were added to a tube, proportionate to the volume of the MSC-CM (e.g. 10mL of MSC-CM + 5mL of reagent). The MSC-CM/reagent mixture was thoroughly mixed until a homogenous solution was achieved, and subsequently incubated at 2-8°C overnight. The precipitated exosomes were then recovered using standard centrifugation at

10,000 x g for 1 hour. Following centrifugation, the supernatant was carefully discarded, leaving the exosomal pellet which was subsequently resuspended with normal saline. The isolated exosomes were then stored at -80°C until experimental use.

2.7 Injection of Normal Saline, MSC-CM, or MSC-CM-Exo

We used 4-weeks-old NOD female mice (Taconic Biosciences [Albany, NY, USA]) as the experimental model. The NOD mouse is an autoimmune strain that develops similar autoimmune characteristics and glandular manifestations, mimicking the human disease progression of SS [49]. Once the mice reached 8-weeks-old, they were divided into three treatment groups, each receiving intravenous injections (IV) twice a week for two weeks: Control: NOD mice treated with normal saline (volume: 100 µl) Group 2: NOD mice treated with MSC-CM (volume:100 µl, protein concentration: 2mg/mL), Group 3: NOD mice treated with MSC-CM- derived exosome (volume: 100 µl, protein concentration: 1mg/mL)

2.8 Secretory Function of the Salivary Glands (Salivary Flow Rate: SFR)

The assessment of SG secretory function (Salivary Flow Rate: SFR) in NOD mice involved the induction of mild gas anesthesia using 1.5–3% isoflurane, 5% halothane, and 1 L/min oxygen. Subsequently, salivary flow was stimulated by subcutaneously injecting 1.0 mg pilocarpine/kg of mouse body weight in the dorsal side of the neck. Whole saliva was collected from the oral cavity using a micropipette placed into a pre-weighed 0.5 mL microcentrifuge tube at the corner of the mouth (Figure 8). Saliva collection, starting 5 minutes after the pilocarpine injection, spanned a 10-minute duration. Saliva volume was determined gravimetrically and stored in a -20°C freezer. The baseline SFR was measured at 8 weeks of age (week 0), followed by measurements at 4- and 8-weeks post-treatment.

Figure 8. Image of mouse during saliva collection of the SFR procedure. The mice are sedated with a mild gas anesthesia while a 0.5 mL microcentrifuge tube is placed at the corner of the mouth for saliva collection. The mice are sedated for approximately 15 minutes, consisting of 5 minutes following the pilocarpine injection to allow for activation and an additional 10 minutes allocated for saliva collection.

2.9 Focus Score

In order to evaluate inflammation, focus scores were analyzed. The focus score is calculated as the number of lymphocytic infiltrates per 4 mm^2 area, where a focus is defined as an accumulation of ≥ 50 lymphocytes. This assessment involved utilizing microscopic examination of serial H&E-stained histological sections obtained from the SGs, particularly the SMGs and PGs, which were cut at multiple levels to ensure a thorough and comprehensive evaluation.

2.10 Characterization of MSC Exosomes

Exosome characterization is crucial because it confirms that exosomes are present in the experimental sample, gives a thorough understanding of their biological roles, and assures the reliability and reproducibility of experimental data. In order to achieve this, we performed three widely used techniques: dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), and transmission electron microscopy (TEM) in order to confirm the presence of exosomes in the MSC-CM-Exo treatment. The use of TEM, NTA, and DLS allows us to effectively characterize the exosomes, providing precise insights into their structural characteristics such as size distribution and morphology. These techniques greatly enhanced the scientific integrity and reliability of our experimental study.

2.11 Nanoparticle Tracking Analysis (NTA) of MSC Exosomes.

Particle tracking analyzes were performed using an NTA 3.4 build 3.4.003 system, which was equipped with a Blue488 nm laser. To prepare the samples, they were diluted tenfold. The camera utilized was an sCMOS set at level 16, with the slider shutter and slider gain adjusted to 1300 and 512, respectively. The frame rate was set to 25.0 frames per second, and a total of 1498 frames were captured during the analysis. The temperature recorded during the process ranged from 26.3 to 26.4°C, and the syringe pump speed was set to 100. All the analyzes were

conducted using the Nanosight 3.0 software, revealing an average particle size of **104.1 ± 1.5 nm.**

2.12 Transmission Electron Microscope (TEM) Analysis of MSC Exosomes

The TEM assay uses formvar-carbon-coated grids. For 15 minutes, a 20 L exosome suspension was put directly onto a carbon grid with the carbon coating facing upwards. Following that, the grids were washed three times with wash buffer, each for 30 seconds. Subsequently, they were stained for 15 minutes with a 2% uranyl acetate buffer, followed by another triple wash of 30 seconds each with the wash buffer. The TEM procedure was carried out after the grids had been allowed to air-dry overnight at room temperature [77].

2.13 Dynamic Light Scattering (DLS) of MSC Exosomes

A DLS analysis was carried out using a Protein Solution DynaPro instrument equipped with the Dynamics (Version 6) software (Wyatt Technology Corp., Santa Barbara, CA). For each exosome measurement, 40 readings of 10-second were recorded in triplicates. The autocorrelation function, which involves convolving the intensity with itself over time, was used to analyse the fluctuations in scattered light intensity. The translational diffusion coefficients of the exosomes were estimated by analyzing the decay of the intensity autocorrelation function in real-time. The hydrodynamic radius (Rh) of the scattering exosomes was determined from the diffusion coefficient using the Einstein-Stokes equation and a hard sphere model (rigid, non-deformable particles). Following that, the particle sizes and mass distribution were plotted. The light source in this DLS instrument was a semiconductor diode laser with an 830 nm wavelength. The scattering angle was measured at 90° relative to the incident laser beam. The sample's scattered light was collected and directed through fiber optics to an actively quenched solid-state single photon counting detector, which transformed photons into electrical pulses and correlated the data.

2.14 Statistical Analysis

Group means and standard deviations were calculated for each measured parameter. To determine statistical significance, one-way ANOVA test by IBM SPSS Statistics Standard

Campus Edition 29 was performed for the control-, MSC-CM- and MSC-CM-Exo-treated groups. A $p \leq 0.05$ was considered to be statistically significant.

3. Results

3.1 MSC-CM and MSC-CM-Exo Preserved Salivary Gland Functions.

SFR serves as an objective measure of glandular function and holds significant value in assessing treatment efficacy. SFR measurements were taken at three consecutive time points: before treatment at week 0 (8 weeks old), and then at 4 and 8 weeks post-treatment. Analysis of SFR revealed that untreated control NOD mice exhibited continuously low SFR throughout the duration of study, reaching its lowest rate at 4 weeks post-treatment (12 weeks old). Conversely, the groups treated with MSC-CM and MSC-CM-Exo exhibited higher SFR values compared to the control group at all examined time-points post-treatment. Statistical analysis revealed that SFR levels of the MSC-CM-Exo treatment groups were significantly higher at 4- and 8-weeks post-treatment compared to the control group with $p < 0.05$.

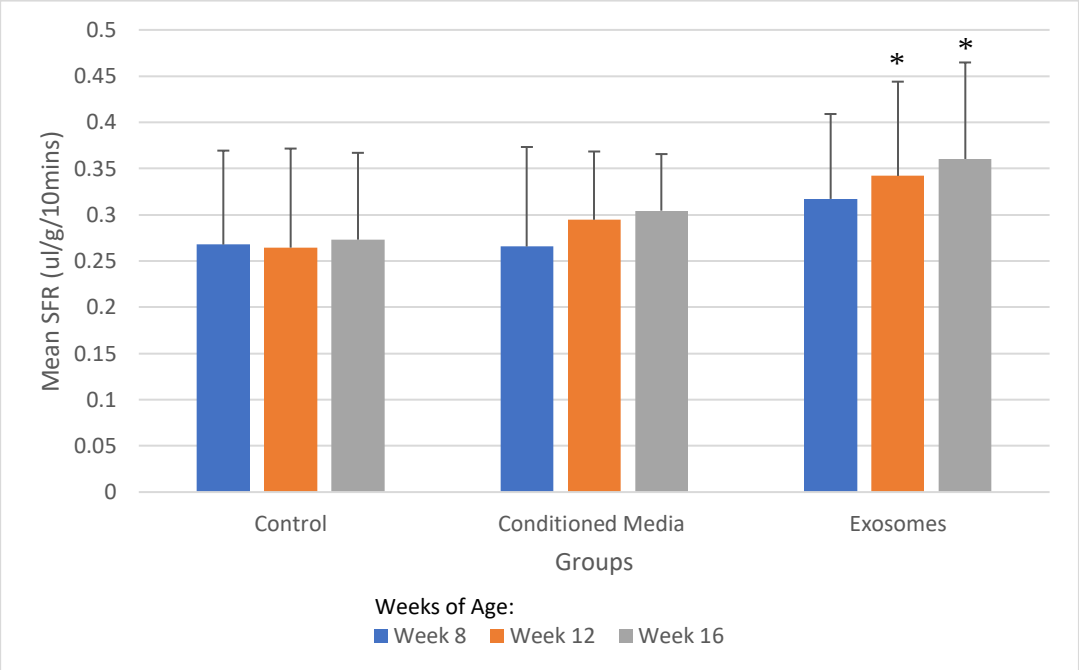


Figure 9. Salivary Flow Rate (SFR) used to represent salivary gland function. SFR was assessed pre-treatment at week 0 (8-week-old) then 4- and 8-weeks post-treatment. SFR was calculated as the volume of saliva per gram of body weight during a 10-minute period. The

control group showed a persistently low SFR, while the MSC-CM- and MSC-CM-Exo-treated groups showed a progressive increase in SFR with each measurement, surpassing the SFR of the control group. * $p < 0.05$. All data were presented as mean ± S.D. Control: normal saline treated.

3.2 None of the mice who received MSC-CM or MSC-CM-Exo treatments exhibited diabetic manifestations over the course of the study.

All mice were normoglycemic at the start of the experiment, at 8 weeks of age (week 0). Control mice (i.e. only receiving injections of normal saline; no MSC-CM or MSC-CM-Exo) developed diabetes during the course of the experiment and 52.6% of them developed diabetes by 8 weeks post-treatment ($p < 0.05$). Two mice in the control group developed diabetes 3 weeks post-treatment. All mice in the two treatment groups (MSC-CM and MSC-CM-Exo), however, showed a relatively stable blood sugar level and survived throughout the observation period of 8 weeks post-treatment (16 weeks of age). Continuing through the course of the study, a greater incidence of diabetes emerged within the control group, culminating in the mortality of three mice at the 8-week post-treatment observation period, which also marks the end of the study.

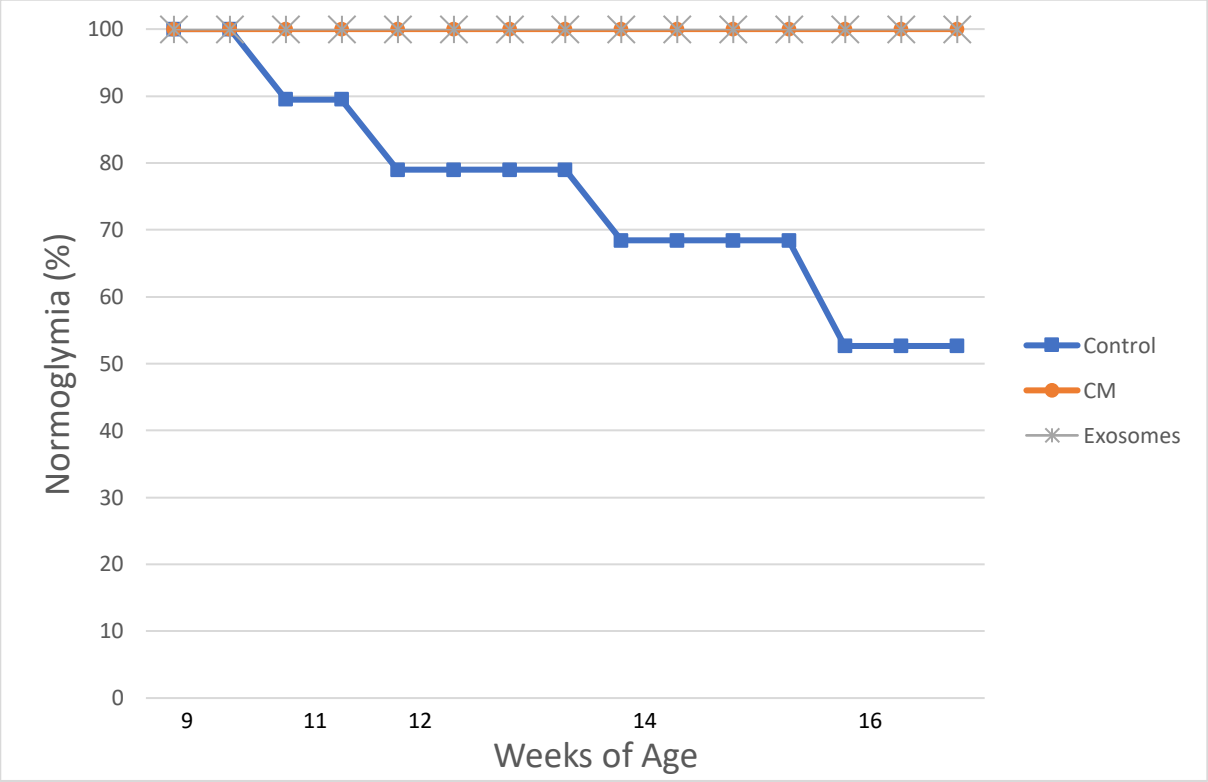
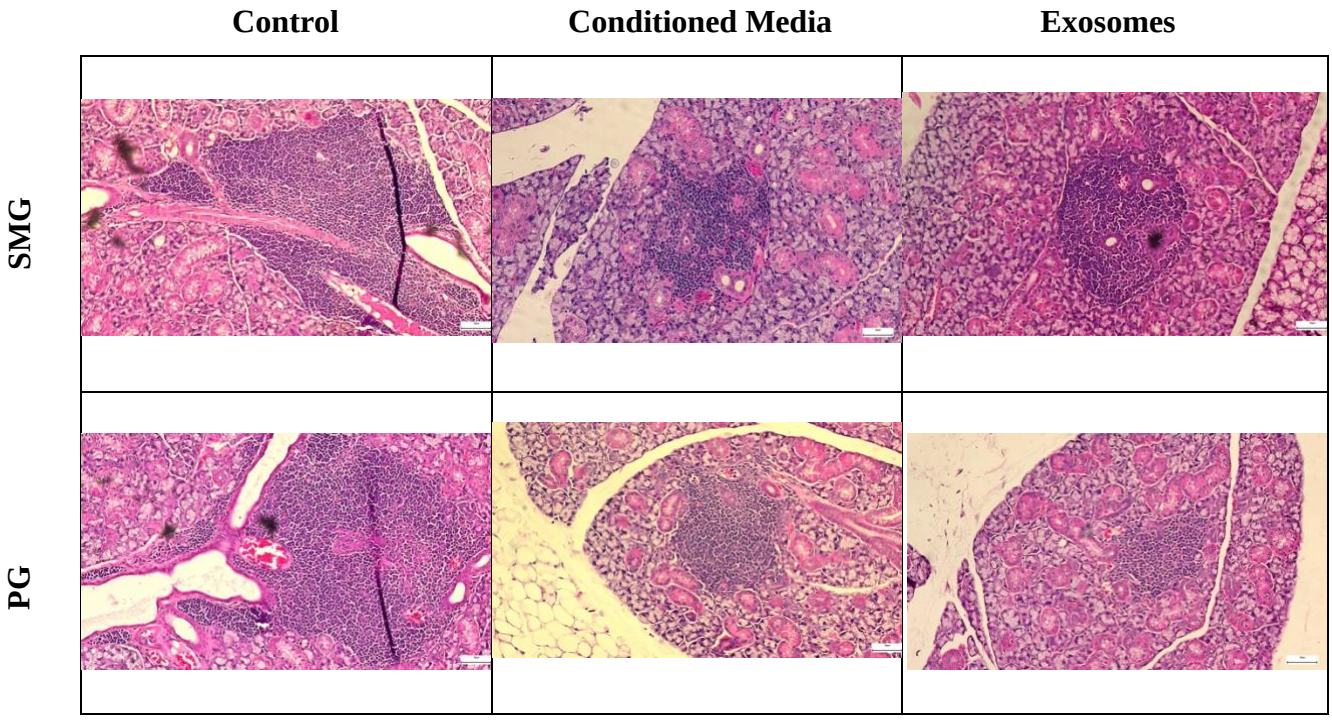


Figure 10. Kaplan–Meier plot for normoglycaemia in control (blue square), MSC-CM (orange circle), and MSC-CM-Exo (grey star) NOD mice monitored for 16 weeks (up to 8 weeks post-treatment). All mice were normoglycemic at the start of the experiment, at 8 weeks of age (week 0). The first diabetic mouse was diagnosed in the control group (blue square) at 3 weeks post-treatment (11 weeks of age) and 52.6% of mice in this group developed diabetes within 8 weeks post-treatment (16 weeks of age) ($p < 0.05$). Mice in the treated groups (orange circle and grey star) on the other hand, had a stable level of blood sugar throughout the course of the study.

3.3 MSC-CM and MSC-CM-Exo Immunomodulatory and Immunosuppressive Functions Were Evidenced by A Decrease in Lymphocytic Influx.

The therapeutic efficacy against autoimmune diseases relies on its ability to intricately modulate immune dysregulation through targeted modulation of pathogenic cells while concurrently preserving the integrity of the remaining immune system and restoring peripheral tolerance [50]. To gauge the extent of lymphocytic infiltration, we performed histopathological analysis using serial H&E-stained sections of the SMGs and PGs, wherein the findings were quantified as the focus score (number of lymphocytic infiltrates per $4mm^2$, where a focus comprises of ≥ 50 lymphocytes). Notably, the MSC-CM and MSC-CM-Exo treatment groups exhibited diminished focus scores in comparison to the control group in both the SMG and PG. Statistical analysis revealed that the focus scores of the MSC-CM and MSC-CM-Exo treatment groups were significantly lower in the SMG compared to the control group with $p < 0.05$. Additionally, both the MSC-CM and MSC-CM-Exo treatment groups manifested conspicuously smaller foci when contrasted with the control group.

(A)



(B)

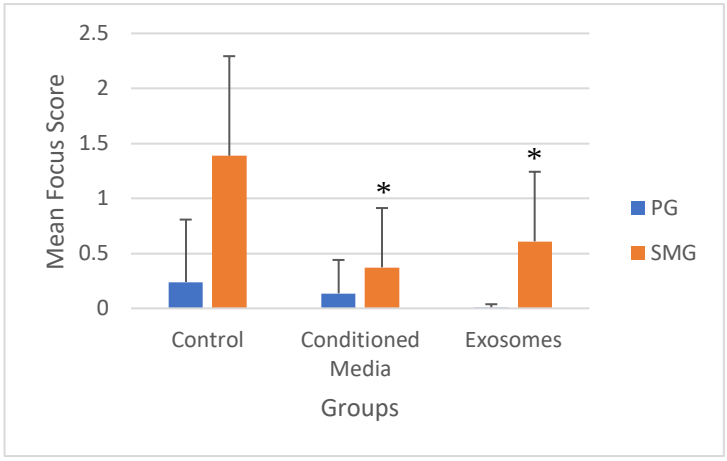


Figure 11. Analysis of the focus score and lymphocyte composition in the SMG and PG at 8 weeks post-treatment. (**A**) The upper panel displays H&E-stained images of lymphocytic infiltrates in the SMGs, while the lower panel shows images of lymphocytic infiltrates in the PGs. The black scalebars in the bottom right corner of all image represents 50 μm. (**B**) Focus score analysis, based on the number of lymphocytic infiltrates per 4mm², was conducted using serial H&E-stained sections cut at various levels under the light microscope. The results indicated a lower mean focus score for the treated groups in comparison to the control group,

with statistical significance observed specifically for the SMG. * $p \leq 0.05$. All data were presented as mean ± S.D. Control: normal saline treated.

3.4 MSC-derived Exosomes Isolated from Bone Marrow Preserved their Double-Membrane Morphology and Size Range.

Exosomes circulating in the MSC-CM were successfully isolated using a commercial kit. The efficiency of the kit's exosome isolation process was thoroughly characterized through NTA (Figure 11.a), TEM (Figure 11.b), and DLS (Figure 11.c) analysis. The TEM images demonstrated that the isolated exosome nanoparticles retained their double-membrane integrity and characteristic cup-shaped morphology. Some exosomes occasionally assembled in aggregates as shown in Figure 11.b.i. The NTA analysis revealed a diameter size distribution of the particles, with a mean size of 104.2 nm and a mode of 87.5 nm, encompassing 1.08 x 108 (± 4.75 x 106) particles per mL (Figure 11.a), which falls within the expected exosome diameter range of 30 to 150 nm [24]. Additionally, the DLS analysis results for the same sample indicated a population of particles with a hydrodynamic radius of approximately 30 nm, consistent with the anticipated diameter range of exosomes (Figure 11.c) [25]. Another residual population of particles with a radius of 415 nm was observed, potentially corresponding to exosome clusters (aggregates).

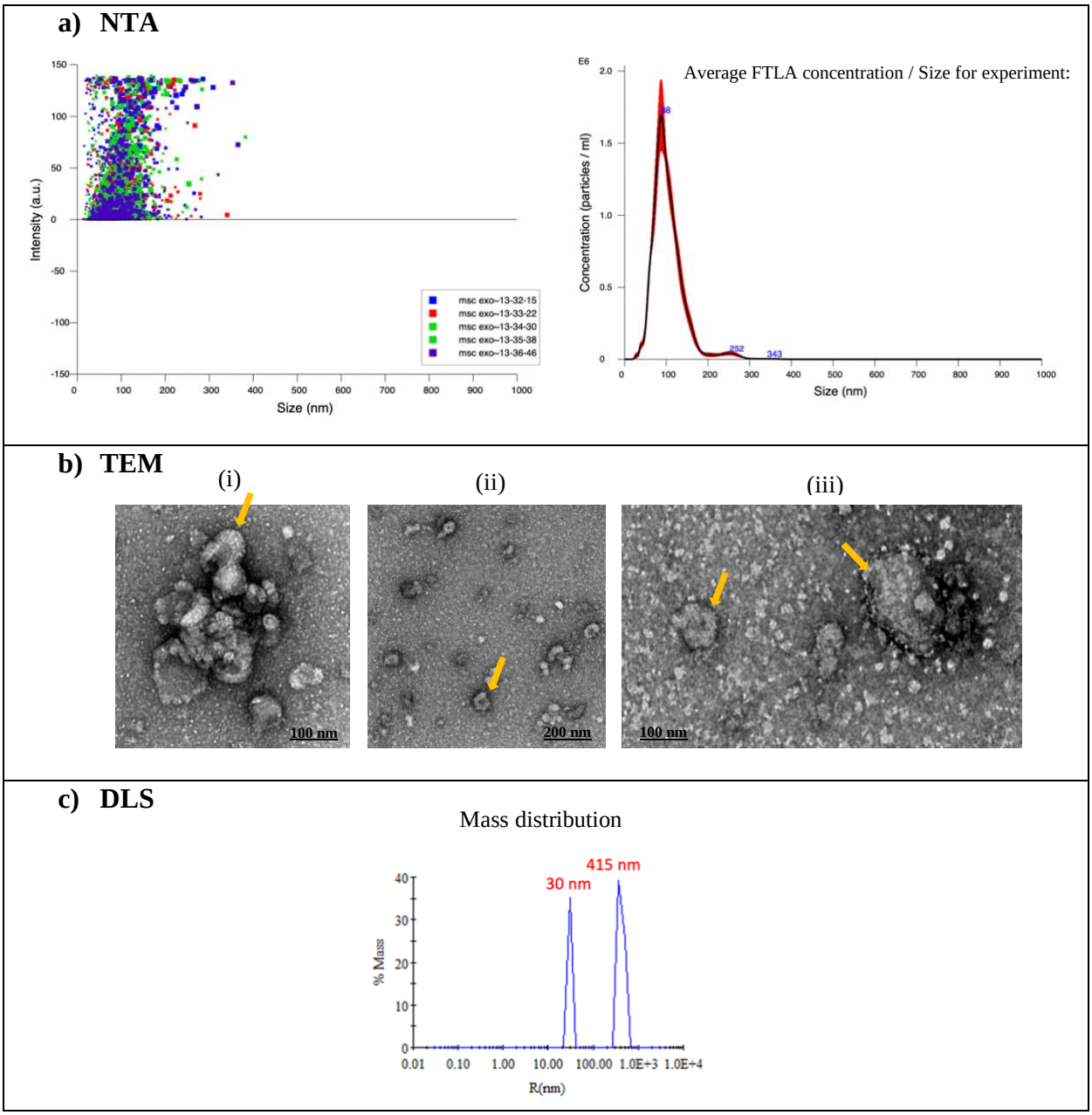


Figure 12. Characterization of exosomes isolated from bone marrow mesenchymal stem cells. (a) The intensity vs nanoparticle size analysis revealed the diameters of exosomes (measured in nm) with a prominent peak at 88 nm. Nanosight software was used to analyze the NTA capture data, presenting the averaged finite track length adjustment (FTLA) concentration/size for the experiment with the use of an exosome isolation kit (The Total Exosome Isolation Reagent by Thermo Fisher Scientific) (**b)** TEM images of mouse BM-MSC exosomes isolated using an exosome isolation kit (The Total Exosome Isolation Reagent by Thermo Fisher Scientific). The black scalebars in the bottom left corner of Figure 11.b.i and

11.b.iii represents 100 μm. The black scalebars in the bottom left corner of Figure 11.b.ii represents 200 μm. **(c)** The size distribution of exosomes, isolated using an exosome isolation kit (The Total Exosome Isolation Reagent by Thermo Fisher Scientific), determined through DLS measurements.

4. Discussion

The results of our study have provided valuable insights into the therapeutic potential of MSC-CM and MSC-CM-Exo in the treatment of SS-like disease in female NOD mice. Our comprehensive investigation led to the discovery of three key findings:

The first key finding pertains to the preservation of SG function in the treated mice. MSC-CM and MSC-CM-Exo treatment restored the exocrine function of the SGs in the female NOD mice. The preservation of SG function is crucial because the loss of salivary function is a defining feature of SS and can result in severe complications such as xerostomia, keratoconjunctivitis sicca, and difficulties masticating and swallowing. Our results showed that these treatments have the potential to improve the exocrine dysfunction that is commonly associated with the disease, thereby enhancing the overall quality of life for those who are affected.

The second key finding revolves around a notable contrast in the incidence of diabetes between the control group and the mice subjected to treatments with MSC-CM and MSC-CM-Exo. Specifically, while the control group demonstrated a gradual susceptibility to diabetes over the course of the study, none of the mice receiving MSC-CM and MSC-CM-Exo treatments show blood glucose levels surpassing 250 mg/dL. This disparity underscores the encouraging prospects of these treatments in mitigating the onset of both diabetes and SS. The observed resilience of treated mice in maintaining normoglycemia suggests a potentially vital role played by factors derived from MSCs in regulating blood glucose levels. Given these findings, it becomes imperative to delve deeper into the mechanistic pathways that underlie this protective effect. Such findings hold the potential to yield insights with far-reaching implications for the advancement of innovative approaches to prevent and manage diabetes and SS effectively.

The third key finding pertains to the partial restoration of peripheral tolerance in salivary tissues resulting from the MSC-CM and MSC-CM-Exo treatments. The observed decrease in lymphocytic infiltration, in combination with fewer and smaller foci, suggests that the immunological response within the SGs has been reduced. Immune-mediated deterioration of the SG tissue is a prominent pathogenic characteristic of SS, resulting in reduced saliva production. The reduction in lymphocyte infiltration in response to the treatments implies a possible immunomodulatory effect, which may have assisted in the preservation SG function.

4.1 Advantages of Exosome Therapy in Sjögren's Syndrome:

The utilization of exosomes as a therapeutic strategy in the context of SS has garnered immense interest among researchers, primarily driven by a myriad of compelling advantages [49]. Several notable advantages include their capability to facilitate natural cell-to-cell communication (refer to Section 1.9), their possession of regenerative properties (refer to Section 1.9), and an array of other remarkable attributes:

Non-immunogenic and low immunogenicity: Exosomes derived from a patient's own cells (autologous exosomes) have been reported to exhibit diminished immunogenicity and a reduced likelihood of adverse reactions upon administration. In contrast to alternative cell-based therapeutic approaches, the utilization of autologous exosomes significantly decreases the probability of rejection or immune-related complications. In a study conducted by Wahlgren et al., the potential of plasma-derived exosomes to transport exogenous short interfering RNA (siRNA) molecules to monocytes and lymphocytes was extensively explored. Their results provided compelling evidence of the effective delivery of siRNA by plasma-derived exosomes, presenting a promising avenue for targeted gene silencing within specific immune cell populations. Moreover, the study demonstrated the feasibility of isolating exosomes from patient's body fluids, such as plasma, or cell cultures, facilitating subsequent modification and reintroduction back into the same patient [62]. In addition, exosomes exhibit an exceptional characteristic of low immunogenicity in comparison to liposomes and virus-based drug delivery systems. This advantageous feature arises from the inherent nature of exosomes as naturally secreted vesicles derived from cells. Unlike liposomes, which are synthetic lipid-based nanoparticles, exosomes bear membrane components that closely resemble those of host cells,

thus minimizing the likelihood of triggering an immune response [62]. In contrast, virus-based drug delivery systems often encounter significant immunogenicity concerns due to the viral components they incorporate, which can activate immune cells and elicit immune reactions [63].

Targeted delivery: Exosomes possess the inherent capacity to undergo engineering (i.e. surface and genetic) or modification processes for the purpose of accommodating specific cargo molecules, including DNAs, RNAs, lipids, metabolites, proteins, nucleic acids, or therapeutic drugs facilitating their transportation to target cells or tissues. Through strategic modifications, engineered exosomes can be equipped with surface-expressed targeting ligands, enabling precise and selective cargo delivery to desired cell types. This targeted delivery strategy not only amplifies the therapeutic efficacy but also mitigates undesired off-target effects, thereby optimizing the overall therapeutic outcome. At present, LAMP-2B emerges as the most extensively employed exosomal surface protein for the exhibition of a targeting motif. LAMP-2B, belonging to the lysosome-associated membrane protein (LAMP) family, predominantly localizes to lysosomes and endosomes, while a minor fraction is distributed to the cell surface. Researchers have substantiated the presentation of the N-terminal region of LAMP-2B on the exosomal surface, permitting the attachment of targeting sequences. By employing phage display, cell-specific binding peptides that target specific organs or tissues can be screened, selected, and genetically modified at the N-terminus of LAMP-2B to achieve their desired targeting effects [51].

Stability and biocompatibility: Exosomes exhibit inherent resilience, displaying robust stability even in challenging conditions, such as acidic environments. Their capacity to maintain biological activity enables prolonged storage without compromising their functional integrity. Moreover, exosomes demonstrate exceptional biocompatibility and carry minimal risk of toxicity, establishing them as a secure and promising therapeutic modality [52].

Potential for crossing biological barriers: Exosomes possess the distinctive capability to cross complex biological barriers, including the blood-brain barrier, blood-cerebrospinal fluid barrier, and blood-lymph barrier, which frequently pose as a difficult challenge in drug delivery. This valuable trait enables exosomes to selectively navigate towards specific target sites, such as the CNS, thus providing a transformative impact on the treatment prospects for neurological

disorders and facilitating the precise administration of therapeutics to areas that were previously inaccessible [53].

Diagnostic and prognostic potential: Exosomes have the ability to carry and transport molecules, including proteins and genetic materials, from their parent cells. This unique and crucial function makes them very promising as biomarkers for both disease diagnosis and prognosis [54]. As mentioned, these exosomes can be extracted from a wide range of biofluids such as blood, urine, and cerebrospinal fluid thus, offering a highly non-invasive approach for performing liquid biopsies and monitoring disease progression [55].

Such remarkable attributes of exosomes have driven them to the forefront of therapeutic research, where they hold great promise for addressing the clinical manifestations and pathophysiological mechanisms of SS [40].

4.2 Limitations and Challenges of Exosome-Based Therapy in Sjögren's Syndrome

While the potential advantages associated to the used of exosomes as a form of therapy for SS are noteworthy, it's crucial to acknowledge the existence of limitations and challenges that must be addressed. These factors must be carefully considered and mitigated to establish the effectiveness, safety, and feasibility of the application of exosomes within clinical settings [46].

Isolation and Purification: Exosome and exosomal miRNA identification, isolation, and purification from biological sources pose complex technological challenges [49]. Various studies have documented a range of methods, used independently or in combination, for the isolation and purification of exosomes. These methods include techniques like immunoaffinity capture, size exclusion, polymeric precipitation, ultracentrifugation, microfluidics, and the utilization of commercially available kits [62]. Amidst these techniques, ultracentrifugation is commonly used for isolation although, there exists a risk of co-purifying additional impurities or inducing alterations in the composition of exosomes through this technique. Hence, there is a pressing requirement to develop more effective, economically sustainable, and standardized methods for

isolation. This development is crucial for ensuring the consistent and dependable production of exosomes tailored for therapeutic applications [49].

Heterogeneity and Variability: Exosomes originate from a wide array of cell types and inherently exhibit heterogeneity in their composition. This intrinsic variability poses a notable challenge when it comes to devising standardized protocols for therapies based on exosomes, given its potential impact on both the therapeutic effectiveness and consistency [63].

Characterization and Quality Control: As mentioned earlier, exosomes exhibit inherent heterogeneity in their composition, a quality influenced by variables like their cellular origin, the surrounding physiological environment, and the methods used for their isolation. It is imperative to accurately characterize exosomes, ensuring the consistent preservation of attributes like size, cargo content, and surface markers to ensure their utmost therapeutic efficacy. Overcoming the hurdles associated with standardized characterization methodologies and implementing strict quality control measures emerge as critical objectives in this realm [64].

Cargo Loading, Stability, and Capacity: Within the realms of exosome-based therapy, the loading of therapeutic cargo, including substances like drugs, nucleic acids, or proteins, stands as a pivotal aspect. Yet, the challenges posed by the limited cargo capacity and small size of exosomes are undeniable. This becomes particularly pronounced when striving to accommodate therapeutic molecules such as nucleic acids, proteins, or drugs. A crucial necessity lies in the efficient loading and preservation of cargo within exosomes, ensuring their stability and integrity throughout storage, transportation, and administration processes. This is imperative to achieve optimal therapeutic outcomes. Moreover, the limited cargo capacity of exosomes may potentially hinder their ability to deliver enough therapeutic agents to targeted cells or tissues impacted by SS. Overcoming these hurdles is vital to improve cargo loading efficiency, sustaining cargo stability, and ultimately maximizing the therapeutic potential of exosomes in the context of SS treatment [65, 66].

Scalability and Production: The mission of generating substantial quantities of exosomes for clinical utilization poses significant challenges. Existing production methodologies, whether rooted in cell culture practices or the isolation from biological fluids,

come with limitations in terms of yield and scalability. Beyond this, establishing well-suited protocols for the production and storage of exosomes becomes paramount to preserve their biological activity and uphold the integrity of these valuable therapeutic entities. Hence, the pursuit of scalable production techniques that guarantee both the quality and characteristics of therapeutic exosomes remains an ongoing challenge [46, 67].

Regulatory Considerations: The realm of utilizing exosomes as therapeutic agents stands as an emerging and dynamic field. Within this context, regulatory challenges concerning their classification, manufacturing standards, and approval processes present significant considerations. These factors hold the capacity to hinder or complicate the clinical transition of exosome-based therapies for SS, potentially leading to delays in their implementation [68].

5. Conclusion

This study contributes to the ever-growing body of evidence supporting the therapeutic benefits presented by MSC-CM and MSC-CM-Exo, in addressing SS-like disease within female NOD mice. The outcomes we've revealed are indeed promising, showcasing that both MSC-CM and especially, MSC-CM-Exo treatments hold promise as plausible pathways for SS therapy. Notably, the observed preservation of SG function and restoration of peripheral tolerance provide invaluable insights into the underlying mechanisms that drive the efficacy of these treatments. Furthermore, the MSC-CM and MSC-CM-Exo treatments come with inherent safety, high bioavailability, ease of administration, and transferability, all of which stand as notable strengths in their application as potential therapeutic options.

Nonetheless, there exists a need for further research to delve into a more comprehensive understanding of the potential underlying mechanisms at play. While our study has provided encouraging outcomes, delving deeper is imperative to attain a full understanding of the molecular and cellular mechanisms that underlie the reported therapeutic benefits observed in the treated NOD mice. This is particularly important given that MSC-CM encompasses an array of constituents, primarily proteins, a more extensive analysis of its composition would offer valuable insights. Beyond this, conducting long-term research and comprehensive safety evaluations hold significant weight in establishing the translational potential of these treatments for clinical use. In conclusion, the results presented here reveal MSC-CM and MSC-CM-Exo as

promising therapeutic options for managing SS. However, they also emphasize the necessity for further investigations to fully comprehend their potential and safety profile within the context of SS treatment.

6. **Publication:** Article submitted for publication during this thesis

cancers

MDPI

Review

An Overview on the Histogenesis and Morphogenesis of Salivary Gland Neoplasms and Evolving Diagnostic Approaches

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Simple Summary: Diagnosing salivary gland neoplasms (SGN) remain a challenge, given their underlying biological nature and overlapping features. Evolving techniques in molecular pathology have uncovered genetic mutations resulting in these tumors. This review delves into the molecular etiopatho-genesis of SGN, highlighting advanced diagnostic protocols that may facilitate the identification and therapy of a variety of SGN.

Abstract: Salivary gland neoplasms (SGN) remain a diagnostic dilemma due to their heterogenic complex behavior. Their diverse histomorphological appearance is attributed to the underlying cellular mechanisms and differentiation into various histopathological subtypes with overlapping fea-tures. Diagnostic tools such as fine needle aspiration biopsy, computerized tomography, magnetic resonance imaging, and positron emission tomography help evaluate the structure and assess the staging of SGN. Advances in molecular pathology have uncovered genetic patterns and oncogenes by immunohistochemistry, fluorescent in situ hybridization, and next–generation sequencing, that may potentially contribute to innovating diagnostic approaches in identifying various SGN. Surgical resection is the principal treatment for most SGN. Other modalities such as radiotherapy, chemotherapy, targeted therapy (agents like tyrosine kinase inhibitors, monoclonal antibodies, and proteasome inhibitors), and potential hormone therapy may be applied, depending on the clinical behaviors, histopathologic grading, tumor stage and location, and the extent of tissue invasion. This review delves into the molecular pathways of salivary gland tumorigenesis, highlighting recent diagnostic protocols that may facilitate the identification and management of SGN.

Keywords: salivary glands; salivary gland neoplasms; epithelial tumors; head and neck cancer; molecular pathology; diagnostic advances

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1. Introduction

Salivary glands are tubulo-acinar exocrine organs that embryonically initiate in the sixth–eighth week of intrauterine life. The parotid gland is believed to arise from the oral ectoderm, while the submandibular and sublingual glands are from the embryonic endoderm [1,2]. Their development is attributed to the physiologic process of 'branching morphogenesis', described as the rearrangement of a single epithelial bud to generate multiple acinar and ductal units, through continuous multi-directional branching [3]. 'Epithelial–mesenchymal interaction', described as a secondary induction of the epithelium

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