ISSN 1948-0210 (online)

World Journal of *Stem Cells*

World J Stem Cells 2023 December 26; 15(12): 1035-1103

Published by Baishideng Publishing Group Inc

World Journal of Stem Cells

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Editorial Board Member of *World Journal of Stem Cells*, Sujeong Jang, PhD, Assistant Professor, Department of Physiology, Chonnam National University Medical School, Hwasun-gun 58128, South Korea.
sujeong.jjang@gmail.com

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Production Editor: *Xiang-Di Zhang*; **Production Department Director:** *Xu Guo*; **Editorial Office Director:** *Jia-Ru Fan*.

NAME OF JOURNAL
World Journal of Stem Cells

ISSN
ISSN 1948-0210 (online)

LAUNCH DATE
December 31, 2009

FREQUENCY
Monthly

EDITORS-IN-CHIEF
Shengwen Calvin Li, Carlo Ventura

EDITORIAL BOARD MEMBERS
https://www.wjgnet.com/1948-0210/editorialboard.htm

PUBLICATION DATE
December 26, 2023

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World J Stem Cells 2023 December 26; 15(12): 1035-1062

DOI: 10.4252/wjsc.v15.i12.1035

ISSN 1948-0210 (online)

REVIEW

Stem cells and pain

Matheus Deroco Veloso da Silva, Maiara Piva, Geovana Martelossi-Cebinelli, Mariana Stinglin Rosa Ribas, Beatriz Hoffmann Salles Bianchini, Olivia K Heintz, Rubia Casagrande, Waldiceu A Verri Jr

Specialty type: Cell and tissue engineering

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): A
Grade B (Very good): 0
Grade C (Good): C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Kim YB, South Korea; Lin L, China

Received: July 28, 2023
Peer-review started: July 28, 2023
First decision: September 27, 2023
Revised: November 6, 2023
Accepted: November 30, 2023
Article in press: November 30, 2023
Published online: December 26, 2023

Matheus Deroco Veloso da Silva, Maiara Piva, Geovana Martelossi-Cebinelli, Mariana Stinglin Rosa Ribas, Beatriz Hoffmann Salles Bianchini, Department of Pathology, Laboratory of Pain, Inflammation, Neuropathy and Cancer, State University of Londrina, Londrina 86057-970, Paraná, Brazil

Olivia K Heintz, Morningside Graduate School of Biomedical Sciences, University of Massachusetts Chan Medical School, Worcester, MA 01655, United States

Rubia Casagrande, Department of Pharmaceutical Sciences, Center of Health Science, State University of Londrina, Londrina 86038-440, Paraná, Brazil

Waldiceu A Verri Jr, Department of Pathology, Laboratory of Pain, Inflammation, Neuropathy and Cancer, Center of Biological Sciences, State University of Londrina, Londrina 86057-970, Paraná, Brazil

Corresponding author: Waldiceu A Verri Jr, PhD, Associate Professor, Department of Pathology, Laboratory of Pain, Inflammation, Neuropathy and Cancer, Center of Biological Sciences, State University of Londrina, Rodovia Celso Garcia Cid/Pr 445 Km 380, Londrina 86057-970, Paraná, Brazil. waverri@uel.br

Abstract

Pain can be defined as an unpleasant sensory and emotional experience caused by either actual or potential tissue damage or even resemble that unpleasant experience. For years, science has sought to find treatment alternatives, with minimal side effects, to relieve pain. However, the currently available pharmacological options on the market show significant adverse events. Therefore, the search for a safer and highly efficient analgesic treatment has become a priority. Stem cells (SCs) are non-specialized cells with a high capacity for replication, self-renewal, and a wide range of differentiation possibilities. In this review, we provide evidence that the immune and neuromodulatory properties of SCs can be a valuable tool in the search for ideal treatment strategies for different types of pain. With the advantage of multiple administration routes and dosages, therapies based on SCs for pain relief have demonstrated meaningful results with few downsides. Nonetheless, there are still more questions than answers when it comes to the mechanisms and pathways of pain targeted by SCs. Thus, this is an evolving field that merits further investigation towards the development of SC-based analgesic therapies, and this review will approach all of these aspects.

Key Words: Inflammation; Neuropathy; Nociceptive; Pain; Pain treatment; Stem cells

Core Tip: Since the discovery of stem cells (SCs), they have emerged from a distant dream into a routine therapeutic approach depending on the field. Nowadays, the use of SCs in pain management is mainly based on their anti-inflammatory capacities, releasing neurotrophic factors and providing cellular support to replace damaged neural cells. Evidence supports that SCs can influence nociceptor neuron sensitization building a foundation for the application of these versatile cells in the treatment of neuropathic and inflammatory pain.

Citation: Silva MDVD, Piva M, Martelossi-Cebinelli G, Stinglin Rosa Ribas M, Hoffmann Salles Bianchini B, K Heintz O, Casagrande R, Verri Jr WA. Stem cells and pain. *World J Stem Cells* 2023; 15(12): 1035-1062
URL: https://www.wjgnet.com/1948-0210/full/v15/i12/1035.htm
DOI: https://dx.doi.org/10.4252/wjsc.v15.i12.1035

INTRODUCTION

Pain is a major cause of suffering and disability, and can be characterized as a distressing experience that usually signals the presence of injury or disease, generating complex physiologic and emotional responses[1]. According to the Global Pain Index Study by GlaxoSmithKline released in 2020, interviews with 19000 individuals around the world indicate that 34% of the globe's population is in pain every day[2]. Consistently, United States's National Center of Health and Statistics revealed that 20.4% of adults suffered from chronic pain in 2019, and indicated the three major consequences of the condition were: Decreased quality of life, opioid dependence, and poor mental health[3]. This alarming scenario highlights the urgency to pinpoint the physiopathological mechanisms underlying pain and how they interconnect with other systems, which are essential to developing and improving the availability of therapeutic approaches. In this review, we highlight the stem cell (SC)-based therapies aiming to reduce pain. Despite the existence of review articles on SCs and specific types of pain, we observed that there is a gap in the literature regarding comprehensive review articles in this topic approaching various types of pain, the mechanisms of action of SC-based analgesic therapies, and pre-clinical and clinical articles. Therefore, the present review article aims to fill this gap. Data supports that SC-based approaches will revolutionize the field of pain treatment of varied etiologies as we will discuss.

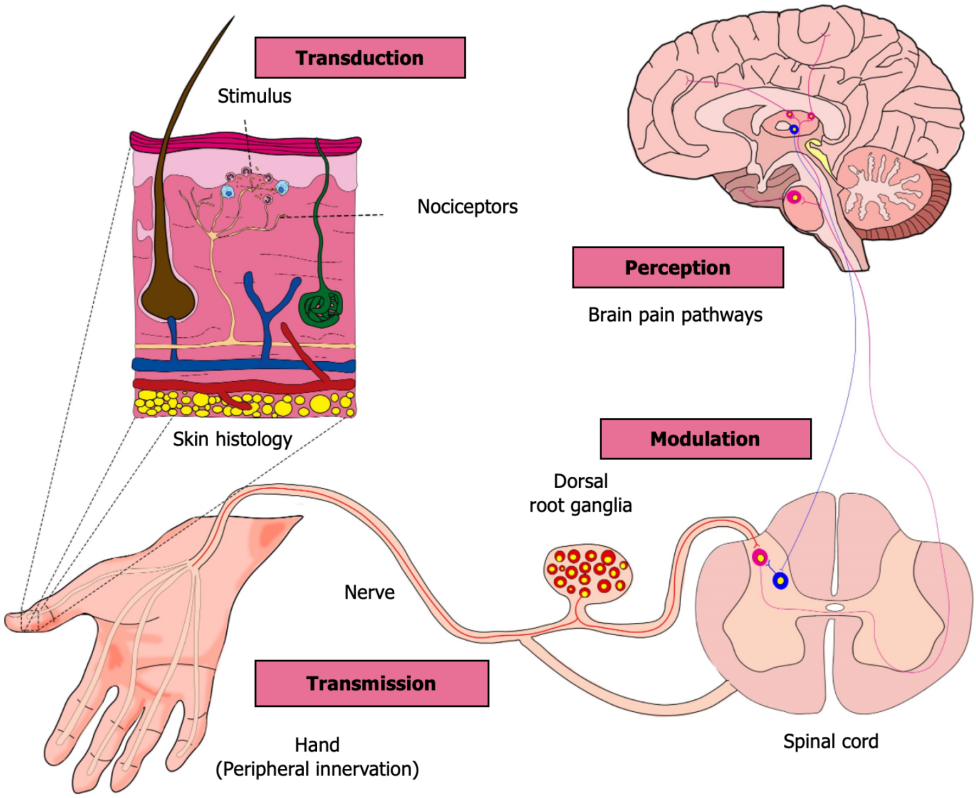
GENERAL VIEW OF PAIN MECHANISMS AND DEFINITIONS

According to the International Association for the Study of Pain (IASP), pain can be defined as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage"[4]. The mechanisms underlying the physiology of pain are extremely complex, involving at least two types of neurons; one whose cellular bodies are in the dorsal root ganglia (DRG) and axons projecting to peripheral tissues and the spinal cord, which are specialized in the perception of potentially harmful stimuli, and then neurons which are present in the spinal cord and in the cortex of the brain, responsible for interpreting the harmful stimuli[5] (Figure 1).

To fully comprehend the pain-related states that can affect the human body, it is imperative to define their source or the causal initiators. The present review will divide pain scenarios as: (1) Inflammatory pain, which involves the presence of inflammation as the primary cause of pain and is responsible for nociceptor neuron activation and plasticity to induce chronic pain[4]; (2) Pathogen-induced pain, a painful state caused by microbial pathogens that directly activate pain-related receptors, which also involves inflammation, but with the presence of a microorganism initiating the process[6]; and (3) Neuropathic pain (NP), a consequence of damage to the nervous system and extensive tissue repair, leading to residual nerve-healing pain[7].

Usually, pain begins with the recognition of possible damage or potentially harmful molecules. When facing a noxious stimulus, our body is able to respond, at cellular and molecular levels, through the immune and nervous system in an attempt to neutralize and repair the damage caused by such stimulus[8]. The immune system and nervous system are responsible for mediating the inflammatory process, generating edema, heat, redness, pain, and loss of function depending on the intensity of those cardinal signs of inflammation[9,10].

For an inflammatory response to occur, the harmful agent must cause tissue damage in the host, or possess molecules that are recognized by immune cells or neurons to trigger either a pro-inflammatory cascade or neurogenic inflammation, respectively[9]. After recognition, a complex cell signaling process begins, inducing vascular alterations to recruit leukocytes that will reach the primary inflammatory foci by diapedesis[10]. Several molecules secreted by immune cells (*e.g.*, cytokines, chemokines, prostanoids) as well as receptors present in their cell membranes and molecules expressed by the pathogen itself are capable of activating nociceptors[9,11]. Nociceptors consist of a subset of sensory neurons, which innervate peripheral tissues (*e.g.*, joints, skin, respiratory and gastrointestinal tract) and have a role in sensing nociceptive stimuli that will be interpreted in the cortex as pain with all its affective and cultural aspects[12].



DOI: 10.4252/wjsc.v15.i12.1035

Figure 1 Pain mechanism. Representative diagram of anatomical levels involved in the steps of pain from detection of stimulus up to its interpretation and modulation. Starting from the left to the right sides of the figure. The initiation of pain processing starts with the sensing of a noxius stimulus on the skin. This stimulus is detected by nociceptors (whose cellular bodies are in dorsal root ganglia and axons that project to the peripheral tissues and spinal cord) and then relayed to the spinal cord before ultimately reaching the brain (depicted by the ascending pathways in pink). In the brain, the stimulus is interpreted and converted into the sensation of pain. Subsequently, descending pathways (represented in blue) become active, limiting nociceptive input at the spinal cord level. It is crucial to emphasize that glial cells play a significant role in the transmission and modulation of pain. They are activated by neuropeptides released by neurons as well as inflammatory molecules released by immune cells. The recognition of these molecules, whether they are pro-nociceptive or anti-nociceptive, can stimulate glial cells to alter their behavior and contribute to the persistence of the stimulus by releasing nociceptive molecules. Nociceptive neurons can also interact with immune cells that release molecules capable of activating and sensitizing these neurons as well as cause neurogenic inflammation. Varied pathogens (*e.g.*, bacteria, parasites, virus) present virulence factors that can activate nociceptor neurons. For a further in-depth understanding of pain mechanisms in varied conditions we recommend the following review articles[125,174-177].

These stimuli (*e.g.*, inflammatory molecules, pathogen virulence factors) can activate receptors present in nociceptors triggering the phosphorylation of ion channels controlled by ligands [transient receptor potential (TRP) channels] or modify sodium channels controlled by voltage [Voltage-gated sodium (Nav) channels]. Those stimuli, therefore, cause changes in the ion channels facilitating and/or inducing nociceptive neuron depolarization resulting in their sensitization to mechanical and thermal stimulation as well as neuronal firing to transduce the nociceptive information, respectively[9,13]. It is important to stress that the expression of these channels can also be increased due to chronic stimulus, so that neurons that initially express low levels of an ion channel or cytokine receptor start to express them at higher levels[14].

Another important fact, as touched on above, is that the receptor/ion channel activation and pattern of expression can lead to pain sensitization. According to IASP, pain sensitization is defined as increased responsiveness of nociceptors to their normal or subthreshold afferent, and can be divided as hyperalgesia, characterized by increased pain due to a noxious stimulus, or allodynia, a painful response to normally innocuous mechanical or thermal stimuli[15]. Thus, the modifications of what is expressed by nociceptive neurons, and modulating their activation state and responsiveness to stimuli are plastic changes potentially leading to chronic pain.

The functions of the TRP ion channels are related to thermal and mechanical perception[16]. For example the TRP cation channel subfamily V member 1 (TRPV1), involved in heat hypersensitivity and activated by capsaicin; TRP melastatin 8 (TRPM8), involved in cold hypersensitivity; and TRP ankyrin 1 (TRPA1), involved in hypersensitivity to chemical and mechanical stimulation[17]. Furthermore, Nav channels (Nav1.7, Nav1.8 and Nav1.9) are directly responsible for neuronal depolarization, and their expression and activation state can also be modulated during inflammation, infection, and nerve lesions leading to acute and chronic pain[18].

HOW CAN PAIN BE QUANTIFIED IN ANIMAL MODELS?

As this review will approach in great part pre-clinical data using animals, it is important to briefly discuss how "pain" is quantified in animal models. The usage of the term pain to define the quantification of nociceptive behavior in animal models is not widely accepted, because pain assessment involves an emotional component that is often lost in the evaluation of animal responses in most tests. On the other hand, simplifying the terminology by using the word pain, facilitates the understanding by the non-specialized reader about what is under discussion. As an essential physiological mechanism that helps to guarantee the integrity of the organism, pain triggers behavioral responses, for example, moving the hand or paw from a noxious stimulus that can cause tissue damage[19]. In fact, there are subjective components associated with the painful sensation that can only be assessed in humans, since there are variations in the quality and intensity of pain experienced by different individuals for similar injuries, influenced by culture, sex, age, personal experiences, comorbidities and genetic factors[20].

There are different methods to analyze the presence of pain, as well as methods to quantitate it. Most laboratory studies use experimental rodent models, thus, the methodologies are focused on the evaluation of behaviors that can be quantified, such as paw withdraw, paw flinching, paw licking, and abdominal contortions[21], with or without the combination of other methodologies (*i.e.,* place preferences to temperatures or even self-administration of treatment).

Nociceptive assessment methods can be divided into stimulus-evoked and non-stimulus-evoked (spontaneous) behaviors. Spontaneous pain occurs regardless of the presence of an additional evoking stimulus and can be assessed using grimace scales, burrowing assays, gait analysis and weight-bearing methods[21]. Pain evoked by a stimulus can be described as hyperalgesia or allodynia[22]. This parameter is evaluated according to the type of stimulus, being subdivided into mechanical, heat and cold stimuli. Methods that assess pain evoked by mechanical stimuli seek to assess nociceptive sensitivity to a mechanical stimulus (*i.e.,* mechanical pressure on the paw), normally using the von Frey filament method (allodynia indicator), electronic pressure meter test (hyperalgesia indicator), and Randall & Sellito tests (which use increasing pressure or constant pressure over time, assessing hyperalgesia). The analysis of pain evoked by temperature stimuli seeks to assess thermal nociception, either by a heat source (*i.e.,* hot plate, Hargreaves test, tail flick), cold (*i.e.,* cold plate, cold plantar assay and acetone application/evaporation test) or both (*i.e.,* temperature place preference)[21].

Furthermore, behavioral tests are usually analyzed in conjunction with data obtained from cellular and molecular experimental approaches. For instance, neuronal function, activity, and phenotype can be assessed *in vivo* and *in vitro* using electrophysiology studies, intracellular calcium levels, immune staining of neuronal populations and their markers of activation and/or function, release of neuropeptides, cytokines and neurotransmitters, patterns of mRNA, protein and lipid profiles, and optogenetics. Additionally, it is also possible to study the contribution of non-neuronal cells in the nociceptive processes by staining glial immune and parenchymal cells, phenotype markers, and quantitating their production of mediators and functions. Thus, behavioral assays can be accompanied by a great variety of non-behavioral methods to demonstrate a specific hypothesis[23,24].

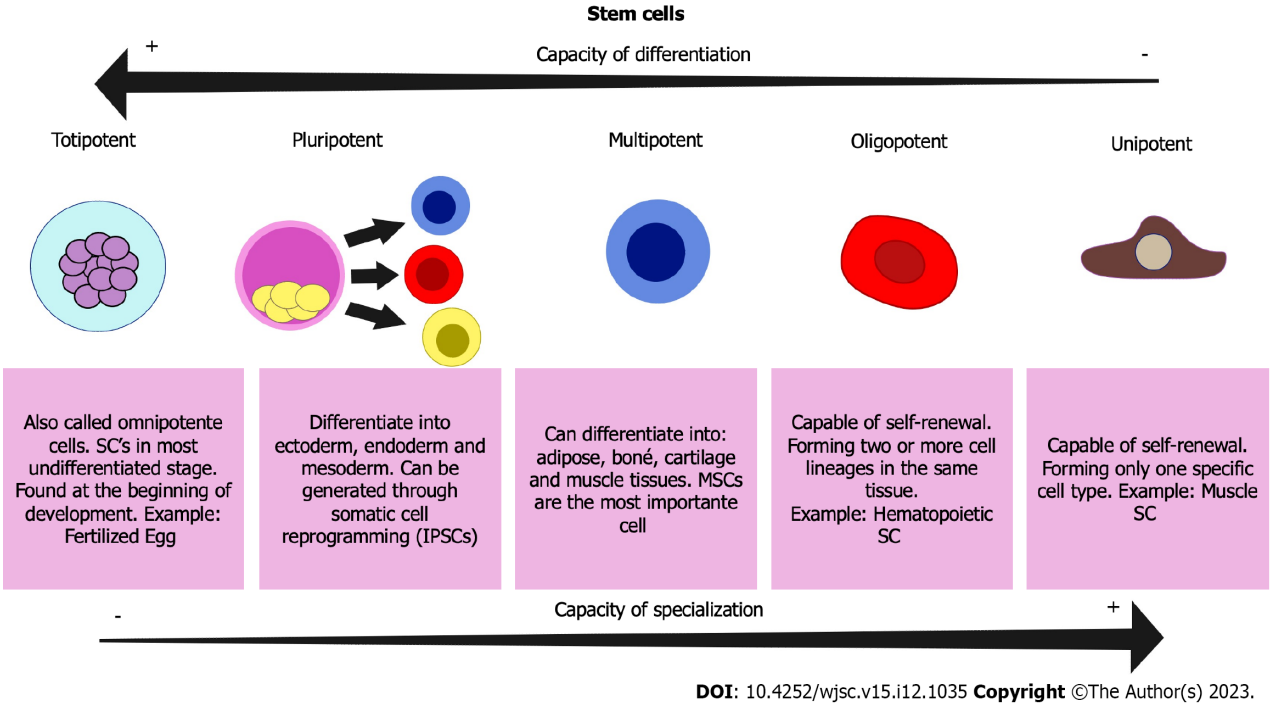
SCS: TYPES AND SOURCES

SCs are non-specialized cells with a high capacity for replication and self-renewal that have a wide range of differentiation possibilities[25]. These cells are present in all stages of life (embryonic, fetal, and adult), they give rise to differentiated cells in organs[26] and are involved in the development, maintenance, repair and renewal of tissues[27].

SC can be categorically divided into five distinct groups (totipotent, pluripotent, multipotent, oligopotent, and unipotent) according to their ability to differentiate, which varies according to the origin and derivation of the cell[26]. Totipotent SC, also called omnipotent, are the cells in the most undifferentiated stage and are found at the beginning of development (*i.e.,* fertilized oocyte)[28]. Pluripotent SC are cells that differentiate from the three germ layers (ectoderm, endoderm and mesoderm)[29]. These cells can be generated through somatic cell reprogramming and are called induced pluripotent SC (iPSC)[26]. Multipotent SC are found in most tissues and have the ability to differentiate into varied tissues, such as adipose, bone, cartilage, and muscle[30]. Within this group, mesenchymal SC (MSC) are the most important cells as will be discussed[26]. Oligopotent SC (*i.e.,* hematopoietic SC) are cells capable of self-renewal, forming two or more cell lineages in the same tissue[31]. Finally, unipotent SC are cells capable of self-renewal and differentiation into only one specific cell type[26] (Figure 2).

Although it might seem that specialization could mean restriction in some tissue types, totipotent SC have virtually no boundaries for differentiation, whereas pluripotent SC demonstrate some degree of specialization. As for the multipotent subgroup, MSC have the advantage of originating in different tissues. MSC have a differentiation rate compatible with the potential application as a pain treatment, particularly for inflammatory and autoimmune diseases[25,32,33].

In addition to classification according to their ability to differentiate, SC can also be classified according to their origin, forming four distinct groups (embryonic, fetal, adult and induced). Embryonic SC (ESC) are pluripotent SC derived from the blastocyst after fertilization (5 to 6 d)[34]. Similar to pluripotent SC, ESC differentiate into the three germ layers or remain in an undifferentiated stage[29,35]. Fetal SC are cells that remain in tissues in a quiescent state until local stimulus induces their proliferation and differentiation into specific cells of the tissues in which they are located[26]. Adult SC are cells derived from the three germ layers and the placenta[26] and depend on specific signals to enter cell division[36]. These cells are important resources in the cell repair and healing processes, as they help to maintain tissue homeostasis by replacing senescent or damaged cells[36]. Finally, iPSC are pluripotent SC produced from the genetic reprogramming of adult somatic cells[26], developing a state similar to ESC, both in morphology, proliferation and gene expression[37].



DOI: 10.4252/wjsc.v15.i12.1035

Figure 2 Types of stem cells: Different types and characteristics. Representative scheme of the different subtypes of stem cells and their main characteristics. SC: Stem cells; MSC: Mesenchymal stem cell; iPSC: Induced pluripotent stem cell.

Currently, MSC are the main subset of SC used in therapeutic approaches, as they can be isolated from any source of human tissue and reprogrammed. MSC have the advantage of being derived from the patient and, therefore, are adequate for the donor/patient, minimizing the possible ethical issues that could arise from the use of cells from a third-party donor[38,39].

In this review, we discuss recent studies that illustrate the advantages and disadvantages of SC technology to treat painful conditions. Although SC studies have become a popular field of research, there are still a lot of unanswered questions regarding the possible application of SC in pain treatment as well as data supporting their therapeutic benefit.

HOW CAN SC TREATMENT BE USED IN PAIN MANAGEMENT?

Currently, analgesic treatment strategies include acetaminophen, nonsteroidal anti-inflammatory drugs, antidepressants, antiepileptics, local anesthetics, and opioids; and their use is closely linked to significant side effects such as: High renal and hepatic toxicity, headaches, mood swings, constipation, nausea, weight gain and even dependence[40]. Furthermore, these drugs have limited efficacy (Table 1). The statistical measurement for this effectiveness is the number needed to treat (NNT), which is the number of people who must be treated with a given drug for the desired effect to be observed in one person. Thus, the closer the NNT is to 1 the higher the treatment efficacy is[41]. Along with concerning adverse effects, the present scenario highlights the need of additional options to control and treat painful conditions[40].

In this context, the use of SC as a therapeutic approach for pain treatment has great potential due to their unique properties. In general, the use of SC for pain treatment is based on their ability to: (1) Modulate the inflammatory process, switching the pro-inflammatory profile into a pro-resolving state; (2) Interacting directly on the peripheral nervous system, promoting changes on neuronal excitability of primary afferent nociceptor neurons; and (3) Acting on the central nervous system (CNS), *via* alteration of neuronal excitability in the spinal cord and brain (Figure 3). These mechanisms will be discussed in detail throughout the following paragraphs.

The first mechanism of action of SC to control pain is to modulate the inflammatory process. SC can shape the activity of neutrophils, macrophages, B cells, T cells, natural killer (NK) cells and dendritic cells[42] (Figure 3). In innate immunity, SC regulatory action is based on their ability to produce soluble human leukocyte antigen G5 [capable of inhibiting NK cell-mediated cytolysis and interferon-gamma (IFN-γ) secretion][43]. Another important point is that SC are able to increase the production of interleukin-10 (IL-10), an important cytokine involved in the polarization of M2 macrophages, inducing tissue repair effects[44]. In adaptive immunity, the effects of SC actions involve increased nitric oxide (NO) production (suppressing T cell activity)[45], reduced prostaglandin E_2 (PGE_2) levels[46] and increased indoleamine 2,3-dioxygenase (IDO) activity[47]. Furthermore, it has also been described that treatment with SC increase the expression of IL-4 (by type 2 helper cells)[46] and IL-10[44].

On the other hand, SC can also produce soluble mediators to shape the inflammatory response. SC can be stimulated by inflammatory cytokines such as IFN-γ, IL-1α, IL-1β and tumor necrosis factor alpha (TNF-α)[45,48]. However, in response to those inflammatory cytokines, SC produce transforming growth factor-beta (TGF-β) and IL-10, which are

Table 1 Painkillers and numbers needed to treat

Drug	Drug class	NNT	Ref.
Acetaminophen 650 mg + oxycodone 10 mg	NSAID + opioid	2.7	Gaskell *et al*[178], 2009
Acetaminophen 500 mg + ibuprofen 200 mg	NSAID combination	1.6	Moore and Hersh[179], 2013
Aspirin 1200 mg	NSAID	2.4	Bandolier Extra[180], 2003
Codeine 60 mg	Opioid	16.7	Maxwell and Bateman[181], 2007
Diclofenac 100 mg	NSAID	1.8	Gaskell *et al*[178], 2009
Ibuprofen 400 mg	NSAID	2.5	Lyngstad *et al*[182], 2021
Morphine 10 mg (intramuscular)	Opioid	2.9	Bandolier Extra[180], 2003
Naproxen 500 mg	NSAID	2.7	Derry *et al*[183], 2009
Oxycodone 15 mg	Opioid	4.6	Gaskell *et al*[178], 2009

NSAIDs: Nonsteroidal anti-inflammatory drugs; NNT: Number needed to treat.

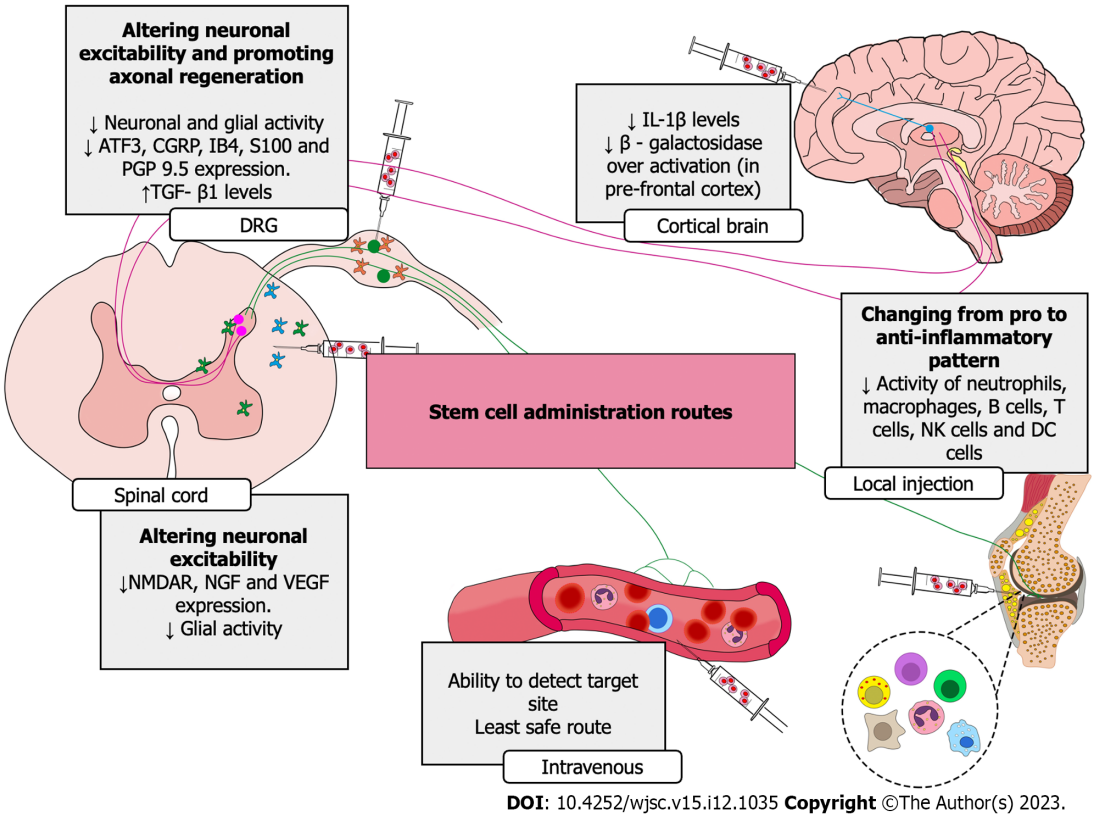


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Figure 3 Analgesic mechanisms of stem cells depending on the route of administration and targets/tissues. This scheme summarizes the mechanistic changes caused by stem cell (SC) treatment that resulted in analgesia. The explanation of analgesic mechanisms of SC treatments in the cerebral cortex, spinal cord, dorsal root ganglia, intravenous and local treatment (intra articulary) (indicated by the syringes) can be observed by up and down arrows plus the changed parameter. ATF3: Activating transcription factor 3; CGRP: Calcitonin gene-related peptide; IB4: Isolectin B4; NMDAR: N-methyl-D-aspartate receptor; NGF: Nerve growth factor; PGP9.5: Protein gene product 9.5; VEGF: Vascular endothelial growth factor; TGF: Transforming growth factor; IL: Interleukin.

anti-inflammatory and analgesic cytokines[49,50].

Added to their anti-inflammatory capabilities, SC become promising potential candidates to reduce peripheral sensitization of afferent sensory neurons[51] (Figure 3). SC can decrease the production of cytokines (IFN-γ[46], IL-2[46], IL-17[52] and TNF-α[46]) capable of sensitizing nociceptors and leading to hyperalgesia. SC can also increase the level of cytokines capable of decreasing nociception such as IL-4[46] and IL-10[53,54]. On the other hand, treatment with SC can increase the level of molecules indirectly linked to the promotion of hyperalgesia, such as NO (has a dual role in pain)[45,55], PGE_2[46,56] and IDO[47,57].

These data provide evidence that the applicability of treatment in inflammatory conditions directly depends on the inflammatory microenvironment, which can be positively influenced by SC both by decreasing molecules linked to nociceptive sensitization and by increasing molecules indirectly linked to reducing pain (Figure 3). However, specific cellular targets and the source of soluble mediators were not fully investigated in the experimental settings. We envisage that the field would present a huge evolution if the studies focused not only in quantitating the modulation of soluble mediators, but also investigating the cellular targets and interactions explaining the activity of SC.

In addition to their action on the neuroimmune axis (DRGs and the immune system), an interesting fact that has not been explored much is that SC have a key structural similarity with primary afferent nociceptive sensory neurons. Primary afferent nociceptive sensory neurons and bone marrow-derived mesenchymal stromal cells express the TRPM8 receptor. Recent studies demonstrate that the influence of this receptor on neurons involves the detection of cold temperatures (18-23 °C), and that TRPM8 inhibition reduces pain[58]. In addition, this channel is also capable of modulating cell differentiation in SC, as its activation increases osteogenic differentiation in human bone marrow MSC[59].

The use of SC in pain treatment is also based on the ability of these cells to act on the CNS since they have the ability to desensitize the CNS by inhibiting glutamate-related pathways (reduction of NMDAR expression and TGF-β1 secretion) [60]. Furthermore, SC can decrease central sensitization, *via* reduced glial cell activity, once again contributing to the attenuation of hyperalgesia[61] (Figure 3), these points will be discussed in further details in the following topics.

SC IN THE TREATMENT OF INFLAMMATORY DISEASES AND INFLAMMATORY PAIN

The number of articles investigating the relationship between pain and SC is still small, so this section was divided into two parts: (1) The use of SC to treat inflammatory diseases. This part is of interest in terms of the perspective of application to inflammatory pain since inflammatory mediators can induce nociceptor sensitization mechanisms; and (2) Articles that analyze the analgesic activity of SC treatment.

SC in the treatment of inflammatory diseases without the assessment of pain

In this topic we will present the mechanisms behind the ability of SC to modulate the inflammatory response, and the most recent discoveries involving SC and inflammation. Reducing pain is a potential outcome since inflammation was reduced in these studies, however, this specific disease symptom was not tested.

As previously discussed, the inflammatory process is composed of a series of signals. Through the release of inflammatory molecules (cytokines, leukotrienes, and prostanoids), which are used for cellular communication, tissue resident immune cells start the inflammatory response process. Examples of these inflammatory molecules include, for instance, IL-1β, IL-5, IL-6, IL-17A, TNF-α, nerve growth factor (NGF), LTB_4, 5-HT and PGE_2. They have a role in the recruitment and activation of leukocytes and some can also activate receptors expressed by the primary afferent nociceptor sensory neurons inducing the activation (causing depolarization) or sensitization (causing an enhancement of response upon other chemical, mechanical or thermal stimulation) of these neurons. This neuro-immune interaction is relevant to pain and inflammation[62] and we will discuss how SC can interfere with it.

Neutrophils are the most abundant cell type in the blood and large numbers are recruited in acute inflammation[63]. SC activity on neutrophils may present two distinct patterns[64]. MSC can suppress hydrogen peroxide production in activated neutrophils in vitro. On the other hand, tissue resident glandular MSC seem to play an early role in lipopolysaccharide (LPS)-triggered inflammation by producing cytokines and chemokines to recruit neutrophils. These polymorphonuclear leukocytes presented an increase in their lifespan, chemokine production and response to LPS stimulation[65]. This MSC-dependent response is protective in the sense that LPS stimulation represents part of an infection. On the other hand, neutrophils can participate in the induction of pain by producing LTB_4 and PGE_2, which activate and sensitize nociceptor neurons[66,67]. Reactive oxygen species can also activate nociceptor neurons[9].

One of the most important anti-inflammatory mechanisms of SC is to induce a class switch in the pattern of macrophages from M1 phenotype to M2 phenotype. This ability was observed in models of osteoarthritis using the treatment with exosomes from iPSC and MSC. The M2 macrophage phenotype is involved in tissue repair as well as in the resolution phase of inflammation[68]. Upon inducing this macrophage phenotype switch, there is a decrease in the production of pro-inflammatory cytokines IL-1β and TNF-α produced by M1 macrophages[68,69]. Interestingly, this activity seems to be related to SC-derived PGE_2. Contrasting with the hyperalgesic role of PGE_2 in inflammation by sensitizing primary nociceptive sensory neurons[70], this prostanoid also has other regulatory functions. For instance, when dendritic cells are stimulated to produce PGE_2 and IL-10, these antigen presenting cells reduce the expression of major histocompatibility complex (MHC)-II and CD86, thus, reducing their function of presenting antigens. As a result, there is a reduction of lymphocyte proliferation and adaptive immune response[71]. This evidence points to specific roles of PGE_2 and how somewhat opposing effects can be triggered by this prostanoid depending on the site of production and cellular target as well as explain anti-inflammatory activities of SC. Furthermore, PGE_2 can induce the production of TSG-6[72], which is capable of converting the macrophage phenotype from pro-inflammatory to anti-inflammatory[73]. Finally, MSC can recruit monocytes and macrophages through the production of chemokines such as C-C motif chemokine ligand (CCL)2, CCL3 and CCL12 in inflamed tissue, which contributes to the tissue repair[72,74].

Another characteristic of SC is their ability to interfere with the lymphocytic pattern. In Crohn's disease patients, it was observed that treatment using MSC was able to decrease lymphocyte proliferation, the proportion of CD4+ T cells, and decreases the levels of TNF-α and IL-6. The authors also demonstrated an increase in regulatory T cells (Tregs) and IL-10 production, thus, suggesting that an MSC would shift the T cell population towards an increase of Tregs that produce IL-10 to limit inflammation and reducing CD4+ T cells[75]. It has also been reported that SC are able to inhibit the prolif-

eration of B lymphocytes[76]. The mechanism by which SC can affect these changes in both T and B lymphocytes is not fully elucidated. However, Lin *et al*[77] demonstrated that SC express adhesion molecules vascular cellular adhesion molecule-1 and intracellular adhesion molecule 1, which lead to adhesion to lymphocytes indicating a possible SC contact dependent regulation of lymphocyte function[78]. In addition, SC release NO, PGE_2 and hepatocyte growth factor[79], as well as activate the programmed cell death 1 death receptor[80], suggesting that SC could reduce lymphocyte proliferation and survival[81-83].

In addition to its influence on the immune system, it is also interesting to note the opposite. One of the reasons why most studies use MSC is due to their ability to evade the immune system explained by their lack of HLA class I and II surface markers. Both molecules are necessary for recognition by immune cells, thus, lacking such molecules is an essential characteristic to avoid the rejection of the transplanted SC and also leaves open the possibility of transplanting cells from one donor to a patient, and not solely autologous transplantation[77]. Despite this characteristic, most human studies involving SC and inflammation use MSC in a non-randomized manner. It is also noteworthy that MSC are believed to be the only SC with immunoregulatory and regenerative capabilities, in addition to presenting almost all of the effects mentioned above in this topic[77].

Application of SC in the treatment of inflammatory pain

Inflammatory pain occurs when afferent sensory neurons detect specific molecules that are able to sensitize or activate these neurons, such as cytokines, peptides, and other molecules[84]. Most of these molecules are secreted by immune and glial cells, which normally communicate in a controlled and homeostatic manner. In inflammatory diseases, there is an imbalance between pro and anti-inflammatory molecules, leading to the activation of membrane receptors in nociceptive neurons and the consequent activity of sodium channels and TRP channels resulting in neuronal sensitization and activation[9]. Inflammation also leads to neuronal plasticity in which they express higher levels of ion channels, additional receptors, and present enhanced activity and response when compared to a non-sensitized neuron. These plastic alterations cause the transition from an acute to a chronic pain state[85].

Table 2 summarizes the current literature in which SC treatment was applied to reduce pain; taking as a principle that these cells can reduce the production of pro-hyperalgesic molecules. It is noteworthy that studies on the analgesic activity of SC in inflammatory pain have been increasing in the last 10 years. Before that, the articles were mainly focused on the mechanisms of inflammation control using SC, but not necessarily on pain. Evidence supports that the mechanism by which SC reduce thermal and mechanical hypersensitivity is based on three perspectives: (1) Targeting the inflammatory response: By having the ability to reduce the secretion of pro-inflammatory cytokines (capable of sensitizing nociceptors) such as IL-6, TNF-α and IL-17[69,86,87] as well as increasing IL-4 levels, a cytokine capable of mediating analgesia[88]; (2) Modifying cell phenotype: the effects of these cytokines (mentioned in the perspective 1) are also based on the ability of SC to induce a change in the macrophages phenotype from M1 to M2 (non-phlogistic macrophage)[88], in addition to decreasing CCL2, CCL5 and CXC10 (macrophage recruiting factors)[87]. Additionally, SC participate in the reduction of mast cell degranulation, which would result in the secretion of varied molecules with nociceptive activities such as 5-HT, histamine, LTB_4 and cytokines[89]; and (3) Neuronal and glial effects: The treatment with SC can decrease the expression in the spinal cord of calcitonin gene-related peptide (CGRP)[90] and ionized calcium-binding adaptor molecule 1 (IBA-1) (marker of glial activation, related to central sensitization)[91] and decrease the activity of immune cells. The current understanding is that SC activity occurs through the secretion of TSG-6[92], a soluble chemokine-binding protein. In turn, TSG-6 acts through inhibiting the expression of protein kinase C-γ[87] and suppressing the Toll-like receptor 2 (TLR2)/myeloid differentiation factor-88 adaptor protein (MyD88)/nuclear factor kappa B (NF-κB)[91] signaling pathway. This TLR2/MyD88/NF-κB cascade occurs in glial cells in the spinal cord and its activation leads to the production of pro-inflammatory/hyperalgesic cytokines.

The studies that address treatment in humans do not address possible signaling mechanisms of SC. However, SC treatment decreases pain for 6 mo [autologous bone marrow concentrate, one treatment, (0.5-1) × 10^6 cells][93] to 1 year (autologous adipose-derived stromal vascular cells, one treatment, 14 × 10^6 cells)[90] in knee osteoarthritis patients and for 2 years for discogenic back pain patients[93]. Thus, although the analgesic mechanisms of SC in humans remains elusive, the data supports the analgesic effect of clinical SC treatment.

SC TREATMENT OF PATHOGEN-INDUCED PAIN

In general, treatment using SC has great analgesic and anti-inflammatory potential. Similarly to sterile inflammatory diseases, infections also cause inflammation, however, there is a dual role in which inflammation involves the immune response against the pathogen as well as being responsible for tissue damage. Finding the balance between these two effects is difficult[94]. Studies involving SC and infections caused by bacteria and viruses are restricted only to the inflammatory context of infections. On this topic we will present data that demonstrate a potential analgesic effect in diseases caused by SC in bacterial and viral infections.

Bacteria-induced pain

Bacterial infections can commonly cause discomfort and pain[95]. It is believed that pain caused by bacteria and their bacterial components can occur in two different manners: The first and most classic one occurs through the activation of immune cells, production of pro-inflammatory cytokines, and the consequent nociceptor sensitization by neuronal effects of inflammatory molecules[96,97]. The second occurs through the direct activation of nociceptor neurons by bacterial virulence components, such as α-hemolysin, capable of forming pores (as its primary activity), thus activating both

Table 2 Articles that used stem cells to treat inflammatory pain (stem cell-based treatment of inflammatory pain or in models where pain is certainly involved, but was not investigated)

Ref.	Stem cell therapy design	Key findings	Pain-related highlights	Route of administration	Number of cells or amount of extracellular vesicles and exosomes
Hsueh *et al* [69], 2023	iPSC-derived EVs for the treatment of rabbit articular cartilage OA in an *in vivo* model and an *in vitro* interleukin (IL)-1 β-induced model	Improvement in both *in vivo* and *in vitro* models of OA by stimulation of chondrocytes proliferation and decreasing senescence were accompanied by: Decreasing of TNF-α); IL-6; protein 21 (p21); MMP 13; ADAMTS5; and increasing of collagen II	Indirect: Specific pain receptors/pathways weren't investigated	i.a.	100 μg iPSC-EV
Gao *et al* [184], 2022	Small EVs from iPSC-derived mesenchymal stem-cells ameliorate tendinopathy pain by inhibiting mast cell activation	The treatment was able to decrease acute pain in tendinopathy, as well as inhibit infiltration of activated mast cells and interactions with nerve fibers *in vivo*. In the *in vitro* experiments, the treatment decreased mast cell degranulation and the expression of pro-inflammatory cytokines and genes involved in the hypoxia inducible factor-1 signaling pathway	Pain behavior was measured by the von Frey method. And the weight distribution on the knees by SWB; immunofluorescence staining of tendon sections for tryptase (mast cell marker) and PGP9.5 (nerve fiber marker) was performed to assess the number of activated mast cells and the anatomical interaction between mast cells and nerve fibers. In addition, the SWB and CatWalk test was also. carried out	Local injection (quadriceps tendon)	1×10^9 particles
Yu *et al* [185], 2022	Intravital imaging and single cell transcriptomic analysis for engraftment of mesenchymal stem-cells in an animal model of interstitial cystitis/bladder pain syndrome	The transplanted cells formed a perivascular-like structure. They were also shown to express cyclin-dependent FOSe kinase-1 which played a key role in modulating the migration, engraftment and anti-inflammatory functions of multipotent MSCs, which determined their therapeutic potency *in vivo*	*In vivo* two-photon intravital microscopy and single-cell transcriptome analysis were used to assess the effects of stem cell treatment on interstitial cystitis/bladder pain syndrome	Injected into the outer layer of the anterior wall and dome of the bladder	10^6
Zhang *et al* [186], 2022	EVs derived from MSCs alleviate neuroinflammation and mechanical allodynia in interstitial cystitis rats by inhibiting NLRP3 inflammasome activation	SC treatment decreased suprapubic mechanical allodynia and frequent urination in rats with interstitial cystitis. It also decreased glial cell activity as well as neuroinflammation in the spinal cord. Furthermore, the treatment was able to decrease the activation of NLRP3 inflammasomes and the TLR4/NF-κB signaling pathway	Behavioral test (von Frey) was performed to measure allodynia and western blot and immunofluorescence for protein related to inflammation and central sensitization analysis: CD9, CD63, CD81, ALIX, TNF-α, IL-1β, IL-6, IBA-1, GFAP, NLRP3, Caspase-1, IL-18, TLR4, p65 NK-κB, phospho-p65 NK-κB (western blot). NLRP3, neuron-specific nuclear protein, GFAP and OX-42 labeling (immunofluorescence)	i.t.	20 μg
González-Cubero *et al* [86], 2022	EV and soluble fractions of adipose tissue-derived MSCs secretome induce inflammatory cytokines modulation in an *in vitro* model of discogenic pain	There was a decrease in the expression of IL-6, IL-8 and IL-17	Indirect method: The authors measured the regulatory capacity of EVs on the inflammatory molecules IL-1α, IL-1β, IL-6, IL-8, IL-17, nerve growth factor, brain-derived neurotrophic factor, IFN-γ, NF-κB and TNF and MMP-1, MMP-2, MMP-3, MMP-13 and ADAMTS-5	*In vitro* model	1×10^6
Yang *et al* [91], 2020	Anti-inflammatory protein TSG-6 secreted by bone marrow MSCs attenuates neuropathic pain by inhibiting the TLR2/MyD88/NF-κB signaling pathway in spinal microglia	Stem cells are capable of secreting TSG-6. This article demonstrated that i.t. administration of this protein leads to a decrease in mechanical allodynia and heat hyperalgesia. In addition to inhibiting neuroinflammation in the spinal cord (IBA-1), the protein administration inhibited the activation of the TLR2/MyD88/NF-κB pathway in the dorsal horn of the ipsilateral spinal cord by the secretion of TSG-	The activation of the TLR2/MyD88/NF-κB signaling pathway was evaluated by western blot and by immunofluorescence. Mechanical allodynia and heat hyperalgesia were observed by behavioral tests	i.t.	5×10^6

		6 and reduced the production levels of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α			
Zhang *et al* [187], 2019	MSCs exosomes alleviate temporomandibular joint OA by attenuating inflammation and restoring matrix homeostasis	It was observed that the treatment led to repair of the temporo-mandibular joint, along with a reduction in inflammation and pain. Treatment increased IL-1β-impaired sulfated glycosa-minoglycan synthesis and suppressed IL-1β-induced nitric oxide and MMP13 production. These effects were partially abrogated by inhibitors of adenosine receptor, protein kinase B, ERK and adenosine monophosphate activated protein kinase phosphorylation	Mechanical hyper-nociception was assessed using the von Frey microfilament. The expression of inflammatory mediators and other components was measured using quantitative polymerase chain reaction	i.a.	100 μg
Ebbinghaus *et al*[88], 2018	A promising new approach for the treatment of inflam-matory pain: Transfer of stem cell-derived tyrosine hydroxylase-positive cells (mouse model)	It has been demonstrated that the administration of endogenous tyrosine hydroxylase positive cells (iTH+) cells, prior to the induction of antigen-induced arthritis, was not sufficient to suppress the disease. However, the treatment was able to decrease pain behavior evoked by inflammation, largely due to the production of IL-4 induced by iTH+ cells. Furthermore, the treatment was able to reduce the levels of pro-inflammatory molecules, in addition to increasing the number of M2 macrophages in dorsal root ganglia	Inflammatory molecules were quantified, such as: IFN-γ, IL-2, IL-4, IL-6, IL-10, CCL3, CCL5, CXCL1, CXCL2, CXCL10, and CXCL12. Additionally, pain-related behavior tests and IBA-1 and arginase 1 labeling in the dorsal root ganglion *via* immunofluorescence was performed	i.v.	10^6
Ichiseki *et al* [92], 2018	I.a.-injected MSC stimulate anti-inflam-matory molecules and inhibits pain related protein and chondrolytic enzymes in a monoiodo-acetate-induced rat arthritis model	The treatment was able to inhibit central pain sensitization (decreased expression of CGRP in the spinal cord) and increase the secretion of TSG-6 by stem cells, an anti-inflammatory factor and cartilage protector	For the evaluation of central sensit-ization, CGRP staining was performed by immunofluorescence. And the histochemical technique was also used for the evidence in the joint of ADAMTS5 and TSG-6	i.a.	5.0×10^6
Fodor and Paulseth [90], 2016	Adipose derived stromal cell injections for pain management of OA in the human knee joint	After 3 mo of treatment, patients showed improvement in WOMAC and VAS scores, which were maintained for 1 yr. ROM and TUG improved until the third month. All patients achieved full activity with decreased knee pain and no infections or adverse effects reported	Patients were evaluated by following scores on the WOMAC, VAS pain scale, ROM, TUG, and magnetic resonance imaging	i.a.	14.1×10^6 nucleated stromal vascular fraction cells per knee
Pettine *et al* [93], 2016	Treatment of discogenic back pain with autologous bone marrow concentrate injection with minimum two-year follow-up (humans)	Stem cell treatment reduced visual analog scale and Initial Oswestry Disability Index scores. In addition to reducing pain in patients. The treatment proved to be effective for up to 2 yr after the injection	Pain was assessed using scores provided by patients	Intradiscal injection	$(0.5\text{-}1) \times 10^6$
Durand *et al* [89], 2015	Persistent visceral allodynia in rats exposed to colorectal irradiation is reversed by MSC treatment	Induced a time-dependent reversion of the visceral allodynia and a reduction of the number of anatomical interactions between mast cells and PGP9.51 nerve fibers	Spinal sensitization (was available for labeling of phospho-ERK neurons), colonic neuroplasticity (as increased density of substance P1 nerve fibers); s, visceral sensitivity was evaluated by studying the contraction of the abdominal muscles in response to colorectal distension	i.v.	1.5×10^6
Watanabe *et al*[87], 2015	Early transplantation of MSC after SCI relieves pain hypersensitivity through suppression of pain-related signaling cascades and reduced inflammatory cell	The treatment was able to decrease thermal and mechanical hypersens-itivity. Improvements in pain were mediated by suppression of PKC-γ and p-CREB expression in dorsal horn neurons. The authors also reported a decrease in the levels of	Mechanical allodynia and thermal sensitivity were recorded. In addition, immunofluorescence was performed on spinal cord sections, labeling for: PKC-γ or p-CREB, GFAP, cD11B and phospho-protein 38. For immunoblot analysis,	Injection into the middle of the contusion site, identified as the middle point of the laminectomy area	2.0×10^5

	recruitment	pro-inflammatory cytokines (TNF-α, IL-6), mediators of early secondary vascular pathogenesis (MMP9) and macrophage recruitment factors (CCL2, CCL5 and CXCL10). All in addition to increased levels of a microglial stimulating factor GM-CSF)	components of the mitogen-activated protein kinase family, inflammatory mediators (TNF-α, IL-6, MMP-9), macrophage recruiting factors (CCL2, CCL5, and CXCL10) and GM-CSF (a microglial stimulating factor) were analyzed		
Emadedin *et al*[188], 2012	Intra-articular injection of autologous MSC in six patients with knee OA	The treatment was able to improve scores related to pain, the functional status of the knee and the distance covered up to six months after the injection	VAS which is a subjective assessment that represents the patient's perception of the current pain state with a higher score reflecting more severe pain. Functional status of the knee was assessed by WOMAC OA index. This index evaluates pain, joint stiffness, physical and social function of patients with OA of the knee	i.a.	(20-24) × 10^6

iPSC: Induced pluripotent stem cells; OA: Osteoarthritis; IL: Interleukin; TNF-α: Tumor necrosis factor alpha; p21: Protein 21; MMP: matrix metalloproteinase; ADAMTS5: A disintegrin and metalloproteinase with thrombospondin motifs 5; i.a.: Intra-articular; EV: Extracellular vesicle; SWB: Static weight bearing; PGP9.5: Protein gene product 9.5; CDK1: Cyclin-dependent FOSe kinase 1; MSC: Mesenchymal stem cells; NLRP3: NOD-like receptor protein 3; i.t.: Intrathecal; TLR: Toll-like receptor; NF-κB: Nuclear factor kappa B; IBA-1: Ionized calcium-binding adapter molecule 1; GFAP: Glial fibrillary acidic protein; CD: Cluster of differentiation; IFN-γ: interferon-gamma; TSG-6: Tumor necrosis factor alpha-stimulated gene 6; MyD88: Myeloid differentiation primary response 88; ERK: Extracellular signal-regulated kinase 1; iTH+: Tyrosine hydroxylase positive cells; CCL: C-C motif chemokine ligand; CXCL: C-X-C motif chemokine ligand; DRG: Dorsal root ganglion; i.v.: Intravenous; CGRP: Calcitonin gene-related peptide; WOMAC: Western Ontario and McMaster Universities Arthritis Index; VAS: Visual Analogue Scale; ROM: Range of motion; TUG: Timed ascent and descent; PKC-γ: Protein kinase C gamma; GM-CSF: Granulocyte-macrophage colony stimulating factor; p-CREB: Phospho cyclic AMP response element binding protein; p-p38: Phospho-protein 38.

Nav1.8+ and TRPV1+ neurons[98,99]; or by LPS, which is capable of activating TLR4 expressed by neurons or be sensed by TRPA1 (at lower doses) and even TRPV1 (at higher doses)[100,101]. Thus, bacteria can induce nociceptor sensory neuron sensitization indirectly by activating immune cells that will produce nociceptor sensitization molecules or directly by activating neuronal receptors and triggering nociceptor depolarization by forming membrane pores[96].

As discussed in the topic "SC in the treatment of inflammatory diseases and inflammatory pain", the effects of SC activity on bacterial infections have two main characteristics. The first is that treatment with SC can increase phagocytic activity and neutrophil survival in bacterial infections[102,103]. Second, SC treatment can attenuate exacerbated immune responses, as seen in a murine model of endotoxemia induced by LPS, the administration of MSC by the intraperitoneal route is capable of reducing the severity of the disease, mainly by reducing the levels of IL-1β, IL-6, IL-8 and TNF-α and increasing IL-10 in the plasma, as well as reducing the recruitment of neutrophils in the liver[104].

Despite demonstrating great analgesic potential by reducing the levels of cytokines causing hypersensitivity and increasing bacterial clearance, no articles were found that investigated a possible decrease in pain in models that use bacteria and SC treatment. This fact highlights a gap in the literature and potential field to be explored.

Viral infection-induced pain

In general, viral infections can cause pain. Depending on the type of virus and site of infection, an inflammatory process begins, characterized by the high release of inflammatory mediators. The detection of these mediators can occur through the central or peripheral nervous system[11]. A recent study demonstrated that most of the symptoms of intranasal H1N1 infection (reduction in food intake, water intake, and mobility during early-stage infection and improved survival) come from the detection of PGE_2. This prostanoid activates EP3 receptors, which are expressed in both the hypothalamus and circumventricular organs, as well as in nerve endings in the nasopharynx[105]. It has also been demonstrated that the herpes simplex virus 1 can also infect the neurons present in the DRG, and the persistence of the virus leads to the recruitment of leukocytes to the region, TNF-α secretion and neuronal hypersensitivity[106]. While the pain caused by Chikungunya virus depends on its envelope protein E2 activation of TRPV1+ nociceptor sensory neurons[107].

As discussed earlier, SC have a high anti-inflammatory capacity. This characteristic is also observed against viruses since treatment with SC can decrease the levels of cytokines[108] that sensitize nociceptors. An interesting fact about SC is that these cells have IFN-responsive genetic machinery. Therefore, upon detecting the presence of the signal produced in viral infections (flavivirus, dengue and Chikungunya virus), they express genes related to IFN-induced transmembrane proteins, which prevents the contamination of these cells[109]. In murine models, treatment with MSC reduces the levels of IL-1α, IL-6, TNF-α and IFN-γ in response to H9N2 infection[110]. It has also been described that the administration of extracellular vesicles derived from MSC was able to reduce the viral load in lung epithelial cells of pigs infected with H1N1/H7N2/H9N5[111]. Recent evidence demonstrates that extracellular cells derived from MSC have the *in vitro* capacity to inhibit the replication of the Influenza virus and severe acute respiratory syndrome coronavirus 2[112]. It is also important to report that recently a number of clinical studies have been carried out in humans using MSC treatment in coronavirus disease (results not yet reported)[113-117]. Despite the potential of SC treatment to reduce pain and the fact that viral infections can cause pain, there is a gap in the literature of studies investigating the analgesic effectiveness of SC in the treatment of viral infection-triggered pain.

SC-BASED TREATMENTS FOR NP

Studies involving inflammation and pain are still somewhat restricted when compared to the volume of articles that study NP and treatment using SC. On this topic, we will present an overview of the mechanisms involved in the development of NP and then explain the mechanisms underpinning SC therapy.

What mechanisms are involved in the development of NP?

To fully comprehend the mechanisms that modulate NP, it is essential to understand how our body processes the external and internal stimuli that can lead to nociceptive alterations. Pain sensation is the product of higher brain center perception and can be influenced by a number of factors like attention, affective dimensions, autonomic variables, immune variables, and hormones[118].

In normal tissue, pain is triggered by intense or noxious stimuli that leads to the activation of high threshold transmembrane ion channels, a process defined as nociceptive pain. These ion channels present on nociceptor neurons convert mechanical, thermal or chemical stimuli - that can vary from pinpricks and light touch to vibrations, indentations, gravity and sound waves - into biochemical regulated electrical signals that are directed to the brain through the generation and conduction of action potentials, characterizing mechanotransduction[119].

NP, on the other hand, occurs when a pathological process leads to nerve damage, and the healing process of the nervous system results in maladaptation observed by the lower unbalanced threshold of neuronal activation that can involve numerous pain-related processes, from the detection by the nociceptor neuron to the acknowledgement of nociceptive signaling by the brain[120]. NP comprises peripheral neuropathy, postherpetic neuralgia, trigeminal neuralgia, nerve root pain, and phantom limb pain; and can be caused by lesions or diseases involving the primary afferent sensory neurons of the somatosensory nervous system, including peripheral fibers (Aβ, Aδ and C fibers) and CNS neurons[121-123].

The extensive modulatory possibilities that can unravel during the healing process of nervous tissue make evident the importance of neuroplasticity, a phenomenon that can be defined as the ability of the nervous system to adapt its responses according to intrinsic or extrinsic stimuli by reorganizing its structure, functions, or connections after injuries. Thus, neuroplasticity is a key factor in the development of NP[124].

NP typically arises from an incorrect healing process due to an imbalance between neuroimmune interactions, glial cells, and neurotrophic factors. Briefly, a nervous system lesion triggers inflammatory and repair responses that are not always successful. Unsuccessful nerve repair will lead to plastic changes causing the sensitization of nociceptive neurons, sympathetic sprouting forming basket structures that explain sympathetic maintained chronic pain, incorrect formation of novel synapses causing the stimulation of second order neurons upon touch (causing allodynia). Maladaptive tissue repair can also involve the activation of glial cells that further stimulate nociceptive neurons causing retrograde sensitization and boosting second order nociceptive signaling to the brain when a nervous fiber is sectioned. Cellular events occur at the site of injury and in the neuronal soma corresponding to the area in the DRG. These cellular events include, for instance, local immune cell signaling *via* purinergic P2 receptors-ATP signaling[125]. Nerve damage is, therefore, an initiating event, but it is not the sole orchestrating factor. Neuronal plastic changes include the alteration of ion channel properties, affecting spinal and brain sensory signaling, shifting pain perception so that normal innocuous stimuli can result in pain by facilitating neuronal depolarization upon thermal and mechanical stimulation as a result of nociceptor neuron sensitization owing to increased membrane excitability. Spontaneous neuronal firing can also be observed[119,125,126].

The regeneration process can be divided into five steps: (1) Fluid phase; (2) Matrix phase; (3) Cellular migration phase; (4) Axonal phase; and (5) Myelination phase[127]. After injury, neuronal genetic expression is altered to induce the release of neurotrophic and angiogenic factors like NGF, brain-derived neurotrophic factor, glial cell-derived neurotrophic factor (GDNF), vascular endothelial growth factor-A and angiopoietin-1[128], and to upregulate the expression of their corresponding receptors. These growth factors support the axonal lengthening of the injured nerve from its proximal fragment, as the damaged axons in the distal nerve fragments undergo degeneration, a process known as Wallerian degeneration[127]. Infiltrating macrophages and Schwann cells also take part by clearing myelin debris and retro feeding the secretion of neurotrophic and pro-angiogenic factors, enabling the formation of connective tissue bridging the nerve clefts, as Schwann cells create an endoneurial tube that guides the axonal regeneration process starting from a growth cone located at the Ranvier's node[129].

The accurate balance between compensatory and decompensatory reactions of the nervous system when facing neural damage is of the utmost importance, because many of the changes that occur in response to neural injury are potentially adaptive, such as the removal of cell and myelin debris, regulation of receptors that counterbalance the loss of input, and appropriate signaling in order to dampen ion fluxes and metabolic stress after the acute injury[130]. Among the adaptive modifications, we can also cite anti-apoptotic signaling to prevent neuronal cell death, induction of axonal growth and sprouting, synaptic remodeling, and remyelination[131].

A defining characteristic that is often present in NP is the absence of an identifiable stimulus upon spontaneous pain. Such abnormal sensitization can be generated at any anatomical level related to the nociceptive sensory experience: (1) The site of the injury that induced the NP in the first place and underwent maladaptive healing - or neuroma - where the regenerated axon can get misdirected and become unable to reach the desired target[132]; (2) Cell death of the corresponding DRG neurons; (3) Alterations in gene regulation and expression of the surrounding intact afferent fibers; (4) Central sensitization and altered connectivity in the spinal cord of low-threshold large myelinated afferents *via* synaptic facilitation and loss of inhibition at multiple levels of the neuraxis; and (5) Voltage-gated channel-related generation of spontaneous ectopic activity in nociceptors, a mechanism whose importance is supported by the effectiveness of nonselective sodium channel blockers as local anesthetics[133]. Increased synaptic strength enables previously sub-

threshold inputs to activate nociceptive neurons, reducing their threshold and enhancing their responsiveness, which results in the expansion of their receptive fields. In addition, phenomena like conduction slowing or blocking, reduced inhibition, inappropriate connectivity, altered processing of both nociceptive and innocuous afferent input, abortive growth, neuronal loss, and glial scarring can be decisive factors underlying the pathogenesis of NP and the onset of spontaneous pain[126,134].

It is important to note that once NP is generated, the sensory hypersensitivity tends to persist for prolonged periods, even though the original initiator factor may have long since disappeared[126]. The complexity and abundance of factors involved in the central and peripheral nervous system modifications, make the treatment of NP challenging and expensive[51].

The use of SC as a therapy for NP

Given the fact that current treatments for NP are not fully effective, there is a need for improving NP treatment. Among the characteristics that stand out in the use of SC for NP is the ability of these cells to migrate to the injured site and repair damaged cells, even when administered systemically[135]. Most literature reports address SC treatment in the context of NP models, in which strategies are based on replacing damaged nerve cells and induce the production of neurotrophic factors. In addition, SC inhibit apoptosis and degeneration processes, and increase the survival of both injured and uninjured nerves[136] (Table 3). Furthermore, the mechanisms of action of SC modulating NP vary according to the stimulus in question (disease model/condition), since each disorder presents its own alterations that can be classified as peripheral and/or central, we will follow this rationale in the next sections.

Peripheral nervous system-related analgesic mechanisms of SC

The peripheral actions of SC focus on their anti-neuro-inflammatory capacity and their neuroprotective potential and ability to promote growth. SC anti-neuro-inflammatory capacity (described in the previous topics) includes the ability to reduce the levels of pro-inflammatory cytokines IL-1β, IL-6[137] and TNF-α[87]. These cytokines are extremely important in peripheral sensitization, because they are capable of sensitizing nociceptors and increase the expression of TRP and Na channels, consequently leading to mechanical and thermal sensitization[9]. In addition to reducing the production of hyperalgesic cytokines, SC can increase IL-10 levels in NP models[137], which also explains the decrease in the pro-inflammatory cytokine production[138] and induction of the class switch of macrophages from an M1 to an M2 profile. M1 macrophages are responsible for proinflammatory responses, overexpress CD80, CD86, and CD16/32 which are essential for activating lymphocytes and thus adaptive immunity. Moreover, M1 phenotype macrophages are capable of secreting pro-inflammatory cytokines[139]. In contrast, M2 macrophages express chemokines CCL17 and CCL22 that mediate the control of Treg cell biology[140], mannose receptor (CD206) that induces endocytosis[141], as well as anti-inflammatory responses and contribute to the repair of damaged tissues by the phagocytosis of debris[138]. Moreover, M2 macrophages can be manipulated *in vitro* to produce an opioid-mediated analgesic effect, demonstrated by its complete blockade by the opioid receptor antagonist naloxone methiodide in an *in vivo* model of chronic constriction injury (CCI) of the sciatic nerve induced NP[142]. Although there is no data on SC and microglia polarization, considering the activity of SC to polarize macrophages towards a M2 profile, if this activity is also true for microglia polarization to an M2 profile, this would be an important analgesic mechanism in NP. M1 microglia are actively involved in NP by producing mediators that sensitize nociceptors[143].

SC are also able to directly interact with the DRG neurons through GDNF production[144]. GDNF is of great importance for studies where there is neuronal damage, such as in sciatic nerve injury[136]. Since the administration of GDNF is capable of decreasing pain-related behaviors, due to its inhibitory action on the molecules such as activating transcription factor 3 - marker of neuronal injury and IB4 (a noceptive neuron marker of a neuronal population that do not express CGRP, and that downregulate NGF and receptor tyrosine kinase)[145,146].

The actions of SC on DRG neurons also affect the production of neuropeptides related to pain signaling. In models of CCI-induced NP, SC treatment was able to decrease nociceptive behavior by releasing TGF-β1, which activates neuronal TGF-β1R[147], as well as reducing the production of hyperalgesic cytokines IL-1β and TNF-α[148].

CNS-RELATED ANALGESIC MECHANISMS OF SC

The actions of SC treatment targeting mechanisms in the CNS environment are based on three hypotheses: (1) Desensitization of the CNS; (2) Inhibition of glial cells; and (3) Reduction in apoptosis and autophagy[149].

The SC mechanisms that are dependent on: (1) Desensitizing the CNS in NP mainly involve the downregulation of glutamate neurotransmission. After the damage of peripheral neurons, glutamate is released in the spinal cord as well as N-methyl-d-aspartate (NMDA) and an increase of its receptor (NMDAR) expression is seen. This mechanism aims to maintain the transmission of the captured noxious signal to the cerebral cortex, where it will be interpreted as pain[150]. Treatment with SC decreases the CNS expression of NMDAR, interrupting the maintenance of nociceptive signaling [150]. In addition, SC can secrete TGF-β1, which inhibits the signaling carried out by glutamate that would otherwise stimulate nociceptive signaling by the activation and proliferation of microglia and astrocytes in NP. TGF-β1 also reduces the expression of proinflammatory cytokines in the CNS in NP, thus, reducing neuroinflammation and nociceptor neuron sensitization[147].

Second, glial cells play a key role in central sensitization. As previously explained, after the detection of noxious stimuli by nociceptors, primary sensory afferent neurons secrete neuropeptides in the spinal cord dorsal horn, where central signaling takes place. If the noxious stimulus persists, glial cells can be activated by these neuropeptides (CGRP and

Table 3 Articles that used stem cells for neuropathic pain treatment (stem cell-based treatment of neuropathic pain)

Ref.	Stem cell therapy design	Key findings	Pain-related highlights	Delivery route	Number of cells or amount of extracellular vesicles and exosomes
Gao *et al* [189], 2023	Huc-MSCs-derived exosomes attenuate neuropathic pain by inhibiting activation of the TLR2/MyD88/NF-κB signaling pathway in the spinal microglia by targeting radical S-adenosyl methionine domain containing 2	Huc-MSCs-derived decreased protein levels of TLR2, MyD88, and p-p65 that were significantly upregulated in the CCI group in model rats	The protein levels of TLR2, MyD88, p65, and p-p65 were examined by western blotting	i.t.	5 μg
Miyano *et al* [190], 2022	I.v. administration of human MSCs derived from adipose tissue and umbilical cord improves neuropathic pain *via* suppression of neuronal damage and anti-inflammatory actions in rats	Both the mechanical threshold and the differences in weight bearing of the right and left hind paws improved significantly. In addition, the authors also reported a decrease in the ATF-3 and IBA-1 in DRG. The authors also reported that the treatment significantly improved the partial sciatic nerve ligation-induced decrease in the level of myelin basic protein in the sciatic nerve	Was performed by von Frey and dynamic weight bearing. Also, the authors did I against ATF-3, IBA-1, myelin basic protein, NeuN, neurofilament (NF) 200	i.v.	5×10^6
González-Cubero *et al* [191], 2022	Application of adipose-derived MSCs in an *in vivo* model of peripheral nerve damage	Rat sciatic nerve damage models both *ex vivo*, on TNF-induced Schwann cells, and *in vivo* using biomaterial implants containing TNF. Upregulation of c-Jun and downregulation of early growth response protein 2 myelin-associated transcription factors were induced by TNF-related damage, but the addition of ASCs or ASC-conditioned medium (secretome) were able to revert the profile	qPCR, western blot, and confocal microscopy were chosen to quantify nerve healing-related protein expression and production *in vivo* and *ex vivo*. The sciatic functional index was calculated to assess nerve regeneration, but no pain-specific mechanisms were investigated	Sciatic nerve	*ex vivo* 0.5×10^6 cells; *in vivo* 4×10^6 ASCs
An *et al* [192], 2022	Immortalized bone MSCs with inducible galanin expression produce controllable pain relief in neuropathic rats	hTERT-BMSCs/Tet-on/GAL cells were able to induce controllable pain relief by spared nerve injury of sciatic nerve under the transcriptional control of doxycycline	To determine the analgesic efficacy acted through GalR1, GalR2, and phospho-protein kinase Mζ expression levels in spinal dorsal horn were analyzed by western blot assay	Subarachnoid space	10^6
Lee *et al* [193], 2022	MSCs spheroids alleviate neuropathic pain by modulating chronic inflammatory response genes	The authors report a decrease in mechanical allodynia, related to a decrease in TNF-α and IFN-γ levels. In addition to a smaller number of cells marked with cluster of differentiation (CD) 68 in the region	The von Frey test was performed to assess mechanical allodynia, while immunofluorescence was used to observe changes in CD68 and IBA-1 levels. TNF-α and IFN-γ levels were assessed by the ELISA assay	Intramuscular	10^6
Chen *et al* [194], 2022	Synergic Effect of early administration of probiotics and adipose-derived MSCs on alleviating inflammation-induced chronic neuropathic pain in rodents	The authors demonstrate that treatment with stem cells alone can reduce thermal hyperalgesia and mechanical allodynia, with the potentiated effects after combined treatment with probiotics. Interestingly, they found a reverse correlation between protein expressions of inflammatory (phospho-NF-κB, IL-1β, TNF-α and MMP-9), apoptotic (cleaved-caspase-3, cleaved-PARP), oxidative-stress (NOX-1, NOX-2), deoxyribonucleic acid (DNA)-damaged (γ-H2AX) and MAPK-family (p-P38, p-JNK, p-ERK 1/2) biomarkers as	To observe pain-related behavior alterations, Hargreaves and von Frey tests were applied. Immunofluorescence was performed for p-p38; NF200; peripherin, 53BP1, β3 Tubulin analysis. Western blot was chosen to identify alteration of p-NF-kB, IL-1ß, TNF-α, MMP-9, NOX-1, NOX-2, caspase 3, cleaved-PARP, γ-H2AX, p-ERK1/2, p-JNK, p-p38, Nav.1.3, Nav.1.8, Nav.1.9 and immunoglobulin G	i.v.	3.0×10^5

		well as the protein levels of voltage-gated sodium channels (Nav.) 1.3, Nav.1.8, and Nav.1.9 in L4-L5 in DRG to the pain-behavior results obtained by thermal hyperalgesia and mechanical allodynia testing, characterizing a set of "pain-connived cells" presenting the following profiles: Nav1.8+/peripherin+, p-ERK+/peripherin+, p-p38+/peripherin+ and p-p38+/NF200+. Mainly by suppressing inflammation and oxidative stress, the combination of probiotic and ASCs therapy was found superior for alleviating CCI-induced neuropathic pain			
Zhang *et al* [195], 2021	Lncenc1 is identified as a novel regulator in neuropathic pain by interacting with EZH2 and downregulating the expression of Bai1 in mouse microglia	Virgin embryonic stem cells express Lncenc1, which can activate microglia in DRG and induce the production of TNF-α, IL-1β, and MCP-1. Lncenc1 silencing reduced mechanical and thermal hyperalgesia, as well as lower levels of pro-inflammatory cytokines	The mechanical withdrawal threshold was measured by von Frey filaments and thermal hyperalgesia *via* hot plate assay. Immunofluorescence was performed to analyze OX-42, western blot to assess EZH2, suppressor of zeste 12, embryonic ectoderm development, BAI1, tri-methylation of histone 3 lysine 27 (H3K27me3), H3K27ac, total histone H3, glyceraldehyde-3-phosphate dehydro-genase and OX-42. RT-qPCR was performed to identify expression alterations on Lncenc1, EZH2, BAI1, OX-42, inflammatory factors TNF-α, IL-1β and chemokine MCP-1. TNF-α, IL-1β and MCP-1 protein changes were assessed by ELISA	Not informed	Not informed
Masoodifar *et al*[161], 2021	Effect of the conditioned medium of MSCs on the expression levels of P2X4 and P2X7 purinergic receptors in the spinal cord of rats with neuropathic pain	Animals treated with the conditioned medium (stem cells secretome) showed a reduction in mechanical and thermal hyperalgesia. A decrease in the expression of P2X4 and P2X7 receptors was related to the interaction of neurons and glial cells in neuropathic pain	The von Frey and hot plate tests were applied to measure mechanical and thermal hyperalgesia, respectively. In addition, qPCR was performed to measure the expression of P2X4 and P2X7 receptors	i.p.	1×10^5
Kotb *et al* [196], 2021	Preemptive stem cells ameliorate neuropathic pain in rats: A central component of preemptive analgesia	MSCs-treatment increased allodynia, mechanical hyperalgesia, and thermal hyperalgesia thresholds. Stem cells were able to reach the cerebral cortex, as the CCI group had few stem cells expressing PCNA, CD117 and nestin in the cerebral cortex. The treated group had numerous CD117-, nestin-, PCNA-positive stem cells recently proliferated in the cerebral cortex. Together, the results indicate a potential central analgesic effect of i.v. MSC-treatment	To evaluate pain behavior, von Frey, Randall and Selitto, and hot plate tests were performed. Immunohistochemical analyses of GFAP, PCNA and nestin were also performed	i.v.	1×10^6
Zhang *et al* [197], 2021	Therapeutic effects of peripherally administered neural crest stem cells on pain and spinal cord changes after sciatic nerve transection	The treatment was able to induce thermal and mechanical analgesia, possibly by decreasing the expression of TRPV1, cFOS, p-ERK, ERK, iNOS and NF-κB, p65 and increasing BDNF and GAP-43 in the spinal cord	To assess mechanical allodynia, the authors used the BEM-404 device (similar to the von Frey). For thermal withdrawal latency, they use Hargreaves. In the western blot, they had the following targets: BDNF, cFOS, GAP-43, p-ERK, ERK 1/2, TRPV1 and iNOS. Immunofluor-escence: IBA-1, GFAP and CGRP were assessed	Local injection	2×10^6
Yang *et al* [91], 2020	Anti-inflammatory protein TSG-6 secreted by BMSCs attenuates neuropathic pain by inhibiting the TLR2/MyD88/NF-κB	I.t. administration of TSG-6 secreted from stem cells decreases mechanical allodynia and thermal hyperalgesia, inhibiting IBA-1 and the activation of the	The activation of the TLR2/MyD88/NF-κB signaling pathway was evaluated by western blot and immunofluorescence, while allodynia and hyperalgesia were assessed by the behavioral tests Dynamic Plantar Aesthesiometer, Hargreaves and	i.t.	5×10^6

	signaling pathway in spinal microglia	TLR2/MyD88/NF-κB pathway in the dorsal horn of the ipsilateral spinal cord. Levels of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, were also reduced	rotarod system		
Jwa *et al* [198], 2020	ASCs alleviate cold allodynia in a rat spinal nerve ligation model of neuropathic pain	ASCs or ASC-derived culture medium decreased neuropathic pain behaviors in a rat model with L5 spinal nerve ligation	Mechanical and cold allodynia were assessed by von Frey filaments and acetone assay, respectively. Mechanisms were not assessed	Intrathecal or injection into the right retro-orbital sinus	10^6
Gama *et al* [164], 2018	Conditioned medium of BMSCs as a therapeutic approach to neuropathic pain: A preclinical evaluation	The animals showed improvement in thermal hyperalgesia and mechanical allodynia. They also showed reduced levels of IL-1β, TNF-α and IL-6 and increased IL-10 in the spinal cord and sciatic nerve	To evaluate thermal hyperalgesia, the Hargreaves test was performed, and von Frey mechanical allodynia. To evaluate the motor function test, the rotarod test was performed. Using the ELISA method, TNF-α, IL-1β, IL-6 and IL-10 were quantified	i.v.	10^6
Lin *et al* [165], 2017	Autologous ASCs reduce burn-induced neuropathic pain in a rat model	There was no difference between the groups regarding thermal hyperalgesia, whereas in mechanical allodynia, the treated group presented analgesia from the 3rd wk of the first treatment. Western blot analyses revealed a decrease in p-Akt/Akt and Bax/Bcl-2 and levels of LC3B-II and Beclin 1 in the spinal cord, suggesting that the treatment also decreased apoptosis and autophagy. This effect was accompanied by a reduction in COX-2, iNOS and nNOS. The treated group also showed lower expression of p-JNK (an inflammatory marker), TUNEL (apoptosis marker), phospho-NFκB (inflammatory marker) and increased p-IκB (an inhibitor of NFκB activation)	Immunofluorescence were performed to analyze p-IκB; NeuN, GFAP, phospho-NFκB and p-JNK; and western blot for COX-2, iNOS, nNOS, Akt/protein kinase B, p-Akt, B-cell lymphoma 2, Bcl-2-associated X protein, β-actin, LC3B and Beclin 1	Subcutaneous into the scar tissue of the right hind paw	10^6
Vaquero *et al*[199], 2018	I.t. administration of autologous bone marrow stromal cells improves neuropathic pain in patients with SCI	Treatment with mesenchymal stromal cells for human chronic SCI: Pain scores demonstrated a continuous decrease in neuropathic pain from the first month until the 10th	Intensity of neuropathic pain was evaluated by standard numerical rating scale (visual analogue scale) from 0 to 10. Mechanisms were not assessed	i.t.	10^6
Sun *et al* [200], 2017	I.t. administration of hBMSCs genetically modified with human proenkephalin gene decrease nociceptive pain in neuropathic rats	hBMSCs engineered with human proenkephalin gene were used on sciatic nerve (CCI)-induced model to reduce neuropathic pain in rats	Mechanical withdrawal threshold (von Frey filaments) and paw thermal withdrawal assays were used to assess the changes in pain-related behavior. Levels of Leu-enkephalin, a neurotransmitter that activates opioid receptors and is released by hBMSCs were found augmented *via* ELISA assay in genetically modified BMSCs compared to secretions released by naıve BMSCs	i.t.	6×10^6
Fischer *et al* [201], 2017	Inhibition of neuropathic hyperalgesia by i.t. BMSCs is associated with alteration of multiple soluble factors in cerebrospinal fluid	BMSCs decrease the levels of intracellular adhesion molecule 1, IL-1β, hepatocyte growth factor), IL-10, and Nope protein relacionated by Tibial nerve injury	Antibody array analysis was performed and the levels of cytokines and other soluble factors in cerebrospinal fluid samples was measured	i.t.	2.5×10^5
Xie *et al* [202], 2017	Active nerve regeneration with failed target reinnervation drives persistent neuropathic pain	Semaphorin 3A, an inhibitory axonal guidance molecule, reduces functional regeneration, spontaneous activity, and pain behaviors when applied to the injury site in vivo. Silencing of the upregulated GAP43 with interfering RNA injected into the axotomized sensory ganglion reduced pain behaviors	Behavior assays: von Frey filaments acetone cold sensitivity, dynamic tactile allodynia with a wisp of cotton across the plantar surface of the hindpaws, and spontaneous guarding behavior score. Immunohistochemistry for GAP43 tracer methods to assess anatomical nerve regeneration	Injury site	

Brini *et al* [203], 2017	Therapeutic effect of human ASCs and their secretome in experimental diabetic pain	Treatments with both human ASC and their secretome were able to reverse mechanical, thermal allodynia and thermal hyperalgesia inducing high IL-1β, IL-6 and TNF-α and low IL-10 levels, restoring cytokine balance, Th1/Th2 balance and preventing skin innervation loss in neuropathic STZ-diabetic mice model	Mechanical allodynia was tested using the Dynamic Plantar aesthesiometer, a drop (50 μL) of acetone was placed in the middle of the plantar surface of the hind paw to evaluate cold allodynia and the hot-plate test was used to assess thermal hyperalgesia. Immunohistochemistry and ELISA were performed for cytokines assessment	i.v.	1×10^6
Watanabe *et al*[87], 2015	Early transplantation of MSCs after SCI relieves pain hypersensitivity through suppression of pain-related signaling cascades and reduced inflammatory cell recruitment	BMSC improved SCI model *via*: Down of protein kinase C-γ and phosphocyclic AMP response element binding protein on DRG neurons, both of which are upregulated in association with at-level allodynia after contusion spinal cord. Decreased activation of MAPK signaling in injured spinal cord by p-p38 and p-ERK1/2 decrease. Decreasing macrophage recruitment through. Down TNF-α, IL-6, MMP-9, CCL2, CCL5, and C-X-C motif chemokine ligand 10. Decreased microglia stimulation factor, granulocyte-macrophage colony stimulating factor, platelet-derived growth factor receptor α	For behavioral and sensory testing, the Basso Mouse Locomotor Scale, the Dynamic Plantar Aesthesiometer (allodynia), and the Plantar Test Apparatus (thermal sensitivity) were assessed. immunohistochemistry, flow cytometry and immunoblot assays were performed to determine protein levels	BMSCs were injected into the middle of the contusion site, identified as the middle point of the laminectomy area	2×10^5
Zhang *et al* [204], 2014	I.t. administration of MSCs reduces the ROS and pain behavior in neuropathic rats	I.t. rat MSCs injection reduced pain response and ROS production in the dorsal horn of neuropathic rats induced by spinal nerve L5 ligation model	Mechanical sensitivity was assessed using von Frey filaments and production of ROS *via* dihydroethidium fluorescent staining	i.t.	10^5
Liu *et al* [205], 2014	MSCs inhibit lipopolysaccharide-induced inflammatory responses of BV2 microglial cells through TSG-6	Anti-inflammatory effects of MSCs and TSG-6 in an *in vitro* LPS-induced BV2 microglial activation model inhibiting NF-κB and MAPK pathways. MSCs can modulate microglia activation through TSG-6 and TSG-6 attenuates the inflammatory cascade in activated microglia	RT-qPCR, western blot, electrophoretic mobility shift assay, immunofluorescence and laser-scanning confocal microscopy techniques were used	*In vitro*	1.0×10^5 LPS-activated MSCs
Vicker *et al* [206], 2014	A preliminary report on stem cell therapy for neuropathic pain in humans	Treatment led to a reduction in stem cell treatment pain intensity scores in 7/9 patients (two with marginal improvement and five subjects with good to excellent pain reduction). Five of these positive responders also reduced their need for gabapentin medication	Patients were assessed for: Change in pain intensity and the secondary outcome was any reduction in daily consumption of anti-neuropathic medication	Perineural, directly in the center or source of pain, and in the adjacent pain field of the affected branches of the trigeminal nerve	Number of cells not reported, but extracted from 100-200 g of patient tissue
Tao *et al* [154], 2013	Role of NRG1/ErbB signaling in stem cell therapy for SCI-induced chronic neuropathic pain	The treatment induces remyelination in the injured spinal cord and reduces SCI-injury-induced chronic neuropathic pain. In addition to increasing levels of NRG1 and ErbB4 slightly reduced by SCI. Also, the author related that Stem cells differentiated into oligodendrocytes	To evaluate mechanical allodynia, the von Frey filament test was applied. Immunofluorescence for NG2, APC-CC1, GFAP, NeuN, and western blot for NRG1 and ErbB4 levels assessment	i.t.	10^6
Xu *et al* [207], 2013	I.t. transplantation of NSCs appears to alleviate neuropathic pain in rats through release of GDNF	The treatment was able to cause thermal and mechanical analgesia. Accompanied by an increase in GDNF in the DRG and spinal cord. The authors also suspected that these changes occurred due to the transformation of stem cells into astrocytes in the spinal cord	To evaluate the mechanical withdrawal threshold, the Electric von Frey test was used. For thermal withdrawal latency, a method with a high-intensity projection lamp bulb was used. For immunofluorescence: Nestin; βIII-tubulin; GFAP. For ELISA: BDNF and GDNF	i.t.	10^6

Choi *et al* [208], 2013	Core-shell nanoparticle controlled human adipose tissue-derived stem cells neurogenesis for neuropathic pain therapy	Treatment activated biochemical functions of Dicer, Oct4, Sox2, Nanog, and glutathione peroxidase 3 improving stem cells self-renewal and differentiation abilities	von Frey and Hargreaves behavior tests were performed to assess mechanical and thermal hyperalgesia changes, respectively. Immunofluorescence, western blot and RT-qPCR techniques were used to study alterations in protein production/expression/localization	i.t.	Unspecified
Franchi *et al* [137], 2012	I.v. NSCs abolish nociceptive hypersensitivity and trigger nerve regeneration in experimental neuropathy	NSCs administration in CCI mouse model significantly decreased proinflammatory (IL-1 β, IL-6), activated anti inflammatory (IL-10) cytokines in the sciatic nerve, and reduced spinal cord Fos expression in laminae I-VI	Thermal hyperalgesia was tested according to the Hargreaves using a Plantar Test Apparatus, while mechanical allodynia was assessed using the Dynamic Plantar Aesthesiometer. Immunohistochemistry, immunofluorescence, and qPCR plus ELISA assays were performed for Fos and GFPI; substance P and CGRP; and IL-1β, IL-6 and IL-10, respectively	i.v.	10^6
Sacerdote *et al*[209], 2013	Systemic aAdministration of human ASCs reverts nociceptive hypersensitivity in an experimental model of neuropathy	Human ASCs were able to completely revert neuropathic pain symptoms in a murine CCI model by: IL-1β decreased and IL-10 increased in the lesioned nerve. Restored normal iNOS expression	Thermal hyperalgesia was tested according to the Hargreaves, while mechanical allodynia was assessed using the Dynamic Plantar Aesthesiometer (von Frey filament)	i.v.	1×10^6, 3×10^6 and 6×10^6
Choi *et al* [73], 2011	Anti-inflammatory protein TSG-6 secreted by activated MSCs attenuates zymosan-induced mouse peritonitis by decreasing TLR2/NF-κB signaling in resident macrophages	TSG-6 interacts through the CD44 receptor on resident macrophages to decrease zymosan/TLR2-mediated nuclear translocation of the NF-κB	RT-qPCR, ELISA, NF-κB translocation assays and isolation of resident macrophage RNA was performed	i.p.	1.6×10^6
Siniscalco *et al*[210], 2011	Long-lasting effects of human MSCs systemic administration on pain-like behaviors, cellular, and biomolecular modifications in neuropathic mice	The treatment was able to reduce pain-like behaviors such as mechanical allodynia and thermal hyperalgesia. In addition to reducing IL-1β and IL-17 levels and increasing IL-10 in the spinal cord and reducing labeling for alternatively activated macrophages (CD106)	For behavior analysis, the following tests were applied: von Frey filaments, Rotarod and Hargreaves. Immunofluorescence: CD73; IL-1β; IL-17; CD4; GFAP; IBA-1; western blot: IL-1β, IL-17, IL-10 e CD106	i.v.	2×10^6

Huc-MSCs: Human umbilical cord mesenchymal stem cells; TLR2: Toll-like receptor 2; MyD88: Myeloid differentiation primary response 88; NF-κB: Nuclear factor kappa B; Rsad2: Radical S-adenosyl methionine domain containing 2; p-p65: Phospho-protein 65; CCI: Chronic constriction injury; i.t.: Intrathecal; i.v.: Intravenous; MSCs: Mesenchymal stem cells; ATF-3: Activating transcription factor 3; IBA-1: Ionized calcium-binding adapter molecule 1; DRG: Dorsal root ganglion; MBP: Myelin basic protein; NeuN: Neuron-specific nuclear protein; NF: Neurofilament; TNF: Tumor necrosis factor; ASCs: Adipose tissue derived-mesenchymal stem cells; RT-qPCR: Real time quantitative polymerase chain reaction; hTERT-BMSCs/Tet-on/GAL: Rafted human telomerase reverse transcriptase-immortalized bone marrow mesenchymal stromal cells with inducible galanin expression; GalR: Galanin receptor; SDH: Spinal dorsal horn; IFN-γ: Interferon gamma; CD: Cluster of differentiation; ELISA: Enzyme-linked immunosorbent assay; i.m.: Intramuscular; IL: Interleukin; MMP: Matrix metalloproteinase; PARP: Poly ADP-ribose polymerase; NOX: NADPH oxidase; MAPK: Mitogen-activated protein kinase; p-JNK: Phosphorylated Jun N-terminal kinase, p-ERK: Phospho-extracellular signal-regulated kinase; Nav: Voltage-gated sodium channels; LncRNA: Long-chain noncoding ribonucleic acid; Lncenc1: Long-chain noncoding RNA embryonic stem cells expressed 1; EZH2: Enhancer of zeste homologue 2; MCP-1: Monocyte chemoattractant protein-1; SUZ12: Suppressor of zeste 12; EED: Embryonic ectoderm development; BAI1: Brain-specific angiogenesis inhibitor 1; H3K27me3: Tri-methylation of histone 3 lysine 27; P2X4: P2X purinoceptor 4; i.p.: Intraperitoneal; PCNA: Proliferating cell nuclear antigen; GFAP: Glial fibrillary acidic protein; TRPV1: Transient receptor potential cation channel subfamily vanilloid 1; iNOS: Inducible nitric oxide synthase; p65: Protein 65; BDNF: Brain-derived neurotrophic factor; GAP-43: Growth-associated protein 43; CGRP: Calcitonin gene-related peptide; TSG-6: Tumor necrosis factor-α-stimulated gene 6 protein; BMSC: Bone marrow mesenchymal stem cells; p-Akt: Phospho protein kinase B; LC3B: Light chain-3B; COX-2: Cyclooxygenase 2; nNOS: Neural nitric oxide synthase; hBMSCs: Human bone marrow stem cells; CSF: Cerebrospinal fluid; Th1: Type 1 helper; SCI: Spinal cord injury; p-CREB: Phosphocyclic AMP response element binding protein; CCL: C-C motif chemokine ligand; CXCL: C-X-C motif chemokine ligand; GM-CSF: Granulocyte-macropgahe colony stimulating factor; PDGFR-α: Platelet-derived growth factor receptor α; ROS: Reactive oxygen species; LPS: Lipopolysaccharides; NRG1: Neuregulin-1; GDNF: Glial cell derived neurotrophic factor; NSC: Neural stem cells.

substance P)[151]. When activated, glial cells' function will be related to the maintenance and enhancement of the interaction between the peripheral and CNSs, secreting, for instance, cytokines that will activate spinal cord neurons [152]. Interestingly, this glial activation can be detected by the expression of some targets, such as IBA-1 for microglia and glial fibrillary acidic protein (GFAP) for astrocytes[61]. These glial cells are responsible for the subsequent release of cytokines inducing a series of cellular responses, such as upregulation of glucocorticoids and glutamate receptors, leading to spinal cord excitation and neuroplasticity[136].

It has been reported that in experimental conditions involving spinal cord injury and spinal cord treatment with SC, embryonic stem (ES) cells differentiate into oligodendrocytes using positive selection and mechanical enrichment[153],

promoting functional recovery after a spinal cord injury producing myelination[154]. Spinal cord SC treatment also decreases GFAP[155] and IBA1[156] indicating the down-regulation of astrocyte and microglia activation, respectively. SC express high levels of CXCL12[157]. CXCL12 can reduce the activation of astrocytes and microglia in spinal nerve ligation models, thus, potentially contributing to the activity of SC in NP[158]. There are other potential repercussions of glial inhibition. For instance, the decrease of IBA1 also occurs by inhibiting gasdermin D-induced microglia pyroptosis, thus, promoting autophagy[159]. In fact, impaired autophagic flux aggravates NP by increasing neuroinflammation[160]. Moreover, the mitogen-activated protein kinase signal pathway is activated after microglial activation, which promotes long-term potentiation and central sensitization in pain[136].

Another mechanism of action of SC that explains their analgesic activity by down-modulating glial cell activation is the capability they have at reducing the expression of purinergic P2X purinoceptor 4 (P2X4) and P2X7 receptors in a rat model of NP induced by CCI of the sciatic nerve[161]. P2X4 and P2X7 receptor activation mainly by ATP (coming from nerve damage) leads to the activation of glial cells and the release of IL-1β, TNF-α and IL-6, capable of sensitizing nociceptors and perpetuating NP[162,163].

In a different perspective, it has also been shown that SC are capable of increasing IL-10 levels in the spinal cord[164], in addition to secreting TSG-6 in the spinal cord[91], and decreasing IBA-1 activity by inhibiting the TLR2/MyD88/NF-κB signaling pathway in spinal microglia. These activities reduce nociceptor neuron activation and neuronal plasticity.

The third analgesic mechanism of SC in the CNS involves the inhibition of neuronal death in the CNS. SC treatment can reduce the levels of p-Akt/Akt and Bax/Bcl-2, LC3B-II, Beclin 1 and TUNEL (markers of cellular death) in the dorsal horn of lumbar spinal cords in burn-induced NP. Thus, suggesting that SC can reduce the levels of apoptosis, necrosis and autophagy related to inflammation in spinal cord neurons of dorsal horn cells[165].

SC TECHNOLOGY AS A TOOL FOR INVESTIGATING PAIN-RELATED MECHANISMS

Although most of the efforts in scientific research regarding SC and pain are guided towards finding suitable painkillers, it is worth mentioning that SC technology can also be proven valuable to create different experimental models, which can contribute to understanding pathophysiological mechanism of pain and thus, evolving towards therapy. As an example, Kaneski *et al*[166] developed a human ESC (hESC)-based model to study the poorly understood pathophysiology of pain in Fabry disease. This X-linked glycolipid storage disorder that results in a deficiency in the lysosomal enzyme alpha galactosidase A (AGA) can cause recurrent attacks of excruciating pain ("Fabry pain crisis") that occur spontaneously or in response to extreme temperatures, fever, fatigue, stress, overheating, or exercise. The group generated two AGA-deficient hESC clones using CRISPR-Cas9 gene editing techniques and demonstrated that AGA-deficient human SC could be differentiated into peripheral neurons with nociceptor properties, offering a tool for the investigation of cellular mechanisms for this and other peripheral neuropathies[166].

Some studies utilized dental pulp SC to investigate the possible effects of sirtuin 6 (SIRT6) - an NAD-dependent protein deacetylase known for its role as a differentiation regulator - as a modulation factor using a model of LPS-induced pulpitis (inflammation in the dental pulp). LPS is the major virulence component of gram-negative bacteria cell walls and is widely recognized as a potent activator of inflammation[167]. Their results demonstrated that injection of lentiviral vector-expression SIRT6 leads to SIRT6 overexpression in rats, reduced LPS-induced neutrophils infiltration, a marked decrease in proinflammatory cytokines (IL-6, IL-1β, and TNF-α) and deactivation of the NF-κB pathway. LPS-induced pulpitis in turn, upregulated TRPV1 expression and activity, by downregulating SIRT6. Interestingly, CGRP release was induced by pulpitis while the overexpression of SIRT6 inhibited TRPV1 expression and CGRP release. The expression of inflammatory cytokines, dentin matrix acidic phosphoprotein 1, and NF-κB activation were upregulated after the addition of capsaicin, a TRPV1 channel agonist. Taken together, their results suggest that SIRT6 may be both a negative regulator of pulpitis and an inflammatory pain modulator[168].

DOWNSIDES AND POSSIBLE TREATMENT SIDE-EFFECTS

The biggest concern surrounding SC treatment is the onset of tumors as a result of the therapy, particularly *via* systemic administration routes. This concern is due to the proliferation potential that these cells have, thus, raising the possibility of tumor development if they continue to proliferate after transplantation[169]. The literature lacks *in vivo* studies evaluating the tumorigenic effect of SC treatment. However, it is currently acknowledged that tumorigenicity can develop through three different manners. Firstly, the presence of undifferentiated SC anchored in other already differentiated lineages can lead to tumor formation, due to their high replication rate[169]. Second, reprogramming factors may remain active in transplanted SC, promoting the transformation of these cells and, consequently, tumorigenic transplantation[169]. Finally, tumorigenicity can also develop during *in vitro* culture through genetic mutations[169]. Studies demonstrate that a significant rate of SC derived from bone marrow undergo spontaneous transformations towards a malignant profile in long-term cultures (5 to 106 wk), showing an increase in the rate of proliferation and morphological and phenotypic changes[170].

Another disadvantage is the possible development of cellular rejections. This can occur when the individual's immune system recognizes the SC as foreign antigens and develops an immune response against them[25,169]. Polymorphic molecules of the MHC are an example of immunologically recognized molecules that induce rejection[171]. In fact, studies have already demonstrated that the administration of ESC in the myocardium of allogeneic mice results in the development of an immune response with significant infiltration of T lymphocytes and dendritic cells[172]. Likewise,

abnormal gene expression in cells differentiated from iPSC can induce a T cell-dependent immune response[173]. Finally, even though there are studies that demonstrate the effects of systemic administration of SC, there is a lack of data in the literature that assess their distribution and actions in the body after treatment.

CONCLUSION

In this review, we provide evidence on the therapeutic potential of the use of SC in pain treatment. Analgesia was observed with SC administration as well as SC extracellular vesicle administration. This analgesic effect was found to be achieved with a variety of routes, such as intra-articular, intravenous, intrathecal, intramuscular, intraganglionar, perineural, subcutaneous, and local injection. Additionally, the SC load for these administrations varied between 1.0×10^5 to 24×10^6 for SC, and 1×10^6 to 1×10^9 for SC extracellular vesicle. The specific conditions of each experiment can be appreciated in the Tables 2 and 3.

This review discussed that the analgesic mechanism of action of SC treatment can be indirect by acting on inflammation, changing the pattern from pro-inflammatory to anti-inflammatory mediators. SC can decrease the levels of cytokines that have a role in mechanical and thermal hyperalgesia in addition to promoting the secretion of cytokines with analgesic roles. These anti-inflammatory analgesic mechanisms have been demonstrated in peripheral tissue. In the CNS, SC can cause analgesia by inhibiting the effects of glutamate on spinal cord neurons and by its ability to decrease glial activity and therefore central sensitization.

There are still gaps in the elucidation of the mechanism of action of SC, since most of the articles in the literature aim mainly at the treatment of NP. Among the literature hiatus we can cite the lack of studies on the analgesic mechanisms of SC in models using microorganisms as stimuli. Confirmatory studies on SC therapy long-term safety are also missing. SC rejection is also a potential drawback and there are no studies comparing the success of therapy using autologous cells to the patient, and cells of exogenous origin. On the other hand, much of the success of therapy using SC comes from the use of MSC, which have a lower possibility of rejection, as well as greater effectiveness in the treatment.

Even with all the limitations and shortcomings, there are already clinical studies using SC both in the treatment of inflammatory diseases and in NP. In all reports, treatments were able to decrease pain scores and restore mobility-related functions. Therefore, SC treatment is a potential approach for pain relief and to achieve such biotechnological advancements, there is a need to fill the current knowledge gaps in order to develop efficient and safe therapies based on SC.

FOOTNOTES

Author contributions: All authors contributed equally to the elaboration of the article.

Supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq; #203112/2020-2, #307852/2019-9, #309633/2021-4, #405027/2021-4, and #427946/2018-2; PRONEX grant supported by SETI/Fundação Araucária and MCTI/CNPq, and Governo do Estado do Paraná (agreement #014/2017); Fundação Araucária (PBA/PROPPG 13/2021 agreements #276/2022-PBA and #250/2022-PBA); Financiadora de Estudos e Projetos-FINEP; and CAPES (finance code #001).

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Country/Territory of origin: Brazil

ORCID number: Matheus Deroco Veloso da Silva 0000-0001-7414-7919; Maiara Piva 0000-0003-4099-3492; Geovana Martelossi-Cebinelli 0000-0001-6751-950X; Mariana Stinglin Rosa Ribas 0000-0001-7689-7457; Beatriz Hoffmann Salles Bianchini 0000-0002-7073-8276; Olivia K Heintz 0009-0007-6946-0450; Rubia Casagrande 0000-0002-2296-1668; Waldiceu A Verri Jr 0000-0003-2756-9283.

S-Editor: Wang JJ
L-Editor: A
P-Editor: Zhang XD

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World Journal of Stem Cells

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World J Stem Cells 2023 December 26; 15(12): 1063-1076

DOI: 10.4252/wjsc.v15.i12.1063

ISSN 1948-0210 (online)

ORIGINAL ARTICLE

Randomized Controlled Trial

Mid-term outcomes of microfragmented adipose tissue plus arthroscopic surgery for knee osteoarthritis: A randomized, active-control, multicenter clinical trial

Cong-Zi Wu, Zhen-Yu Shi, Zhen Wu, Wen-Jun Lin, Wei-Bo Chen, Xue-Wen Jia, Si-Cheng Xiang, Hui-Hui Xu, Qin-Wen Ge, Kai-Ao Zou, Xu Wang, Jia-Li Chen, Ping-Er Wang, Wen-Hua Yuan, Hong-Ting Jin, Pei-Jian Tong

Specialty type: Cell and tissue engineering

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): 0
Grade C (Good): C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Mahmoud MZ, Saudi Arabia; Oommen AT, India

Received: October 5, 2023
Peer-review started: October 5, 2023
First decision: November 22, 2023
Revised: December 4, 2023
Accepted: December 20, 2023
Article in press: December 20, 2023
Published online: December 26, 2023

Cong-Zi Wu, Institute of Orthopaedics and Traumatology of Zhejiang Province, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou 310053, Zhejiang Province, China

Zhen-Yu Shi, Jia-Li Chen, Ping-Er Wang, Wen-Hua Yuan, Hong-Ting Jin, Pei-Jian Tong, Department of Orthopedic Surgery, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou 310006, Zhejiang Province, China

Zhen Wu, Department of Orthopaedic Surgery, Tongde Hospital of Zhejiang Province, Hangzhou 310012, China

Wen-Jun Lin, Department of Orthopaedic Surgery, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325015, Zhejiang Province, China

Wei-Bo Chen, Department of Orthopaedic Surgery, Ruian Hospital of Traditional Chinese Medicine, Wenzhou 325299, Zhejiang Province, China

Xue-Wen Jia, Department of Orthopaedics, Ningbo First Hospital, Ningbo 315010, Zhejiang Province, China

Si-Cheng Xiang, Hui-Hui Xu, Qin-Wen Ge, Kai-Ao Zou, Xu Wang, The First College of Clinical Medicine, Zhejiang Chinese Medical University, Hangzhou 310053, Zhejiang Province, China

Corresponding author: Pei-Jian Tong, MD, PhD, Professor, Department of Orthopedics and Traumatology, The First Affiliated Hospital of Zhejiang Chinese Medical University, No. 54 Youdian Street, Hangzhou 310006, Zhejiang Province, China. tongpeijian@163.com

Abstract

BACKGROUND

Osteoarthritis (OA) is the most prevalent form of degenerative whole-joint disease. Before the final option of knee replacement, arthroscopic surgery was the most widely used joint-preserving surgical treatment. Emerging regenerative therapies, such as those involving platelet-rich plasma, mesenchymal stem cells, and microfragmented adipose tissue (MFAT), have been pushed to the forefront of treatment to prevent the progression of OA. Currently, MFAT has been

successfully applied to treat different types of orthopedic diseases.

AIM
To assess the efficacy and safety of MFAT with arthroscopic surgery in patients with knee OA (KOA).

METHODS
A randomized, multicenter study was conducted between June 2017 and November 2022 in 10 hospitals in Zhejiang, China. Overall, 302 patients diagnosed with KOA (Kellgren-Lawrence grades 2-3) were randomized to the MFAT group (n = 151, were administered MFAT following arthroscopic surgery), or the control group (n = 151, were administered hyaluronic acid following arthroscopic surgery). The study outcomes were changes in the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) score, the visual analog scale (VAS) score, the Lequesne index score, the Whole-Organ Magnetic Resonance Imaging Score (WORMS), and safety over a 24-mo period from baseline.

RESULTS
The changes in the WOMAC score (including the three subscale scores), VAS pain score, and Lequesne index score at the 24-mo mark were significantly different in the MFAT and control groups, as well as when comparing values at the posttreatment visit and those at baseline (P < 0.001). The MFAT group consistently demonstrated significant decreases in the WOMAC pain scores and VAS scores at all follow-ups compared to the control group (P < 0.05). Furthermore, the WOMAC stiffness score, WOMAC function score, and Lequesne index score differed significantly between the groups at 12 and 24 mo (P < 0.05). However, no significant between-group differences were observed in the WORMS at 24 mo (P = 0.367). No serious adverse events occurred in both groups.

CONCLUSION
The MFAT injection combined with arthroscopic surgery treatment group showed better mid-term clinical outcomes compared to the control group, suggesting its efficacy as a therapeutic approach for patients with KOA.

Key Words: Osteoarthritis; Microfragmented adipose tissue; Lipogems; Arthroscopic surgery; Knee

Core Tip: Microfragmented adipose tissue (MFAT) has been successfully applied to treat different types of orthopedic diseases. To assess the efficacy and safety of MFAT with arthroscopic surgery in patients with knee osteoarthritis (OA). In this study, patients' own MFAT combined with arthroscopic surgery was used to promote recovery from OA. Our findings provide evidence supporting the safety and feasibility of this approach in treating knee OA.

Citation: Wu CZ, Shi ZY, Wu Z, Lin WJ, Chen WB, Jia XW, Xiang SC, Xu HH, Ge QW, Zou KA, Wang X, Chen JL, Wang PE, Yuan WH, Jin HT, Tong PJ. Mid-term outcomes of microfragmented adipose tissue plus arthroscopic surgery for knee osteoarthritis: A randomized, active-control, multicenter clinical trial. *World J Stem Cells* 2023; 15(12): 1063-1076
URL: https://www.wjgnet.com/1948-0210/full/v15/i12/1063.htm
DOI: https://dx.doi.org/10.4252/wjsc.v15.i12.1063

INTRODUCTION

Osteoarthritis (OA) is the most prevalent form of degenerative whole-joint disease, resulting in joint pain, stiffness, and decreased function. It is also a leading cause of disability in adults, with an enormous and ever-increasing burden on society[1,2]. Although several nonsurgical treatments are available for OA, such as physical therapy and medication, current mainstream medicine mainly focuses on relieving symptoms and cannot prevent the disease from progressing to the late stages of arthritis that require knee replacement[3]. Before this final option of knee replacement, arthroscopic surgery was the most widely used joint-preserving surgical treatment. Available evidence suggests that arthroscopic knee surgery temporarily improves pain or function in patients with mild arthritis; however, its long-term efficacy in treating moderate-to-severe knee OA (KOA) has not been generally accepted[4-7].

In contrast to limited symptom relief therapy, emerging regenerative therapies, such as those involving platelet-rich plasma (PRP), mesenchymal stem cells (MSCs), and microfragmented adipose tissue (MFAT), have been pushed to the forefront of treatment to prevent the progression of OA[8-10]. MFAT is obtained with a Lipogems (Lipogems Int Spa, Milan, Italy) device, which concentrates extracted autologous adipose tissue into small fragments using mild mechanical forces[11]. This one-step procedure is simple, time-saving, cost-effective, and minimally invasive; hence, it eliminates the need for complicated and time-consuming cell culture procedures. MFAT retains an intact stromal vascular niche harboring abundant functional cells, including adipose-derived stem cells (ADSCs: CD90+CD45-, CD90+CD45-, CD73+CD45-, CD73+CD45-, CD105+CD45-, and CD105+CD45-) and pericytes (CD146+CD34-), and secretes various

functional molecules, such as growth factors, cytokines, and exosomes[12,13]. MFAT can match and sometimes even outperform ADSCs due to its functional advantages over ADSCs; notably, the use of MFAT preserves the microenvironment and the most primitive state of cells, MFAT can be conveniently and easily acquired, there are a wide range of sources of MFAT, and autologous tissues are safe to use[14].

MFAT was initially used in the field of plastic surgery and then expanded to orthopedic procedures due to its antibacterial, anti-inflammatory, antiapoptotic, angiogenesis-promoting, and tissue repair functions[15-17]. Currently, MFAT has been successfully applied to treat different types of orthopedic diseases, such as diabetic foot minor amputations, spinal cord injury, cartilage defects, and articular disease[18-22]. Previous animal studies have successfully shown the benefit of intra-articular MFAT therapy for OA in pain relief and functional improvement[23,24]. Moreover, several clinical trials have reported an overall positive result for pain relief and functional improvement in patients with symptomatic KOA following MFAT therapy[25-27].

However, these successful single-center clinical trials are insufficient to demonstrate widespread therapeutic utility. Hence, well-controlled, high-quality scientific evidence to support the efficacy of MFAT is needed. This multicenter, prospective, randomized controlled trial aimed to evaluate the efficacy and safety of a single intra-articular injection of MFAT during arthroscopic surgery in a larger and more heterogeneous population of patients with KOA.

MATERIALS AND METHODS

Study design and participants

This was a prospective, randomized, controlled, single-blind (blind observer), multicenter trial performed in 10 hospitals in Zhejiang, China, including the First Affiliated Hospital of Zhejiang Chinese Medical University, the First Affiliated Hospital of Wenzhou Medical University, Ningbo First Hospital, Ruian Hospital of Traditional Chinese Medicine, Taizhou Hospital of Zhejiang Province, Hangzhou Red Cross Hospital, Huzhou First Hospital, the First People's Hospital of Hangzhou Linan District, Xiaoshan Hospital of Zhejiang, and Zhuji First Hospital (Table 1). A 24-mo follow-up was performed between June 2017 and November 2022. The inclusion criteria were participants aged 30-80 years with KOA symptoms for over 6 mo, including mechanical (locking and grinding) or meniscal (pain with pivoting) symptoms; Kellgren-Lawrence grades 2-3; and being unresponsive to conservative treatment. The exclusion criteria included local infection of the knee joint; systemic diseases such as blood disorders or diabetes, rheumatoid arthritis, gout, autoimmune disease, or malignancy in the past 5 years; prior injection or use of oral steroids within 3 wk before screening; and knee surgery within 6 mo before screening. The trial was registered at chictr.org.cn (registration number ChiCTR2200055124). All participants provided informed consent, and this study was approved by the Institutional Review Board of the First Affiliated Hospital of Zhejiang Chinese Medical University.

Interventions

Enrolled patients were randomly assigned to the MFAT or control group at a 1:1 ratio using a computer-generated method, stratified by hospital and patient age (30-55 and 56-80 years). The control group received arthroscopic and hyaluronic acid (HA) treatment, while the MFAT group received arthroscopic surgery and MFAT therapy.

All participants underwent one or more of the following knee arthroscopic treatments: Debridement; abrasion or microfracture of chondral defects; trimming of degenerative meniscal tears, fragments of articular cartilage, or chondral flaps; and trimming of osteophytes that blocked full extension. Patients randomized to the MFAT group received an MFAT injection during the same arthroscopic procedure. After subcutaneous infiltration with a solution (a total of 250 mL of a saline solution mixed with 0.5 mL adrenalin 1:1000 and 25 mL lidocaine 0.02%) and resting for at least 15 min, liposuction was performed to obtain abdominal fat. We then introduced the harvested fat into Lipogems® (Lipogems International SpA, Milan, Italy) to produce MFAT according to the manufacturer's instructions[12]. Finally, the processed final product was transferred to 10 mL syringes and injected into the knee after the arthroscopic procedure. Patients in the control group received three injections of HA at a dosage of 5 mL once every month, as previously mentioned.

Outcomes

All patients were clinically evaluated before surgery and during follow-up visits at 6, 12, and 24 mo. The primary outcomes were the change in the total Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) score from baseline to 24 mo. Secondary outcome measures included the WOMAC pain, stiffness, and function score from baseline to 24 mo, the visual analog scale (VAS) score for pain, the Lequesne index score, and the Whole-Organ Magnetic Resonance Imaging Score (WORMS).

Safety assessment

The safety assessment was based on adverse events reported by the patients and physical findings by the evaluators at each follow-up. Mild adverse events were defined as the presence of infection, significant pain, or swelling. A serious adverse event was defined as one that posed a life-threatening risk, was permanently disabling, or required hospitalization.

Statistical analysis

The sample size was based on the endpoint of the total WOMAC score at 24 mo. According to a previous pilot study, the standard deviation of the total WOMAC score at 24 mo was 12.8 points. Considering a 2-sided error alpha of 0.05, a

Table 1 Baseline characteristics of the patients

Characteristics	MFAT group	Control group	*P* value
No. of knees	146	146	
Age, yr	56.4 ± 10.6	54.8 ± 10.1	0.183
Sex, *n* (%)			0.455
Female	95 (65.1)	101 (69.2)	
Male	51 (34.9)	45 (30.8)	
BMI, kg/m^2	22.6 ± 3.4	23.1 ± 3.1	0.246
Kellgren-Lawrence grade, *n* (%)			0.833
2	80 (54.8)	93 (63.7)	
3	66 (45.2)	53 (36.3)	
Osteoarthritis site, *n* (%)			0.815
Left	70 (47.9)	72 (49.3)	
Right	76 (52.1)	74 (50.7)	
VAS pain	5.3 ± 1.4	5.2 ± 1.3	0.637
WOMAC score			
Pain 0-20	9.8 ± 2.7	9.5 ± 2.6	0.264
Stiffness 0-8	2.3 ± 1.4	2.2 ± 1.3	0.519
Function 0-68	35.3 ± 8.4	33.6 ± 10.2	0.115
Total 0-96	47.5 ± 9.6	45.3 ± 11.0	0.071
Lequesne index	10.7 ± 2.8	10.3 ± 2.6	0.215
WORMS	59.1 ± 9.2	60.8 ± 7.0	0.648

WORMS: Whole-Organ Magnetic Resonance Imaging Score; VAS: Visual analog scale; BMI: Body mass index; MFAT: Microfragmented adipose tissue.

minimum power of at least 0.9, a margin of 5.3 points on the total WOMAC, and a 15% drop-out rate, the final sample size was 290 knees (145 for each group).

SPSS Statistics version 25.0 (IBM Corp., Armonk, NY, United States) was used for statistical analyses. The χ^2 test or Fisher's exact probability test, was used to compare count data. The normal distribution of the data was evaluated using a QQ plot and histogram. Different variables are described as the mean ± SD. When comparing the means between groups, a one-way ANOVA or Student's *t* test was used. Changes in primary and secondary outcome measures among the baseline, 6-, 12-, and 24-mo follow-up evaluations were assessed using repeated-measures one-way ANOVA and the Bonferroni post hoc test. A *P*-value < 0.05 indicated statistical significance.

Outcomes were assessed in an intention-to-treat analysis. The intention-to-treat population comprised all patients who had received the injection and had undergone at least one postbaseline assessment; the last-observation-carried-forward method was used to account for missing data.

RESULTS

A total of 322 patients were assessed for eligibility. Fifteen patients were not eligible, and five declined to participate; hence, a total of 302 participants were randomized to either the MFAT group (*n* = 151) or the control group (*n* = 151). Four patients (two in the MFAT group and two in the control group) withdrew consent, and six patients (four in the MFAT group and two in the control group) were lost to follow-up after randomization. It was decided that two patients assigned to the MFAT treatment group should not receive the procedure; according to the intention-to-treat principle, the results from these patients were evaluated with the results of the MFAT group. Thirty-seven patients did not return for follow-up visits; hence, 255 participants (87.3%) completed follow-up after 24 mo (Figure 1). The baseline characteristics of the two groups were similar (Table 1).

Primary and secondary outcomes

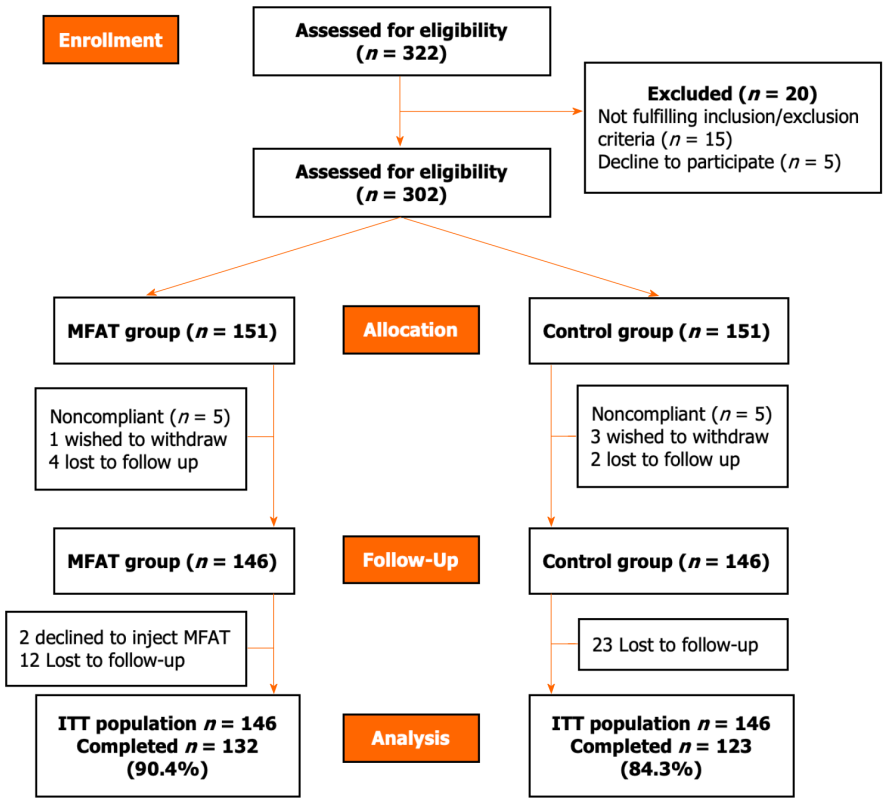
A comparison of the WOMAC scores, VAS scores, and Lequesne index scores between the MFAT group and the control group before and after treatment is shown in Table 2, Figures 2 and 3. In both groups, the WOMAC total score was

Table 2 Primary and second outcomes of the trial from baseline to all follow-ups

	MFAT group (*n* = 146)	Control group (*n* = 146)	*F* value	*P* value
WOMAC total score				
Baseline	48.3 ± 12.9	46.5 ± 13.4	0.406[1]	0.233[1]
6 mo	23.5 ± 8.6	29.8 ± 10.9	20.002[1]	< 0.001[1]
12 mo	22.0 ± 8.1	29.8 ± 10.7	24.479[1]	< 0.001[1]
24 mo	22.8 ± 8.2	31.7 ± 12.6	26.763[1]	< 0.001[1]
F value	379.38[1]	141.916[1]	23.468	
P value	< 0.001[1]	< 0.001[1]	< 0.001[2]	
WOMAC pain score				
Baseline	9.8 ± 2.7	9.5 ± 2.6	1.544[1]	0.264[1]
6 mo	4.3 ± 2.0	5.7 ± 2.7	18.373[1]	< 0.001[1]
12 mo	4.0 ± 2.0	5.7 ± 2.2	32.046[1]	< 0.001[1]
24 mo	4.0 ± 2.0	5.9 ± 2.3	45.115[1]	< 0.001[1]
F value	676.741[1]	240.46[1]	36.69	
P value	< 0.001[1]	< 0.001[1]	< 0.001[2]	
WOMAC stiffness score				
Baseline	3.2 ± 1.7	3.3 ± 2.0	0.003[1]	0.924[1]
6 mo	2.0 ± 1.4	2.4 ± 1.6	9.505[1]	0.054[1]
12 mo	1.7 ± 1.3	2.3 ± 1.4	13.737[1]	< 0.001[1]
24 mo	1.6 ± 1.2	2.2 ± 1.4	13.548[1]	< 0.001[1]
F value	155.117[1]	29.140[1]	9.605	
P value	< 0.001[1]	< 0.001[1]	< 0.001[2]	
WOMAC function score				
Baseline	35.3 ± 12.3	33.8 ± 12.8	0.406[1]	0.304[1]
6 mo	17.1 ± 8.3	21.7 ± 10.2	20.002[1]	< 0.001[1]
12 mo	16.3 ± 7.6	21.9 ± 10.2	24.479[1]	< 0.001[1]
24 mo	17.2 ± 7.8	23.6 ± 11.8	26.763[1]	< 0.001[1]
F value	379.38[1]	141.915[1]	23.468	
P value	< 0.001[1]	< 0.001[1]	< 0.001[2]	
VAS pain				
Baseline	5.3 ± 1.4	5.2 ± 1.3	0.257[1]	0.607[1]
6 mo	2.9 ± 1.3	3.3 ± 1.6	5.355[1]	0.008[1]
12 mo	2.2 ± 1.3	2.7 ± 1.5	5.937[1]	0.007[1]
24 mo	2.2 ± 1.4	2.7 ± 1.6	7.835[1]	0.006[1]
F value	581.206[1]	527.797[1]	11.921	
P value	< 0.001[1]	< 0.001[1]	< 0.001[2]	
Lequesne index				
Baseline	10.7 ± 2.8	10.3 ± 2.7	2.327[1]	0.217[1]
6 mo	7.7 ± 3.2	8.0 ± 3.0	0[1]	0.458[1]
12 mo	7.2 ± 3.2	8.2 ± 3.3	6.684[1]	0.012[1]
24 mo	7.2 ± 2.5	8.2 ± 3.0	8.633[1]	0.004[1]
F value	216.554[1]	45.136[1]	16.293	

P value	< 0.001[1]	< 0.001[1]	< 0.001[2]

[1]*F* statistic and *P* value of the main effect.
[2]*F* statistic and *P* value of the crossover effect.
WORMS: Whole-Organ Magnetic Resonance Imaging Score; VAS: Visual analog scale; MFAT: Microfragmented adipose tissue.



DOI: 10.4252/wjsc.v15.i12.1063

Figure 1 Participant enrollment flow diagram. ITT: Intention-to-treat; MFAT: Microfragmented adipose tissue.

significantly different between time before and after treatment, with separate effect analyses in the MFAT group (F = 379.38) and control group (F = 141.916) (all P < 0.001). The WOMAC total score in the MFAT group was lowest after 12 mo and then increased slightly but remained lower than the values at baseline. The WOMAC total score in the control group decreased, with a maximum decline observed at 6 mo after treatment, and slightly increased at 24 mo. The overall WOMAC total score in the MFAT group was significantly lower than that in the control group after treatment (F = 838.277, P < 0.001). There was also a crossover effect between time and treatment (F = 23.468, P < 0.001). We performed an analysis of the separate effects for each time point. The WOMAC total score in the MFAT group was significantly lower than that in the control group at the same time points during all follow-ups (P < 0.001) (Figure 2A, Table 2). These results indicated the therapeutic benefit of MFAT injection combined with arthroscopy in patients with KOA.

Both groups had a significant reduction in WOMAC pain scores and VAS scores from baseline to posttreatment at 6 to 24 mo ($F_{WOMAC\ pain}$ = 676.741, F_{VAS} = 581.206 for the MFAT group; $F_{WOMAC\ pain}$ = 240.46, F_{VAS} = 527.797 for the control group; all P < 0.001). The most significant decreases in the WOMAC pain score and VAS score from baseline to posttreatment were observed at months 12 and 24 in the MFAT group and at month 12 in the control group. A significant difference in the mean WOMAC pain score and VAS score was observed at all follow-ups in the MFAT group in comparison with the control group (WOMAC pain: All P < 0.001, VAS: All P < 0.01) (Figures 2B and 3A, Table 2). These results indicated that MFAT injection combined with arthroscopy significantly improved pain in patients with KOA.

The WOMAC stiffness score, WOMAC function score, and Lequesne index score at all follow-ups progressively decreased compared with their respective baseline values in both the MFAT group ($F_{WOMAC\ stiffness}$ = 155,117, $F_{WOMAC\ function}$ = 379.38, $F_{Lequesne}$ = 216.554, all P < 0.001) and the control group ($F_{WOMAC\ stiffness}$ = 29.140, $F_{WOMAC\ function}$ = 141.915, $F_{Lequesne}$ = 45.136, all P < 0.001). The WOMAC stiffness score and Lequesne index score in the MFAT group maintained a downward trend up to 24 mo after treatment. Additionally, the WOMAC function score decreased most significantly at 12 mo and slightly increased at 24 mo. The WOMAC function score and Lequesne index score in the control group were lowest after 6 mo and then increased monthly, while the WOMAC stiffness score was lowest at 24 mo. The WOMAC stiffness score, WOMAC function score, and Lequesne index score in both groups were lower than the values at baseline. However, the WOMAC function score of patients in the MFAT group was still lower than that of the control group at all follow-ups (all P < 0.001) (Figure 2D, Table 2). Similar patterns were observed in the WOMAC stiffness score and Lequesne index score

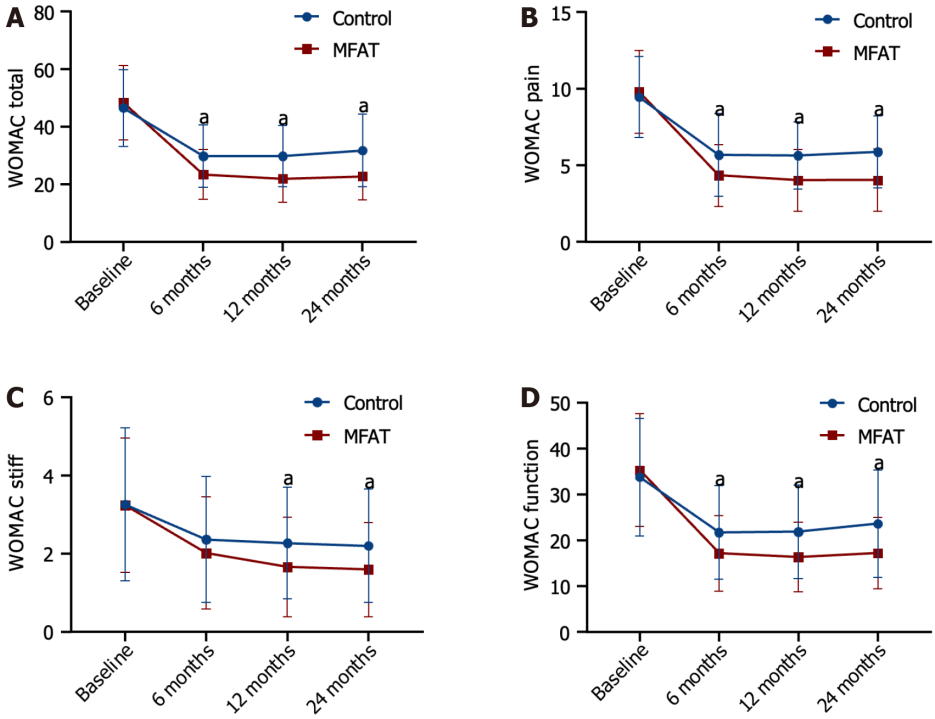


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Figure 2 Trends of Western Ontario and McMaster Universities Osteoarthritis Index scores during the 24-mo follow-up for both groups. A: Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) total score trends; B: WOMAC pain score trends; C: WOMAC stiffness score trends; D: WOMAC function score trends. Statistically significant differences have been reported as $^{a}P < 0.05$ (microfragmented adipose tissue *vs* control). Results are presented as the means ± SD. WOMAC: Western Ontario and McMaster Universities Osteoarthritis Index; MFAT: Microfragmented adipose tissue.

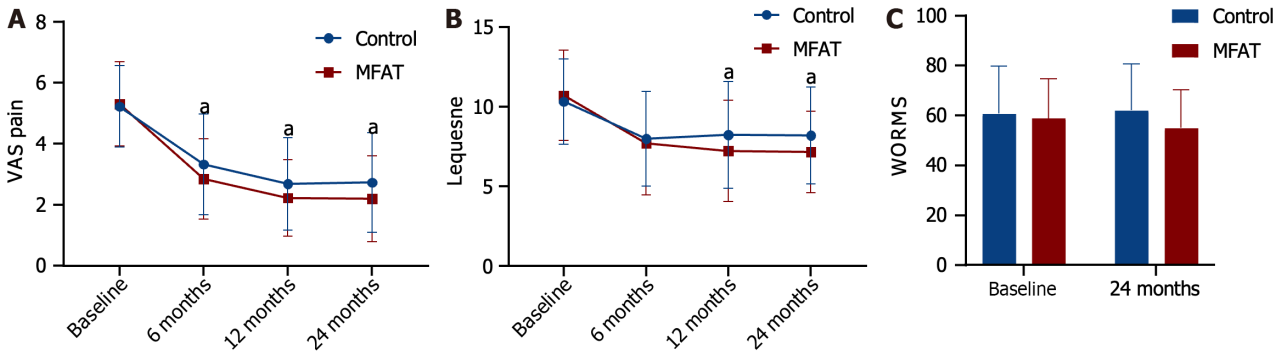

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Figure 3 Trends of the visual analog scale pain score, Lequesne index score, and Whole-Organ Magnetic Resonance Imaging Score during the 24-mo follow-up for both groups. A: Visual analog scale pain score trends; B: Lequesne index score trends; C: Whole-Organ Magnetic Resonance Imaging Score change (n = 10 for each group). Statistically significant differences have been reported as $^{a}P < 0.05$ (microfragmented adipose tissue *vs* control). Results are presented as the means ± SD. WORMS: Whole-Organ Magnetic Resonance Imaging Score; VAS: Visual analog scale; MFAT: Microfragmented adipose tissue.

of the two groups at 12 and 24 mo (all $P < 0.001$), while there was no significant difference between the two groups at 6 mo ($P_{WOMAC\ stiffness\ score} = 0.054$, $P_{Lequesne\ index\ score} = 0.458$) (Figures 2C and 3B, Table 2). The WOMAC stiffness score, WOMAC function score, and Lequesne index score showed that MFAT injection combined with arthroscopy effectively improved function and quality of life in patients with KOA.

The WORMS as an indicator of radiological changes at 24 mo is documented in Table 3. The mean WORMS at 24 mo had significantly greater improvements than at baseline in the MFAT group ($P = 0.020$), while no statistically significant difference was observed compared to the baseline score in the control group ($P = 0.850$). There were no significant between-group differences in the WORMS at 24 mo (55.0 ± 15.5 *vs* 62.1 ± 18.7, $P = 0.367$) (Figure 3C, Table 2). However, a higher proportion of patients in the MAFT group had reduced femoral and tibia bone marrow lesions (BML), repaired meniscus, and improved osteochondral defect compared with the control group (Figures 4 and 5).

Table 3 Comparison of the Whole-Organ Magnetic Resonance Imaging Score before and after treatment in the microfragmented adipose tissue and the control group

	MFAT group	Control group	*t* value	*P* value
Baseline	58.9 ± 15.9	60.8 ± 19.0	0.243	0.811
24 mo	55.0 ± 15.5	62.1 ± 18.7	0.924	0.367
t value	2.814	-0.195		
P value	0.0203	0.85		

WORMS: Whole-Organ Magnetic Resonance Imaging Score; MFAT: Microfragmented adipose tissue.

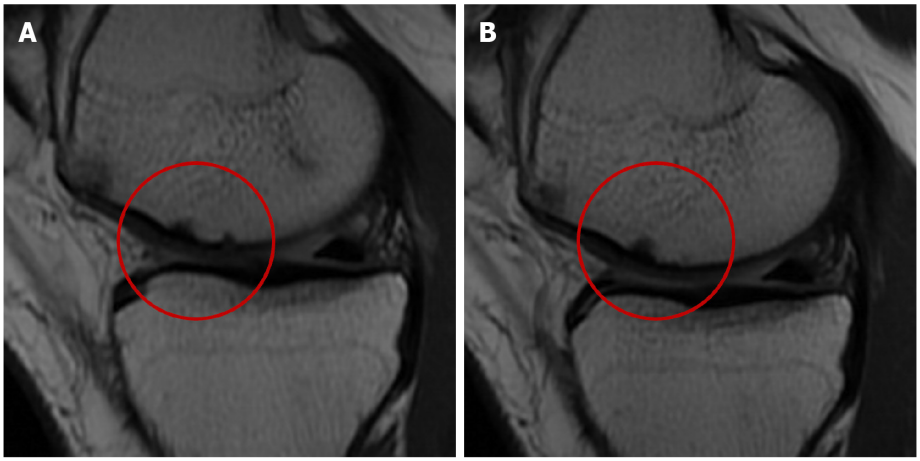

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Figure 4 Magnetic resonance imaging evaluation of osteochondral defect changes at 24 mo. A: Sagittal images of the medial femur and tibia before injection of microfragmented adipose tissue (MFAT); B: Sagittal images of the medial femur and tibia 24 mo after MFAT injections. The osteochondral defect in the circled area was reduced, indicating articular cartilage regeneration at the site of the osteochondral defect.

Safety outcomes

No serious adverse events occurred in the two groups. A total of 17 non-serious adverse events were observed: In eight knees (5.4%) in the MFAT group and nine knees (6.2%) in the control group, joint effusion, pain, or swelling were reported for over 5 d. None of these adverse events led to trial suspension in either group.

DISCUSSION

This multicenter study prospectively analyzed the safety and potential benefits of using MFAT as an adjuvant in the arthroscopic treatment of KOA. The results showed that over a 24-mo follow-up period, the injection of MFAT plus arthroscopic surgery was superior to HA plus arthroscopic surgery across multiple outcome measures, including reductions in the total WOMAC score and the scores for each subscale, VAS pain score, Lequesne index score, and WORMS.

Comprehensive studies and characterization of the injected MFAT have been performed *in vitro*[11-13]. MFAT has been shown to contains large amounts of cells; moreover, various bioactive molecules are released by these cells through a paracrine mechanism to prime and preserve angiogenic, antifibrotic, antiapoptotic, antimicrobial, and immunomodulatory responses in the target tissue. Therefore, MFAT might be a novel source of cell therapy for KOA. Striano *et al*[28] first showed that MFAT transplantation significantly improved the VAS score and expanded the joint space in a 59-year-old male patient with severe knee pain. PRP is an established regeneration therapy for KOA treatment. Recently, a study demonstrated that a single intra-articular injection of MFAT did not differ from an injection of PRP in terms of clinical outcomes at up to 24 mo[29]. This study indirectly reflected the efficacy of MFAT in KOA. Several studies have also confirmed the efficacy and safety of MFAT in the treatment of KOA, including four studies that used MFAT associated with arthroscopic surgery for the treatment of KOA in humans[25,30-39]. The first clinical trial showed that a combination of arthroscopy and a single intra-articular injection of MFAT improved the International Knee Documentation Committee (IKDC) subjective score and total knee injury and OA outcome score (KOOS) in 30 patients affected by diffuse degenerative chondral lesions at 12 mo posttreatment (70% and 67% of the patients improved by at least 10 points for the IKDC subjective score and total KOOS, respectively)[32]. In another study, Cattaneo *et al*[37] reported on 38 patients

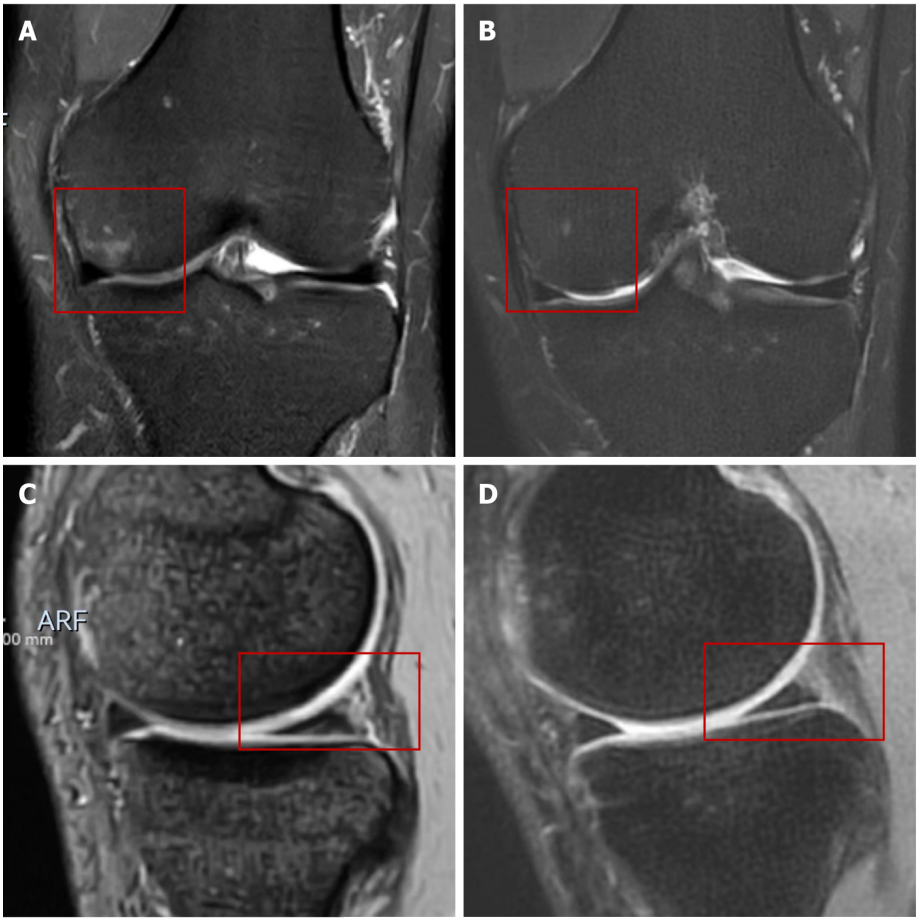

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Figure 5 Magnetic resonance imaging evaluation of bone marrow lesions and meniscus tear changes at 24 mo. A and C: Coronal and sagittal images of the medial femur and tibia before injection of microfragmented adipose tissue (MFAT). The bone marrow lesions (BML) and meniscus tears can be observed in the rectangle; B and D: Coronal and sagittal images of the medial femur and tibia 24 mo after MFAT injections. The BML in the rectangular area was reduced, and the meniscus injury was repaired. ARF: Anterior right inferior.

treated with an arthroscopic procedure combined with an injection of MFAT. The results showed that all the patients were satisfied with the treatment and that 92% of them had improved KOOSs on direct physical examination at the final follow-up. A steady and statistically significant improvement in all KOOSs from the preoperative evaluation to the 1-, 3-, and 6-mo follow-ups was observed. However, the KOOS slightly, but not statistically significantly, decreased at 12 mo. The latest published trial in 2023 included two groups of 78 patients with severe KOA (Kellgren-Lawrence grade 3-4). The study results indicated that compared to arthroscopic debridement (AD) alone, injection of MFAT along with AD was more effective in improving the KOOS score, VAS score, WOMAC score, and KSS score at the 24-mo follow-up. Additionally, magnetic resonance imaging (MRI) T2-mapping scores significantly improved in the medial and lateral condyle compartments[39]. These studies preliminarily confirmed the efficacy of MFAT for treating KOA. However, the results of efficacy trends at different time points following treatment were not consistent. In this multicenter, randomized, placebo-controlled study, patients in the MFAT combined with arthroscopy group achieved better primary and secondary endpoints at 24 mo following treatment than those in the control group. Furthermore, clinical symptoms improved following MFAT treatment in a time-dependent manner, which suggested the efficacy of MFAT treatment for patients with KOA. However, the improvement in clinical outcomes in patients in our MFAT group was smaller than those reported in other studies, which might be explained by the heterogeneity of the enrolled participants.

In our study, arthroscopic surgery in the control group had a favorable mid-term outcome at 6 mo, but this efficacy was not long-lasting, which was consistent with the findings of earlier trials[40]. Interestingly, injecting MFAT could prolong the improving trend in most of the indicators to 24 mo. Regarding the mechanism of MFAT for KOA treatment, we speculate that MFAT may first act through differentiation or paracrine action of the different cells it contains. There is evidence that MFAT itself, not only its derived human MSCs, can directly differentiate into chondrocytes in vitro[20]. Other differentiated cells, such as pericytes in MFAT, may also play important roles since MFAT releases many more growth factors and cytokines involved in tissue repair and regeneration than the enzymatically derived stromal vascular fraction[41]. Second, matrix metalloproteinases and proinflammatory mediators, such as interleukin-1β and tumor necrosis factor-α cause cartilage degradation and synovial swelling, stimulating synovial proliferative and inflammatory responses of both resident synoviocytes and macrophages[42]. After co-cultivation of MFAT and inflamed synoviocytes, the levels of macrophage-specific chemokines [C-C motif ligand 2 (CCL2)/monocyte chemoattractant protein-1 and CCL3/macrophage inflammatory protein-1α) and the degradative marker matrix metalloproteinase-9 were downreg-

ulated, and the mRNA levels of inflammatory factors were reduced, which was partially dependent on Toll-like receptor 4 and nuclear factor-kappaB signaling[43,44]. Additionally, other secreted molecules in MFAT may play anti-inflammatory roles. Ragni *et al*[42] identified extracellular vesicle-shuttled microRNAs (miRNAs) (miR-24-3p, miR-222-3p, and miR-193b-3p) and soluble factors [tissue inhibitor of metalloproteinases-1 (TIMP1), TIMP2, PLG, and CTSS) as both cartilage-protective factors and factors that trigger the switch from M1 to M2 macrophages in the secretome of adipose-derived stem cells cultivated *in vitro* under inflammatory conditions. Third, preserving cartilage function and integrity is a feasible mechanism. Chen *et al*[23] demonstrated that MFAT attenuated pain symptoms and protected chondrocytes and cartilage extracellular matrix from damage in a monoiodoacetate-induced OA rat model. Another clinical trial also indicated that the contents of cartilage glycosaminoglycans significantly increased in specific areas of the treated knee joint by delayed gadolinium-enhanced MRI of cartilage[45]. Finally, MFAT may act as a natural scaffold and display interesting mechanical properties, such as the infrapatellar fat pad[20,46].

MRI can provide a more accurate and reliable basis for assessing factors, such as the lesion location and morphological organization, in patients. A 12-mo follow-up of 64 patients undergoing MFAT therapy revealed that BML was negatively correlated with the therapeutic response rate at 12 mo[47]. Similarly, another study showed that BML was significantly deceased 12 mo after surgery in the treatment group. However, the cartilage scores were nonsignificant between the treatment and placebo groups, although the cartilage layer was thicker in the treatment group at 12 mo[48]. We compared the MRI results of the MFAT group to those of the control group using WORMS, which revealed no significant differences between these two groups at 24 mo. However, similar to the findings of a study by Nguyen *et al*[48], we found that the WORMS at the 24-mo follow-up in the MFAT group had significantly reduced, while it was relatively increased in the control group. Furthermore, MRI of a higher percentage of patients in the MFAT group showed signs of repaired cartilage damage, meniscus repair, and reduced BML, supporting the therapeutic potential of MFAT from the perspective of MRI findings. Additionally, the insignificant results at 24 mo might be partially explained by the limited number of postoperative MRI results in this study due to economic reasons (n = 10 for each group). Although it is well known that BML is closely related to pain in the arthritic knee joint, the current conclusions about the imaging changes in MFAT in the treatment of KOA and the association of imaging with clinical results are inconsistent[49]. Therefore, well-controlled studies with larger sample sizes are needed.

In the current study, we only found a handful of mild adverse events with pain or swelling in the liposuction or injection area, without severe adverse events or complications. This result is consistent with those of a previous study in which only 5.8% of patients who were administered MFAT injections had a transitory hematoma of the abdominal region that had no impact on the knee[50]. Likewise, Bisicchia *et al*[38] reported no adverse events related to MFAT injection at the 12-mo follow-up, and there was only one case of knee effusion 3 d postoperatively in the microfracture control group, demonstrating the safety of MFAT for clinical use.

A major strength of this study is that, as the first multicenter prospective randomized trial in a Chinese population, the study's sample size was relatively large. Moreover, the primary and secondary outcomes included both patient-reported indexes and imaging technologies, and most patients completed the final 24-mo follow-up. However, our study has some limitations. First, we only recruited patients with Kellgren-Lawrence grades of 2-3. We excluded a Kellgren-Lawrence grade of 4 because it indicated marked joint-space narrowing and definite deformity, which may not be suitable for arthroscopic treatment; however, other studies have demonstrated the efficacy of MFAT in patients with severe OA[33,39]. Second, the patients and the surgeons who performed the surgeries could not be blinded to the patients' groups owing to the extra liposuction in the MFAT group. Nevertheless, the operator who processed the data was unaware of the patients' groups. Third, the follow-up time was short, and differences in the volume of MFAT were not examined. Moreover, the differences in the intervention in the control and study groups may have influenced the results. In addition, we should also consider cost-effectiveness since the cost of MFAT might be prohibitive for some patients. Finally, this study was conducted in only Zhejiang, China, as a representative sample of the Chinese population. Long-term studies on the potential dose-dependent effect, including more districts, should be conducted in the future.

CONCLUSION

In conclusion, our study demonstrates that MFAT injection combined with arthroscopic surgery is a safe and effective approach for improving function and alleviating pain in patients with KOA. MFAT therapy produced longer-lasting, statistically significant durations of efficacy at 12 and 24 mo in comparison to those of the control (HA) group. Based on these results, MFAT injection combined with arthroscopic surgery may be considered a potential therapeutic option for KOA.

ARTICLE HIGHLIGHTS

Research background

The most common kind of degenerative whole-joint disease is osteoarthritis (OA). Before the ultimate choice of knee replacement, the most common joint-preserving surgical procedure was arthroscopic surgery. Mesenchymal stem cells, platelet-rich plasma, and microfragmented adipose tissue (MFAT) are examples of emerging regenerative medicines that have been thrust into the forefront of treatment to stop the progression of OA. MFAT is now being used to treat various orthopedic diseases with effectiveness.

Research motivation
Current mainstream medicine mainly focuses on relieving symptoms and cannot prevent the disease from progressing to the late stages of arthritis that require knee replacement. MFAT as a novel way of treatment may be of great significance in alleviating knee OA (KOA).

Research objectives
The present study aimed to assess the efficacy and safety of MFAT with arthroscopic surgery in patients with KOA.

Research methods
Patients diagnosed with KOA (Kellgren-Lawrence grades 2-3) were included in a multicenter, prospective, single-blind randomized trial. In this trial, 302 patients were randomized into the MFAT group (n = 151, were administered MFAT following arthroscopic surgery), or the control group (n = 151, were administered hyaluronic acid following arthroscopic surgery). The study outcomes included changes in the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) score, the visual analog scale (VAS) score, the Lequesne index score, the Whole-Organ Magnetic Resonance Imaging Score (WORMS), and safety, from baseline to 24 mo.

Research results
The changes in the WOMAC score (including the 3 subscale scores), VAS pain score, and Lequesne index score at 24 mo showed significant differences in both groups between the posttreatment visit and baseline ($P < 0.001$). The MFAT group exhibited significant decreases in the WOMAC pain scores and VAS scores at all follow-ups, suggesting the pain-relieving potential of MFAT combined with arthroscopy compared to arthroscopy alone HA treatment ($P < 0.05$). The significant differences in the WOMAC stiffness score, WOMAC function score, and Lequesne index score at 12 and 24 mo after MFAT administration and surgery suggested the potential of MFAT combined with arthroscopy compared to arthroscopy alone control group to improve function and quality of life ($P < 0.05$). There were no significant between-group differences in the WORMS at 24 mo ($P = 0.367$). No serious adverse events occurred in the two groups.

Research conclusions
In summary, our study demonstrates that MFAT injection combined with arthroscopic surgery is a safe and effective approach for improving function and alleviating pain in patients with KOA. Based on these results, MFAT injection combined with arthroscopic surgery may be considered a potential therapeutic option for KOA.

Research perspectives
We demonstrated that MFAT injection combined with arthroscopic surgery had better clinical efficacy than control group for treating KOA at a mid-term follow-up and could be a potential therapeutic approach for patients with KOA.

FOOTNOTES

Co-first authors: Cong-Zi Wu and Zhen-Yu Shi.

Co-corresponding authors: Pei-Jian Tong and Hong-Ting Jin.

Author contributions: Wu CZ, Shi ZY, and Wu Z contributed to the collection and assembly of data, and manuscript writing, and contributed equally to this work; Xiang SC, Xu HH, Ge QW, Zou KA, and Wang X contributed to data analysis and interpretation; Lin WJ, Chen WB, and Jia XW contributed to imaging assessment; Chen JL, Wang PE, and Yuan WH contributed to data analysis and manuscript preparation; Jin HT and Tong PJ contributed to conception and design, imaging assessment, manuscript writing, final approval, and share corresponding authors; and all authors have read and approved the manuscript.

Supported by the National Natural Science Foundation of China, No. 82274547; the Major Program of the National Natural Science Foundation of Zhejiang Province, No. LD22C060002; the State Administration of Traditional Chinese Medicine of Zhejiang Province, No. GZY-ZJ-KJ-23064; and the Zhejiang Provincial Research Foundation for Basic Public Welfare Research, No. LGF20H270005.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of the First Affiliated Hospital of Zhejiang Chinese Medical University (approval No. 22-KL-048).

Clinical trial registration statement: The trial was registered at chictr.org.cn (registration number ChiCTR2200055124).

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding authors.

CONSORT 2010 statement: The authors have read the CONSORT 2010 Statement, and the manuscript was prepared and revised

according to the CONSORT 2010 Statement.

Country/Territory of origin: China

ORCID number: Pei-Jian Tong 0000-0001-5936-4632.

S-Editor: Wang JJ
L-Editor: A
P-Editor: Zhang XD

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World Journal of Stem Cells

Submit a Manuscript: https://www.f6publishing.com

World J Stem Cells 2023 December 26; 15(12): 1077-1092

DOI: 10.4252/wjsc.v15.i12.1077

ISSN 1948-0210 (online)

ORIGINAL ARTICLE

Basic Study

ADSC-Exos outperform BMSC-Exos in alleviating hydrostatic pressure-induced injury to retinal ganglion cells by upregulating nerve growth factors

Zhi-Kun Zheng, Lei Kong, Min Dai, Yi-Dan Chen, Yan-Hua Chen

Specialty type: Cell and tissue engineering

Provenance and peer review: Unsolicited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): C, C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Grawish ME, Egypt; Li SC, United States; Sheykhhasan M, Iran

Received: September 6, 2023
Peer-review started: September 6, 2023
First decision: October 23, 2023
Revised: November 17, 2023
Accepted: December 4, 2023
Article in press: December 4, 2023
Published online: December 26, 2023

Zhi-Kun Zheng, Lei Kong, Min Dai, Yi-Dan Chen, Yan-Hua Chen, Department of Ophthalmology, Affiliated Hospital of Yunnan University/Yunnan Eye Hospital, Kunming 650021, Yunnan Province, China

Corresponding author: Min Dai, PhD, Doctor, Department of Ophthalmology, Affiliated Hospital of Yunnan University/Yunnan Eye Hospital, No. 176 Qingnian Road, Wuhua District, Kunming 650021, Yunnan Province, China. dm9024@163.com

Abstract

BACKGROUND

Mesenchymal stem cells (MSCs) have protective effects on the cornea, lacrimal gland, retina, and photoreceptor cell damage, which may be mediated by exosomes (exos) released by MSCs.

AIM

To investigate the ameliorating effect of exos derived from different MSCs on retinal ganglion cell (RGC) injury induced by hydrostatic pressure.

METHODS

The RGC injury model was constructed by RGC damage under different hydrostatic pressures (40, 80, 120 mmHg). Then RGCs were cultured with adipose-derived stem cell (ADSC)-Exos and bone marrow-derived stem cell (BMSC)-Exos. Cell Counting Kit-8, transmission electron microscopy, flow cytometry, immunofluorescence, real-time quantitative polymerase chain reaction, and western blotting were performed to detect the ameliorating effect of exos on pressure-induced RGC injury.

RESULTS

ADSC-Exos and BMSC-Exos were successfully isolated and obtained. The gibbosity of RGCs was lower, the cells were irregularly ellipsoidal under pressure, and the addition of ADSC-Exos and BMSC-Exos significantly restored RGC morphology. Furthermore, the proliferative activity of RGCs was increased and the apoptosis of RGCs was inhibited. Moreover, the levels of lactate dehydrogenase and apoptosis-related proteins were increased, and the concentrations of antiapoptotic proteins and neurotrophic factors were decreased in damaged RGCs. However, the above indicators were significantly improved after ADSC-

Exos and BMSC-Exos treatment.

CONCLUSION
These findings indicated that ADSC-Exos and BMSC-Exos could ameliorate RGC injury caused by hydrostatic pressure by inhibiting apoptosis and increasing the secretion of neurotrophic factors.

Key Words: Adipose-derived stem cells; Bone marrow-derived stem cells; Exosomes; Glaucoma

Core Tip: We discovered for the first time that adipose-derived stem cell-exosomes (ADSC-Exos) significantly ameliorate retinal ganglion cell (RGC) injury caused by hydrostatic pressure by inhibiting apoptosis and increasing the secretion of neurotrophic factors. ADSC-Exos manifested better ameliorating effects than bone marrow-derived stem cell (BMSC)-Exos in ameliorating the RGC injury induced by hydrostatic pressure. BMSC-Exos ameliorate optic nerve injury caused by hydrostatic pressure by inhibiting apoptosis and increasing the secretion of neurotrophic factors.

Citation: Zheng ZK, Kong L, Dai M, Chen YD, Chen YH. ADSC-Exos outperform BMSC-Exos in alleviating hydrostatic pressure-induced injury to retinal ganglion cells by upregulating nerve growth factors. *World J Stem Cells* 2023; 15(12): 1077-1092
URL: https://www.wjgnet.com/1948-0210/full/v15/i12/1077.htm
DOI: https://dx.doi.org/10.4252/wjsc.v15.i12.1077

INTRODUCTION

Glaucoma is the main cause of irreversible blindness around the world and is a chronic degenerative optic nerve disease [1]. The early pathological changes of glaucoma include reduced axonal transport, axon loss, and dendrite reconstruction, leading to retinal ganglion cell (RGC) apoptosis and thinning of the retinal nerve fiber layer, eventually resulting in visual field defects and loss of vision[2,3]. Apoptosis of RGCs in glaucoma has been demonstrated *in vivo* in several animal models and in humans[4,5]. The mechanism of RGC apoptosis in glaucoma, including oxidative injury[6], inflammatory response[4], and glutamate toxicity[7], particularly how elevated intraocular pressure (IOP) leads to RGC apoptosis, is yet to be fully elucidated. There are three treatment methods for glaucoma caused by high IOP, including using drugs to reduce the production of aqueous humor, surgically aqueous humor drainage, and laser stimulation for cell proliferation and differentiation to repair damaged eye tissue[8]. However, existing IOP-lowering treatments cannot prevent the reduction of RGCs[9], and there is the limitation of repeated treatment, even worse.

The only hope for glaucoma patients with RGC loss to regain vision is preventing RGC apoptosis[10]. Current research is focused on developing neuroprotective measures, particularly the use of stem cell therapy to protect RGCs[11]. Stem cells produce many molecules responsible for cell signaling, such as cytokines, growth factors, morphogens, chemokines, and extracellular vesicles (EVs), improving various cellular mechanisms[12-15]. However, the direct transplantation of stem cells has some risks such as safety, tumorigenesis, and immune rejection[16]. Recent studies have shown that stem cells play a therapeutic role in a paracrine manner, with stem cell-derived exosomes (exos) being the major contributors to stem cell function, carrying and transporting cytokines, proteins, and nucleic acids from stem cells[17]. It also has stronger target specificity and lower carcinogenic and immune risks[18], which can avoid the problems existing in the direct use of stem cell therapy, and increasing the use of stem cell-derived exos is the focus of research to enhance the efficacy of stem cell therapy.

Mesenchymal stem cells (MSCs) are fibroblast-like, self-renewing cells that are present in almost all postpartum tissues and organs, including bone marrow, adipose tissue, blood, umbilical cord, amniotic fluid, and dental pulp and are responsible for regulating the normal development and maintenance of mesenchymal tissue[19]. Bone marrow, adipose tissue, and human MSCs of the umbilical cord have protective effects on cornea, lacrimal gland, retina, and photoreceptor cell damage[20]. This ameliorating effect may be mediated at least in part by exos. MSC-Exos can maintain activity for at least 4 wk after vitreous injection and can integrate into tissues more quickly to provide nutritional factors for RGCs due to their smaller diameter[15]. Bone marrow stem cell (BMSC)-derived exos are also more effective than directly transplanted BMSCs in the treatment of glaucoma because BMSCs have a poor ability to integrate into the retina, remain in the vitreous after injection, and cannot deliver neurotrophic factors to RGCs[21]. Mead *et al*[22] found that the neural function of RGCs was significantly improved after intravitreal injection of BMSC-Exos in a glaucoma rat model. A similar result was reported in the hereditary DBA/2J mouse model of glaucoma[23]. Moreover, studies have shown that BMSC-Exos injected in an optic nerve compression rat model by intravitreal injection can integrate into the cell bodies and axons of retinal neurons and promote axon regeneration by activating the Akt pathway dependent on microRNA (miRNA)[24]. These findings indicate that BMSC-Exos have an ameliorative effect on glaucoma. Moreover, Sheykhhasan *et al*[25] demonstrated that adipose-derived stem cell (ADSC)-Exo and CoQ10 administration could ameliorate memory deficits by modulating SOX2 and brain-derived neurotrophic factor (BDNF) expression in a rat model of Alzheimer's disease. Numerous preclinical studies have confirmed the therapeutic potential of ADSC-Exos in neurodegenerative diseases[26,

27]. Glaucoma is a neurodegenerative disease in which the role of ADSC-Exos has not been studied yet. Therefore, this study aimed to investigate the protective effect of BMSC-Exos and ADSC-Exos on RGC injury, which is the main feature of glaucoma, induced by hydrostatic pressures.

MATERIALS AND METHODS

Extraction of ADSC-Exos and BMSC-Exos

ADSCs and BMSCs were a kind gift from Kunming Cell Bank of Chinese Academy of Sciences (Kunming, China). Cells in the third generation with good logarithmic phase growth were selected. The cell culture medium was discarded, and the cells were washed 3 times with phosphate-buffered solution (PBS) and replaced with serum-free medium. After 48 h in culture, the cells were adjusted to a concentration of 1×10^5 cells/mL. The cells were then centrifuged at 3000 r/min for 10 min at room temperature to collect the supernatant. The supernatant was centrifuged at 20000 r/min for 20 min at 4 °C to remove dead cells and debris, and the supernatant was then filtered through a sterile, 0.22 μm pore size syringe filter to obtain the crude extract of exos. Then the crude extract was centrifuged at 50000 r/min at 4 °C for 1 h. The white precipitate could be seen at the bottom of the tube after centrifugation, and the supernatant was removed. The white precipitate was cleaned with 0.9% sodium chloride injection several times, and the resulting suspension was collected and centrifuged at 4 °C for 10 min at 3000 r/min. Exo protein quantification was performed using a BCA protein assay kit (Beyotime, Shanghai, China). Dynamic light scattering was used to detect the size distribution of the exos. The structure of exos was observed by transmission electron microscopy (TEM) (HITACHI, Japan).

Primary RGCs extraction, culture[28,29], and grouping

Mice 1-3 d old were sacrificed by intraperitoneal injection of 4.3% chloral hydrate, and the eyeballs were removed under aseptic conditions and rinsed three times. Under the microscope, the retinal neuroepithelial layer tissue was isolated. The tissue was rinsed 3 times and digested in 0.5 g/L trypsin for 30 min at 37 °C. The digestion was terminated by the addition of DMEM containing 10% foetal bovine serum (FBS) and filtered through a 40-μm filter. Centrifuge at room temperature for 5 min, discard the supernatant, and add complete DMEM. After adjusting the cell density, the cells were inoculated into cell culture flasks. The cells were placed in a 5% CO_2 incubator at 37 °C for 7-10 d. The obtained cells were taken and identified by immunofluorescence staining using a Thy1.1 antibody. The project was approved by the Yunnan University Ethics Committee (approval number YUN20230463).

Then, the cells were grouped into different experiments as follows: (1) Normal control (NC) group, 40 mmHg group, 40 mmHg + ADSC-exo group, and 40 mmHg + BMSC-exo group; (2) NC group, 80 mmHg group, 80 mmHg + ADSC-exo group, and 80 mmHg + BMSC-exo group; and (3) NC group, 120 mmHg group, 120 mmHg + ADSC-exo group, and 120 mmHg + BMSC-exo group.

Hydrostatic pressure damage model preparation

RGC culture dishes were placed within the pressure chamber[30,31], and the incubating gas mix was pressurized. The cells were exposed to conditions of elevated ambient hydrostatic pressure (40 mmHg, 80 mmHg, 120 mmHg) over and above atmospheric pressure for a period of 2 h. The pressure conditions were then restored to atmospheric, and the culture dishes were removed from the chamber. Under normal cell culture conditions (37 °C, 5% CO_2), the culture was continued for 24 h.

Coculture of ADSC-Exos or BMSC-Exos and RGCs

The concentrations of ADSC-Exos and BMSC-Exos were measured by a BCA kit (P0010, Beyotime). P3-generation SCs with good digestive state were taken from each group to make a uniform cell suspension and put 100 μL per well in 96-well cell culture plates. ADSC-Exos (20 μg/mL)[32] were added after the stem cells were completely attached to the wall. BMSC-Exos (20 μg/mL)[33] were cultured for 24 h, and the cells of each group were collected for subsequent experiments. Then, cell morphology was observed under an inverted phase contrast microscope (Olympus).

Ultrastructure observation of RGCs by transmission electron microscopy

After the cells were treated for 24 h according to the above groups, the cells were digested and collected with Trypsin-EDTA Solution (Beyotime, Shanghai, China), centrifuged in a 1.5 mL EP tube, and the supernatant was discarded. Then, 2.5% glutaraldehyde fixing solution was quickly added to completely cover the cell specimens, which were refrigerated at 4 °C overnight. The fixing solution was poured away, and the samples were rinsed with PBS first and then fixed with 1% osmic acid solution. The samples were subjected to gradient dehydration with an ethanol solution and finally treated with pure acetone for 20 min. The samples were treated with a mixture of equal volume embedding medium and acetone for 1 h. Then, the samples were treated with a mixture of embedding medium and acetone (V/V = 3/1) for 3 h. Next, the embedding medium was used to treat the sample overnight. The samples with osmotic treatment were embedded and heated at 70 °C overnight to prepare the needed samples. The samples were sliced in a Leica EM UC7 ultramicrotome, and slices of 70-90 nm were obtained, double-dyed with lead citrate solution and 50% ethanol saturated solution of uranyl acetate, dried and observed under a transmission electron microscope (Tecnai G2 spirit).

RGCs were labeled by immunofluorescence with β-III tubulin

RGCs were collected in each group, washed twice with PBS, fixed at room temperature for 30 min with 4% paraformal-

dehyde, permeated with 0.1% Triton X-100 for 10 min, washed with PBS 3 times, and sealed with 10% goat serum at room temperature for 1 h. The blocking buffer was removed, and β-III tubulin primary antibody (ab78078, Abcam) was added and incubated overnight at 4 °C. After washing with PBS 3 times, sheep anti-rabbit IgG fluorescent secondary antibody (P0186, Beyotime) was added to the dark, and incubated at room temperature for 2 h. After 3 washes with PBS, 5-10 nonrepetitive visual fields were randomly taken under a fluorescence microscope (Nikon fluorescence microscope), and the number of retinal ganglion cells was quantified by ImageJ software (ImageJ, RRID:SCR_003070).

Detection of cell activity by Cell Counting Kit-8 assay

The cells of each group were digested with Trypsin-EDTA Solution, and the cells were suspended in 10% FBS medium. Each well was inoculated with 1×10^5 cells in 96-well plates. The 96-well plates were cultured in a constant temperature incubator containing 5% CO_2 at 37 °C for 0, 24, 48, 72, and 96 h. At the corresponding time, 10 μL Cell Counting Kit-8 (CCK-8) reagent (Beyotime) was added, and the absorption value at 450 nm was detected by a microplate reader after continuous culture for 2 h.

Detection of the cell apoptosis rate by Annexin V-FITC kit flow cytometry

Cells in each group were digested with trypsin-EDTA solution, collected, washed with PBS, resuspended in 300 μL of binding buffer, and incubated with 10 μL of Annexin V-FITC at room temperature for 15 min in the dark. Before the operation, 5 μL PI was added and then 200 μL binding buffer was added. The apoptosis rate of cells was detected by a flow cytometer.

The localization of CREB and p-CREB detected by immunofluorescence chemical staining

The cells were inoculated into 24-well plates with 5×10^4 cells per well, and each well was cultured with 500 μL medium for 12 h. Then, the cells were fixed with 4% paraformaldehyde at room temperature. PBS containing 0.1% Triton X-100 was added, and permeabilization was performed at room temperature for 10 min. The cells were sealed with 5% BSA at room temperature for 30 min. The liquid was removed and rabbit CREB antibody (Beyotime) and rabbit p-CREB antibody (Beyotime) diluted with 1% BSA were added. Next, the cells were incubated at 4 °C overnight and washed with PBS. The goat anti-rabbit secondary antibody was incubated at room temperature for 1 h and washed with PBS. Finally, the samples were dyed with DAPI solution at room temperature for 5 min and washed with PBS. A fluorescence microscope was used to observe and photograph the cells.

The degree of RGC neuron damage evaluated by the lactate dehydrogenase release test

One hundred microliters of the cell supernatant in each group was collected for the lactate dehydrogenase (LDH) concentration test according to the LDH kit (Keygen, Nanjing, China) instructions. Then, the reaction mixture was added and incubated in the dark for 30 min at room temperature. The LDH concentration was quantified by measuring the absorbance at 490 nm.

Detection of the expression of Bcl-2, Bax, Caspase-3/9, TRkA, TrkB, CREB, and p-CREB by western blotting

The cells of each group were collected and lysed with RIPA lysis buffer containing a protease inhibitor, and the protein concentration was determined with a BCA protein concentration detection kit. The sample was loaded at 30-50 μg/well, separated by 10% sodium-dodecyl sulfate gel electrophoresis for 1 h, transferred to a polyvinylidene fluoride membrane, sealed with PBS buffer containing 5% skim milk powder for 2 h, added to the corresponding primary antibody, and incubated overnight at 4 °C. Then, the cells were rinsed with PBST buffer 3 times for 5 min each, the corresponding secondary antibody was added, and the cells were incubated at 37 °C for 4 h. Next, PBST buffer was used to rinse the cells 3 times. Color development was achieved by using the ECL Western Blot Detection Kit (Thermo Scientific). β-Actin was used as the internal reference. All antibodies were purchased from Abcam Company in Shanghai, China. Image J was used to analyze the gray value of the target protein.

Detection of the relative expression of CNTF, BDNF, NGF, TrkB, TrkA, and CREB mRNA by real-time quantitative polymerase chain reaction

TRIzol reagent (TRIzol Invitrogen) was employed to extract total RNA from cells, which were then treated with DNA enzymes for reverse transcription using a reverse transcription kit (Takara Biotechnology). Two microliters of cDNA product was placed into an EP tube and amplified by a SYBR Green Master Mix reaction system. The Ct value of the template was determined using GAPDH as the internal parameter, and the relative expression level of the target mRNA was quantified by the cycle counting method ($2^{-\Delta\Delta Ct}$). The primer sequences are shown in Table 1.

Statistical analyses

All data in this study were analyzed using SPSS 22.0 statistical software. Data are shown as the means ± SE (the number of samples was 3 per group, and each sample was repeated three times). Comparisons between the two groups were made using independent *t* tests, and comparisons between multiple groups were made using one-way ANOVA. $P < 0.05$ was considered to be a statistically significant difference.

Table 1 Primer sequences

Genes	Primer sequences
GAPDH-F	TGTGACAGTGACTTGGGACA
GAPDH-R	AGGTGGAAGAGTGGGAGTTG
Bcl2-F	TCGCAGAGATGTCCAGTCAG
Bcl2-R	ATCTCCCTGTTGACGCTCTC
Caspase-3-F	AAGGAGCAGCTTTGTGTGTG
Caspase-3-R	TGTCTCAATGCCACAGTCCA
Bax-F	TGCAGAGGATGATTGCTGAC
Bax-F	GATCAGCTCGGGCACTTTAG
CNTF-F	GGTGACTTCCATCAGGCAAT
CNTF-R	TGACACGGAGGTCATGGATA
BDNF-F	TAATGCAGCATGATGGGAAA
BDNF-R	TCACAGTGAAAGCACCTTGC
NGF-F	CATGGGGGAGTTCTCAGTGT
NGF-R	GCACCCACTCTCAACAGGAT
TrkA-F	GTCTGGTGGGTCAGGGACTA
TrkA-R	AAAGCTCCACACATCGCTCT
TrkB-F	ACTCGCTTCTGGCATTGTCT
TrkB-R	TGTTAGTTGTGGTGGGCAAA
CREB-F	TTTTACCCAGGTGCCACTTC
CREB-R	TGGGGCATTATAACCGATGT
Caspase 9-F	TGCCCTTGCCTCTGAGTAGT
Caspase 9-R	AACAAAGAAACGCCCACAAC

Bcl2: B-cell lymphoma 2; Trk: Tropomyosin receptor kinase; BDNF: Brain-derived neurotrophic factor; CREB: Cyclic adenosine monophosphate response element-binding protein; CNTF: Ciliary neurotrophic factor; NGF: Nerve growth factor.

RESULTS

Characterization of ADSC- and BMSC-Exos and RGCs

The immunofluorescence labeled SOX2, which is the marker protein of stem cells was highly positive in the two kinds of stem cells (Figure 1A). Then, exos were further isolated from the stem cell solution, and suborbicular vesicles in the form of complex exo were observed by electron microscopy (Figure 1B). The exo marker molecules CD9, CD63, and CD81 detected by western blot were positive (Figure 1C), which proved that ADSC- and BMSC-Exos were successfully isolated and obtained. Finally, primary RGCs were extracted. Figure 1D shows light microscope images of RGCs. The isolated RGCs were examined by Thy1.1 immunofluorescence (Figure 1E).

ADSC-Exos and BMSC-Exos ameliorate RGCs

RGC growth observation: The RGC damage models under 40, 80, and 120 mmHg pressure were constructed, and cultured with ADSC-Exos (20 μg/mL) and BMSC-Exos (20 μg/mL) for 24 h, and then the morphology of RGCs was observed. The results of phase contrast microscopy showed that the gibbosity of RGCs was lower and that the cells were irregularly ellipsoidal under pressure. The addition of ADSC-Exos significantly restored the morphology of RGCs, but BMSC-Exos failed to restore the morphology of RGCs in the 120 mmHg pressure group (Figure 2A). After pressure was applied, transmission electron microscopy observations showed that RGCs had nuclear swelling, structural disorder, atrophy, malformation, and nucleolar shrinkage or disappearance. The addition of ADSC-Exos and BMSC-Exos significantly restored RGC morphology (Figure 2B).

The number of RGCs: To further explore the effect of ADSC-Exos and BMSC-Exos on RGCs, we performed neuron-specific β III-tubulin expression by immunofluorescence. The results showed that the number of RGCs was significantly reduced after pressure was applied, while the number of RGCs was markedly increased after ADSC-Exos and BMSC-Exos were administered (Figures 3A-C).

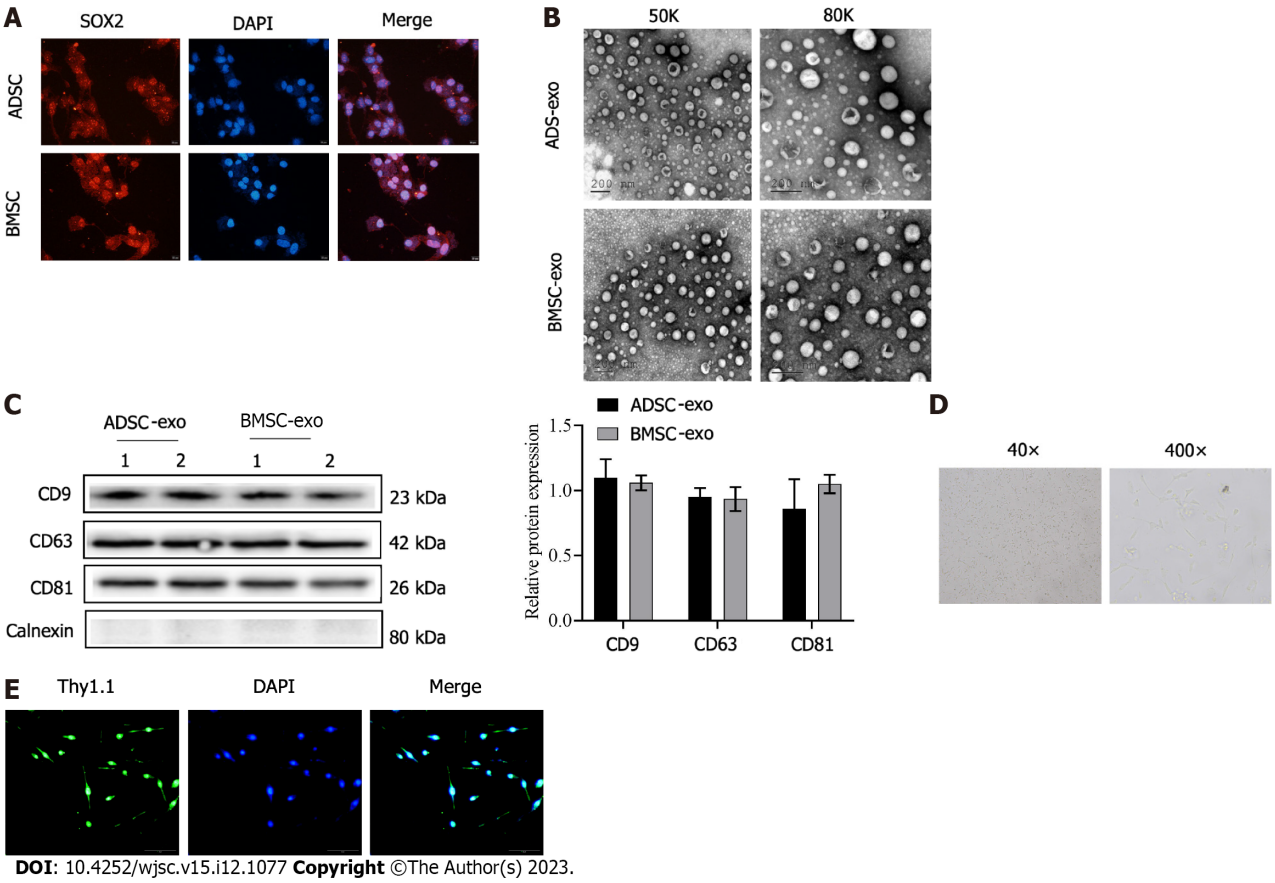

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Figure 1 Adipose-derived stem cell and bone marrow-derived stem cell-exosomes, and retinal ganglion cells were isolated and identified. A: The immunofluorescence staining of stem cell markers SOX2 (Scale bar: 20 μm); B: The morphology of adipose-derived stem cell-exosomes (ADSC-Exos) and bone marrow-derived stem cell (BMSC)-Exos was observed by electron microscopy (scale bar: 200 nm); C: The ADSC-Exos and BMSC-Exos marker molecules (CD9, CD63, and CD81) were detected by western blotting; D: The light microscope images of retinal ganglion cells (RGCs) (magnification: 40 × and 400 ×); E: The isolated RGCs was examined by Thy1.1 immunofluorescence (scale bar: 50 μm). ADSC: Adipose-derived stem cell; BMSC: Bone marrow-derived stem cell; Exo: Exosome.

RGC proliferation and apoptosis: The CCK-8 results showed that RGC proliferation activity in the pressure injury group was significantly lower than that in the NC group, while proliferation activity was elevated after ADSC- and BMSC-Exos treatment (Figure 4A). The flow cytometry results indicated that compared with that in the NC group, the apoptosis rate in the pressure injury group was significantly increased, while the apoptosis rate was significantly decreased after ADSC- and BMSC-Exos treatment (Figure 4B).

The expression of CREB and p-CREB in RGCs

CREB inhibits apoptosis by upregulating the expression of the antiapoptotic genes Bcl-2 and Bcl-xL through a transcription-dependent mechanism. Therefore, this study further explored the effects of exos from different sources on the apoptosis-related protein CREB in RGCs. As shown in Figures 5A-C, the ratio of p-CREB/CREB in the pressure injury group was significantly reduced compared with that in the NC group, while the ratio of p-CREB/CREB was significantly increased after ADSC- and BMSC-Exos treatment.

The expression of LDH, neurotrophic factor and apoptosis-related mRNA and protein

LDH is present in all cells. When RGCs are damaged, they are released rapidly. When LDH is elevated, RGCs are damaged or dysfunctional, which leads to apoptosis. Therefore, this study further explored the effects of exos from different sources on LDH in RGCs. The LDH test kit results showed that compared with that in the NC group, the LDH level was significantly increased in the pressure injury group, while the LDH level was significantly decreased after ADSC-Exos and BMSC-Exos treatment (Figure 6A). western blot and real-time quantitative polymerase chain reaction results indicated that compared with the NC group, the anti-apoptotic protein (Bcl-2) and neurotrophic factor (BDNF, NGF, CNTF, TRKA, TRKB) were significantly decreased and the pro-apoptotic protein (Bax, caspase3, caspase9, CREB) was significantly increased in the pressure injury group, while the above indices could be reversed after ADSC-Exos and BMSC-Exos treatment (Figures 6B and C).

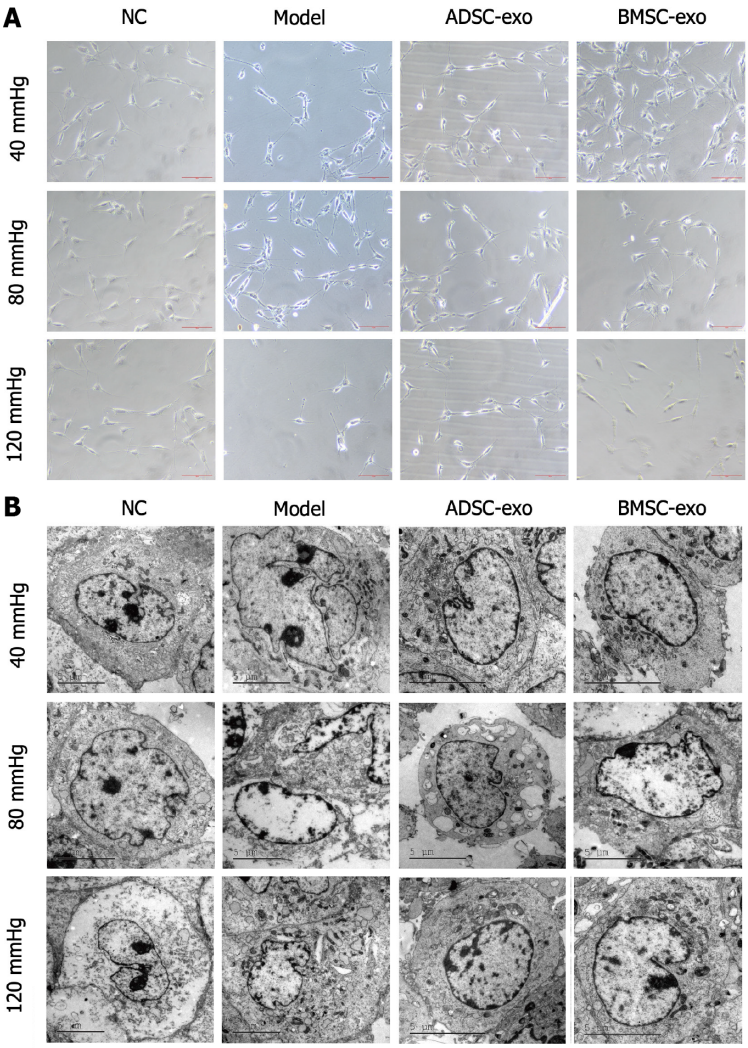

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Figure 2 The growth of retinal ganglion cells. A: The observation of morphology and growth of retinal ganglion cells (RGCs) by phase contrast microscope (Scale bar: 50 μm); B: The observation of RGCs morphology by transmission electron microscope (Scale bar: 5 μm). ADSC: Adipose-derived stem cell; BMSC: Bone marrow-derived stem cell; Exo: Exosome; NC: Normal control.

DISCUSSION

Hydrostatic pressure is known to impact various aspects of cellular anatomy and physiology. Morphological changes in cell shape, alignment and processes and cytoskeletal actin redistribution have been demonstrated in rat RGCs[34,35]. Agar *et al*[36] showed that neurons may undergo apoptosis in direct response to increased pressures at clinically relevant levels. Cell cultures were subjected to elevated hydrostatic pressures in an *in vitro* system based on established pressure chamber models[37,38]. Experimental pressure conditions were selected to be relevant to the IOPs seen in clinical settings, with levels of 100 mmHg analogous to acute glaucoma, 30 mmHg for chronic glaucoma and 15 mmHg for the so-called 'normal' IOP. Therefore, hydrostatic pressures of 40, 80, and 120 mmHg were chosen for this study and the intervention of ADSC- and BMSC-Exos could improve RGC damage by inhibiting RGC apoptosis and promoting neural restoration of RGCs.

Stem cells are a kind of cell population with the potential for self-renewal, high proliferation and differentiation, and multidirectional differentiation. MSCs are derived from connective tissue and can be differentiated into osteoblasts, chondrocytes, fat cells, and so on. In neurodegenerative diseases, MSC secretes a variety of neurotrophic factors that directly promote the survival and growth of nerve cells and act on the microenvironment of nerve tissue[39,40]. MSCs isolated from rat femoral bone marrow injected into a vitreous body can integrate into the retina, survive for at least 5 wk, significantly reduce IOP and have a protective effect on RGCs[41]. Liu *et al*[42] found that BMSC transplantation can significantly reduce photoreceptor cell death and preserve retinal structure in retinal detachment models. Hu *et al*[43] reported that BMSCs promoted the survival of RGCs in the transplanted eye compared with the control eye. MSCs play a role in neuroprotection mainly by regulating the intraocular microenvironment. This suggests that BMSCs have an ameliorating effect. However, the structural integration of MSCs into retinal tissue is complex and difficult due to the barrier action of the inner boundary membrane of the retina and the intercellular junctions[44]. In addition, transplanting MSCs in tissue reconstruction carries risks of tumorigenesis and immunological rejection. Transplanting MSC-Exos may overcome these obstacles in future applications. For example, MSC-Exos have an ameliorating effect on RGCs in some

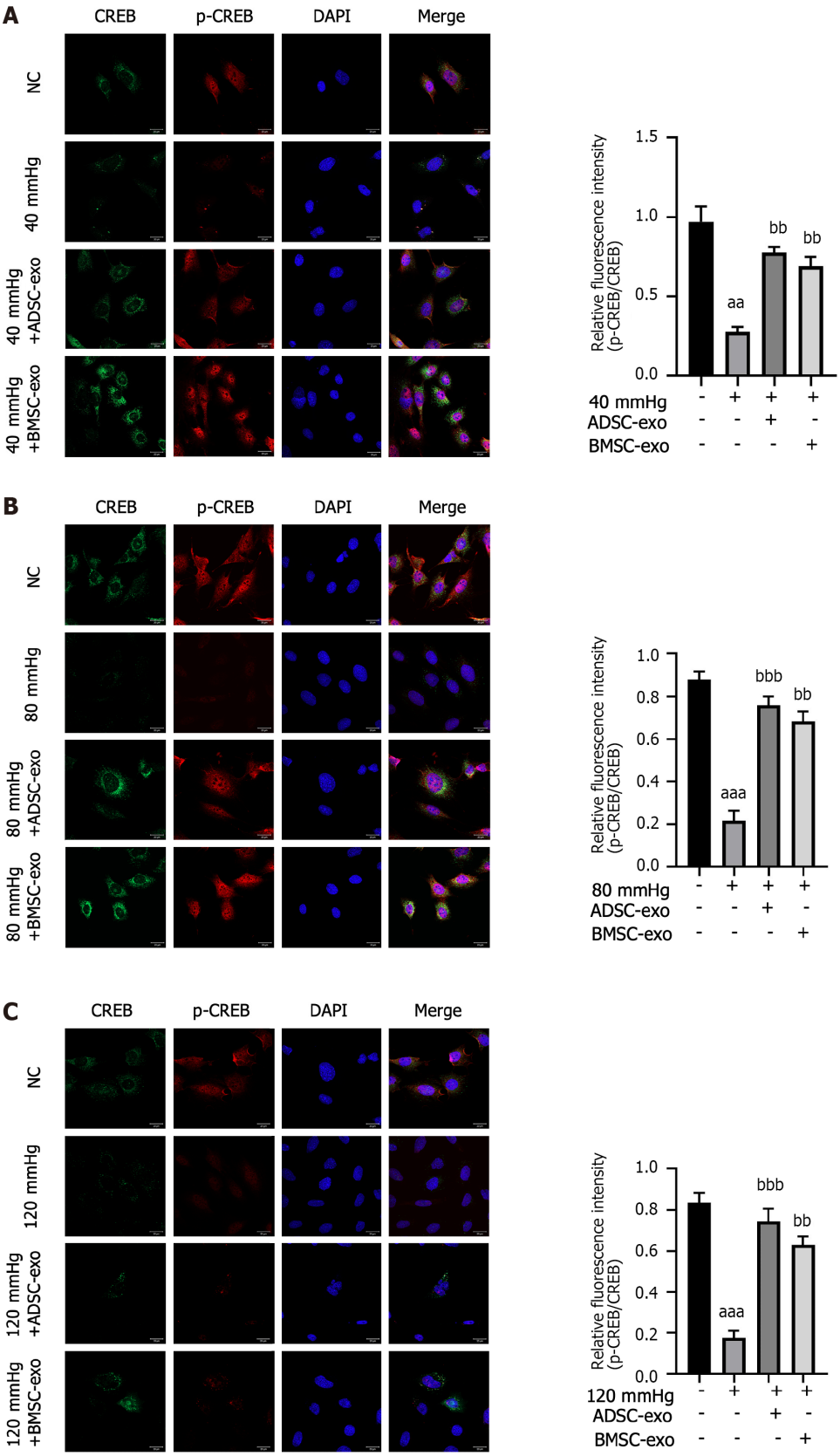

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Figure 3 Immunofluorescence detection of β-III tubulin. A-C: Immunofluorescence staining of β-III tubulin (red) and nuclear staining (DAPI, blue) (left panel) and the number of β-III tubulin-positive retinal ganglion cells (right panel) in different groups after exposure to 40 mmHg (A), 80 mmHg (B), and 120 mmHg (C). [a]*P* < 0.05, compared with the normal control group (scale bar: 50 μm); [b]*P* < 0.05, compared with model (40, 80, and 120 mmHg) group. ADSC: Adipose-derived stem cell; BMSC: Bone marrow-derived stem cell; Exo: Exosome; NC: Normal control; CREB: Cyclic adenosine monophosphate response element-binding protein.

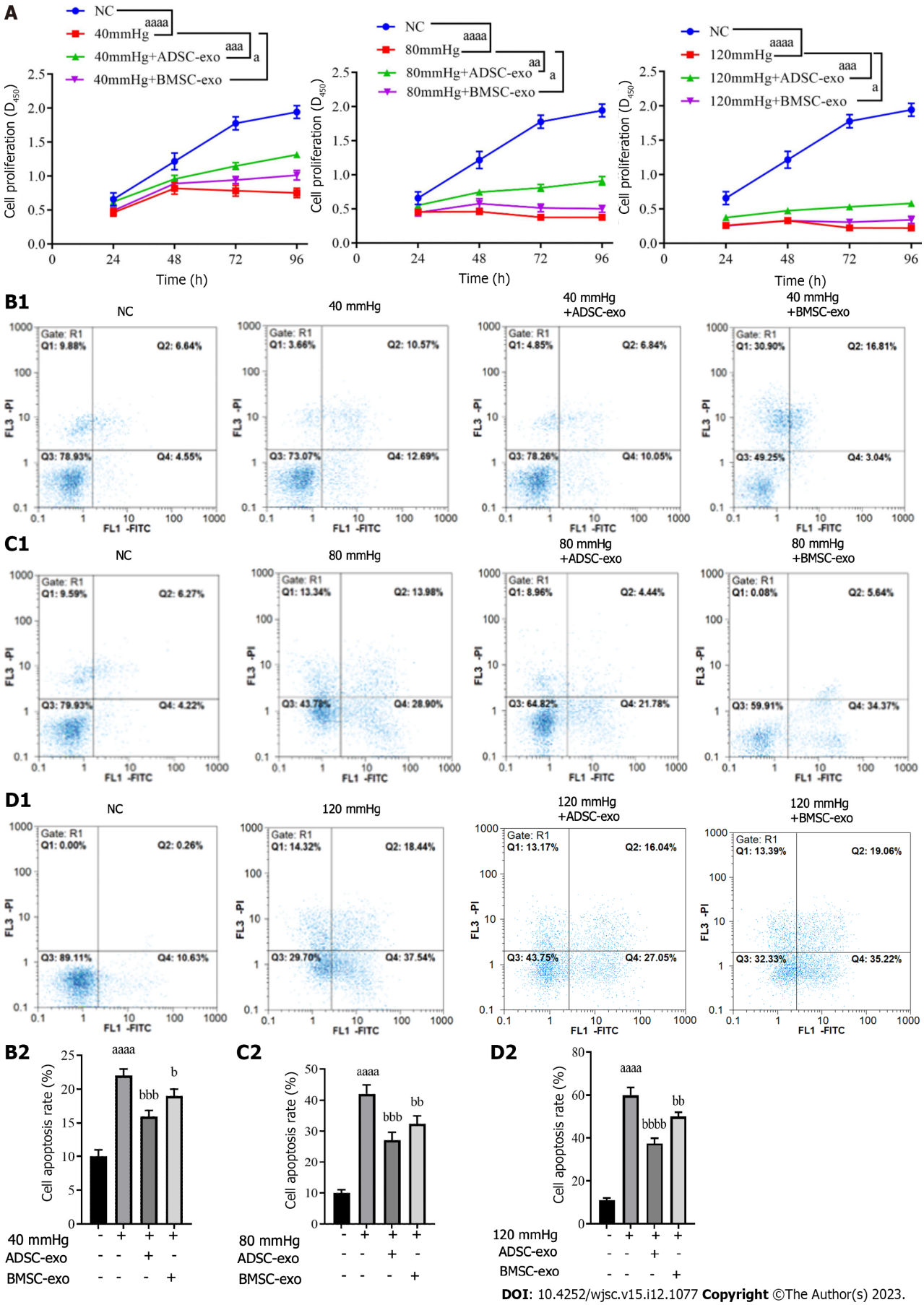

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Figure 4 Retinal ganglion cells proliferation and apoptosis. A: Cell proliferation was detected by using the Cell Counting Kit-8 proliferation reagent; B-D: Cell apoptosis was detected by flow cytometry for apoptosis detection, and representative flow cytometry density plots (left) and statistical bar chart (right). $^{a}P < 0.05$,

compared with the normal control group; [b]*P* < 0.05, compared with model (40, 80, and 120 mmHg) group. ADSC: Adipose-derived stem cell; BMSC: Bone marrow-derived stem cell; Exo: Exosome; NC: Normal control.

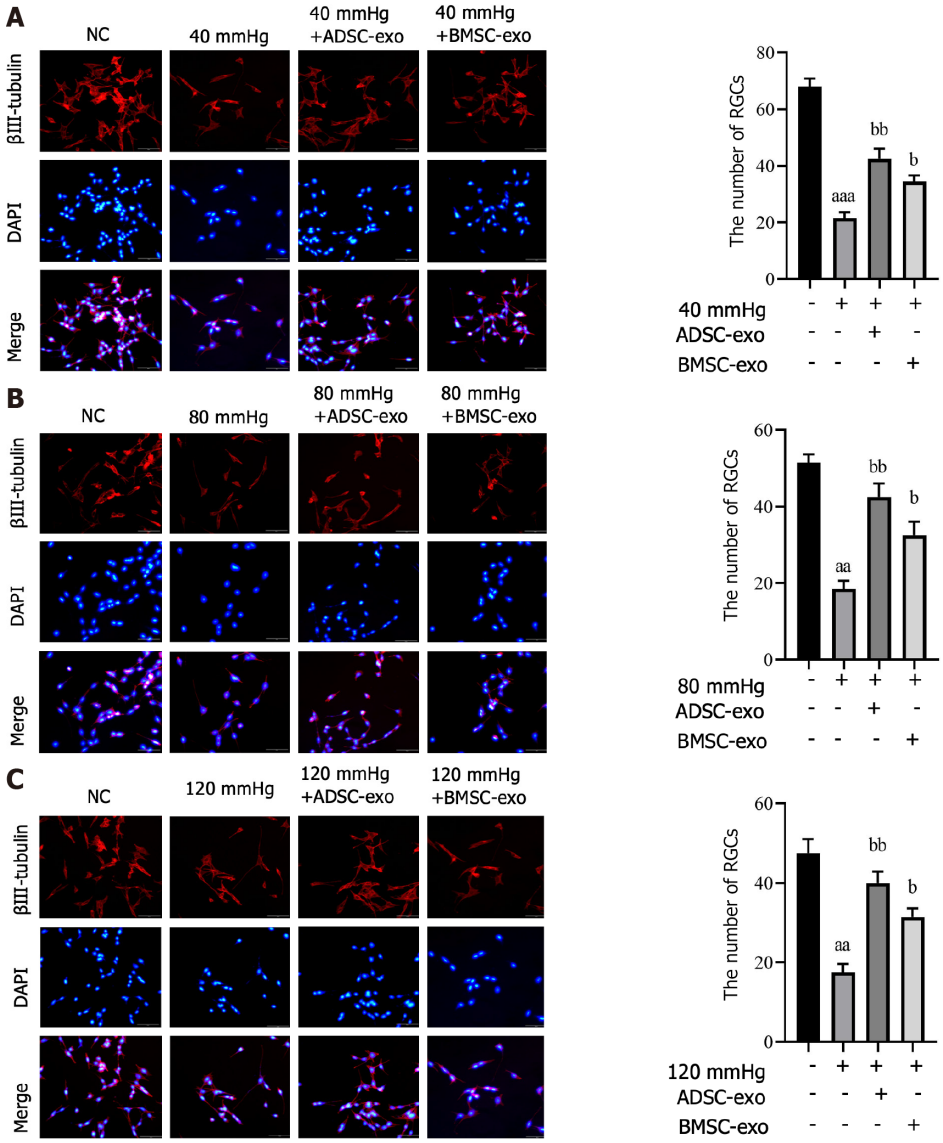

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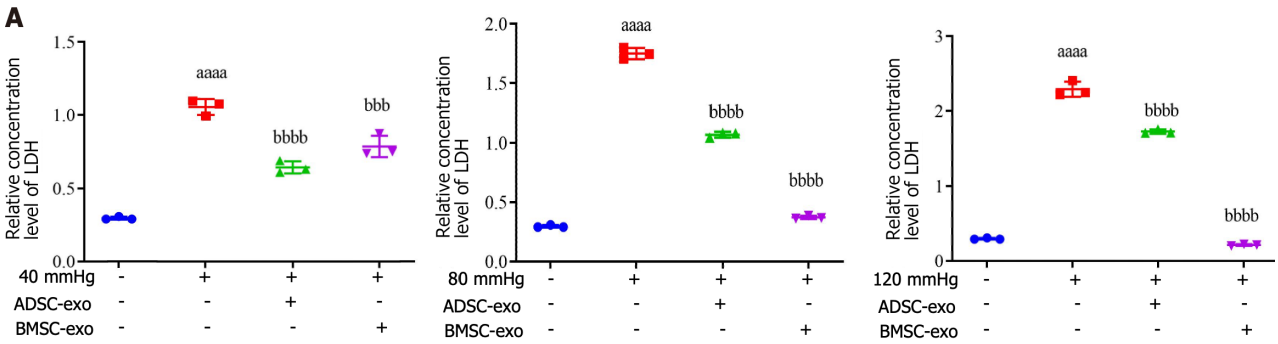
Figure 5 Immunofluorescence detection of p-CREB and CREB. A-C: Immunofluorescence staining of CREB (green), p-CREB (red) and nuclear staining (DAPI, blue) (left panel) and quantification of the ratio of p-CREB/CREB (right panel) in different groups after exposure to (A) 40 mmHg, (B) 80 mmHg, and (C) 120 mmHg (Scale bar: 20 μm). [a]*P* < 0.05, compared with the normal control group; [b]*P* < 0.05, compared with the model (40, 80, and 120 mmHg) group. ADSC: Adipose-derived stem cell; BMSC: Bone marrow-derived stem cell; Exo: Exosome; NC: Normal control.

optic neuropathy rat models[45,46]. Therefore, exos derived from stem cells are emerging as a promising method for glaucoma treatment.

This study elucidated that BMSC-Exos significantly reversed RGC damage induced by hydrostatic pressure in a RGC injury model *in vitro*. The protective effect was mainly reflected in the fact that ADSC-Exos and BMSC-Exos promoted the proliferation of RGCs, the regeneration of RGC axons, and the secretion of RGC neurotrophic factors while inhibiting RGC apoptosis. We emphasized its potential in the prevention or treatment of glaucomatous optic neuropathy. Our study confirmed previous findings that MSC-Exos have an ameliorating effect against RGC damage in some optic neuropathies. BMSC-Exos have been found to increase RGC survival and promote axon regeneration. This ameliorating effect of BMSC-Exos was also observed in a genetic modification DBA/2J glaucoma mouse model[23]. Furthermore, in rats with optic nerve compression, Pan *et al*[47] found that umbilical cord-MSC-Exos enhanced RGC survival. Yu *et al*[48] confirmed that MSC-derived EVs can help alleviate optic nerve injury caused by chronic ocular hypertension, and this effect is achieved by inhibiting cell apoptosis. In addition, we discovered for the first time that ADSC-Exos significantly reversed high-pressure-induced RGC damage. It was reported that after ADSCs and ADSC-the trabecular meshwork (ADSC-TM) were transplanted into the eyes of infantile mice, the cells integrated into TM tissue expressed TM cell markers, and maintained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BMSC-exo
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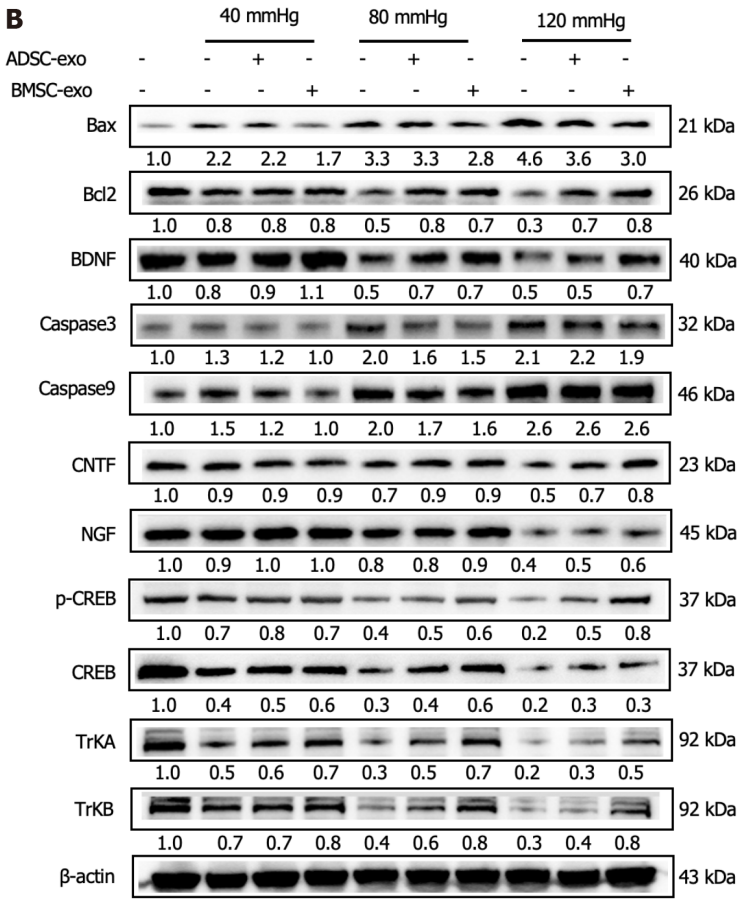
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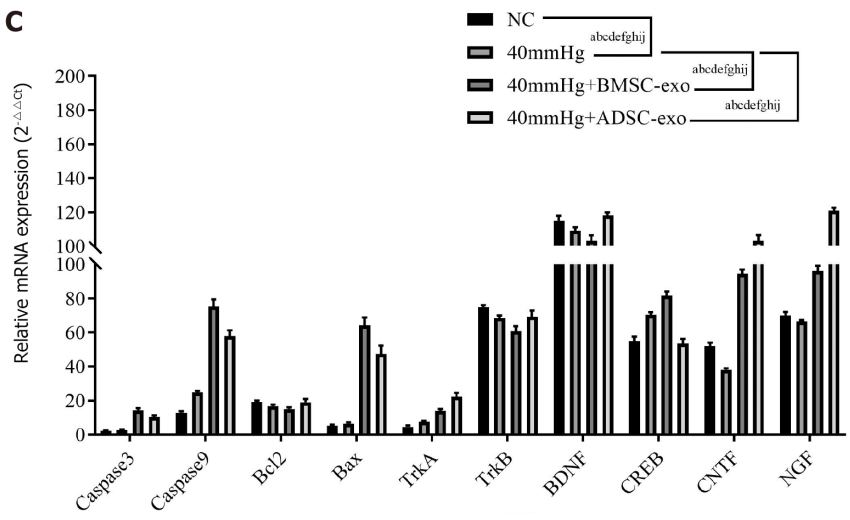
CREB 37 kDa
TrKA 92 kDa
TrKB 92 kDa
β-actin 43 kDa


C
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40mmHg
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Relative mRNA expression (2-△△Ct)
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TrkB
BDNF
CREB
CNTF
NGF

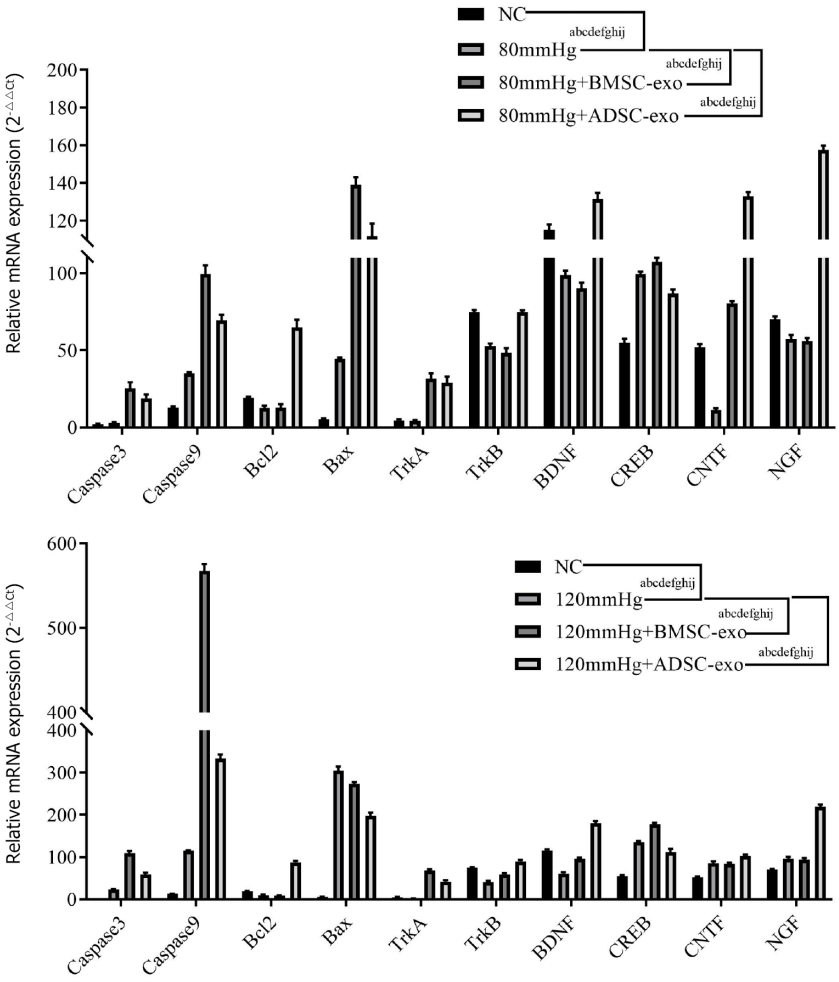

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Figure 6 The expression of lactate dehydrogenase, neurotrophic factor and apoptosis-related protein and mRNA. A: Lactate dehydrogenase (LDH) expression level in each group was measured by the LDH test kit; B: The expression of apoptosis-related proteins and neurotrophic factors were detected by western blotting; C: The mRNA expression of apoptosis-related proteins and neurotrophic factors were detected by real-time quantitative polymerase chain reaction. ^{a}P, ^{b}P, ^{c}P, ^{d}P, ^{e}P, ^{f}P, ^{g}P, ^{h}P, ^{i}P, and $^{j}P < 0.05$, represent the comparison of caspase3, caspase9, B-cell lymphoma 2, Bax, tropomyosin receptor kinase (TrK)A, TrKB, brain-derived neurotrophic factor, cyclic adenosine monophosphate response element-binding protein, ciliary neurotrophic factor, and nerve growth factor proteins between the two groups. ADSC: Adipose-derived stem cell; BMSC: Bone marrow-derived stem cell; Exo: Exosome; NC: Normal control; LDH: Lactate dehydrogenase; Bcl2: polyvinylidene fluoride; Trk: Tropomyosin receptor kinase; BDNF: Brain-derived neurotrophic factor; CREB: Cyclic adenosine monophosphate response element-binding protein; CNTF: Ciliary neurotrophic factor; NGF: Nerve growth factor.

normal IOP, outflow facility, and extracellular matrix[49]. The results demonstrated the possibility of applying autologous or allogeneic ADSC and ADSC-TM cells as a potential therapeutic approach to restore the structure and function of the TM in glaucoma. Therefore, combined with literature reports and our study, ADSC-Exos are expected to be an adjunct therapeutic agent for glaucoma in the future.

Surprisingly, ADSC-Exos manifested better protective effects than BMSC-Exos in the present study. There is evidence that ADSCs are most conducive to clinical utilization. In addition, adipose tissue is relatively abundant in the human body compared with other tissues. ADSCs can be isolated from adipose tissue. In addition, Kern *et al*[50], through comparative analysis of MSCs from bone marrow, umbilical cord blood, or adipose tissue, concluded that 500 times more stem cells were obtained from adipose tissue than from the same amount of bone marrow. Moreover, ADSCs are easier to obtain from adipose tissue due to their subcutaneous location than BMSCs. Patients tend to choose less traumatic sites for collecting tissue. Significant discrepancies are observed in cellular exos from different sources, including the type and content of mRNAs, miRNAs, and proteins[51]. Importantly, ADSCs have a higher proliferation capacity than BMSCs[52]. Moreover, ADSC-Exos possess the advantages of ADSCs. Hence, ADSC-Exos seem to be more favorable than BMSC-Exos because they are easily obtained and have a high proliferation capacity. ADSC-Exos could also be considered for the treatment of glaucoma. The graphic abstract is shown in Figure 7.

Some limitations should be stated. First, we lacked the comparison of neurotrophic factors and miRNAs between ADSC-Exos and BMSC-Exos by bioinformatics and other basic biological methods. Second, the effects of ADSC-Exos and BMSC-Exos on RGCs should be further validated by an *in vivo* experiment. Last but not least, more *in vivo* and *in vitro* experiments are needed to provide theoretical evidence for that MSC-Exos protect RGCs.

CONCLUSION

These findings indicated that ADSC-Exos and BMSC-Exos could ameliorate RGC injury caused by hydrostatic pressure by inhibiting apoptosis and increasing the secretion of neurotrophic factors.

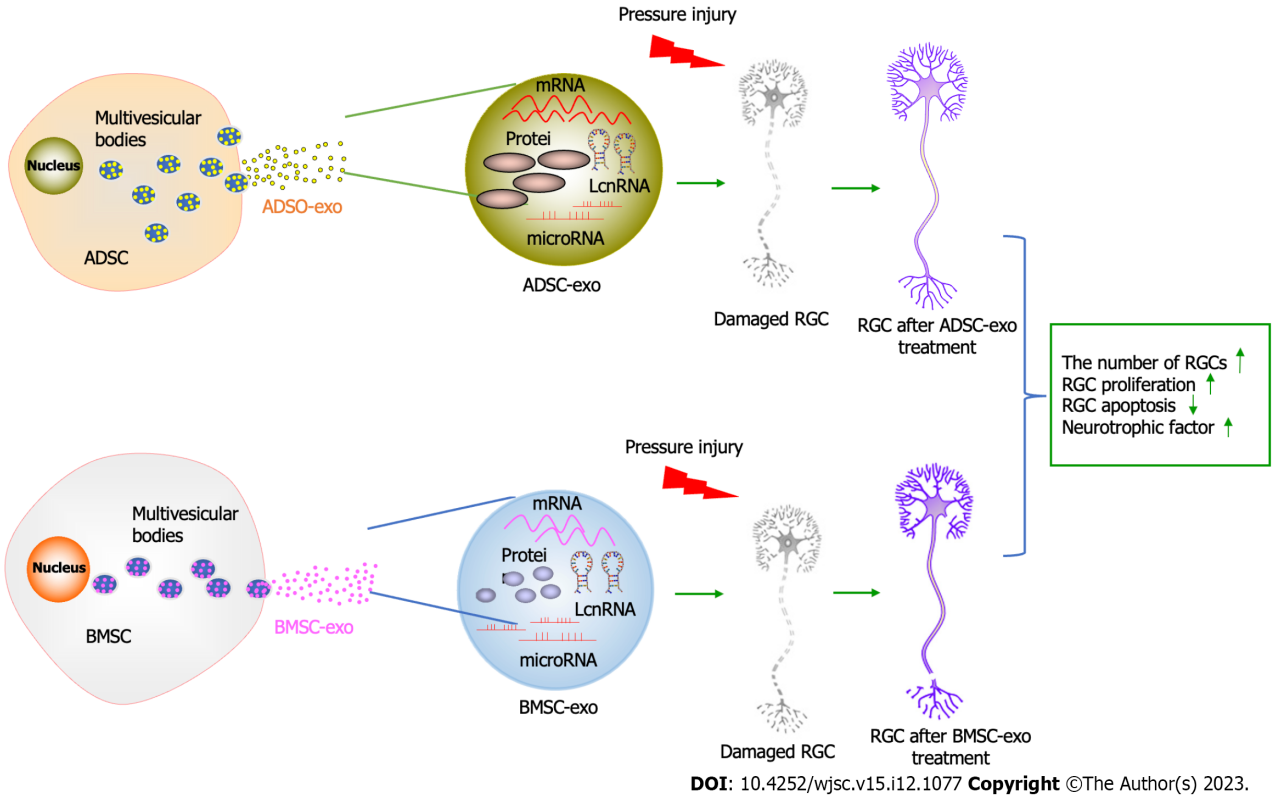

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Figure 7 Graphical abstract. ADSC: Adipose-derived stem cell; BMSC: Bone marrow-derived stem cell; LncRNA: Long non-coding RNA; RGC: Retinal ganglion cell; Exo: Eexosome.

ARTICLE HIGHLIGHTS

Research background

Mesenchymal stem cells (MSCs) have a protective effect against damage to the cornea, lacrimal gland, retina and photoreceptor cells, and this protective effect can be mediated through exosomes (exos) released by MSCs.

Research motivation

Experimental pressure conditions were selected to be relevant to intraocular pressures seen in clinical settings, with levels of 100 mmHg analogous to acute glaucoma, 30 mmHg for chronic glaucoma and 15 mmHg for the so-called 'normal' intraocular pressure. Bone marrow-derived stem cell (BMSC)-Exos have an ameliorative effect on glaucoma. However, the effect of adipose-derived stem cell (ADSC)-Exos which is the ideal source of exos on optic neural degeneration has not been studied yet.

Research objectives

This study investigated the ameliorating effect of exos derived from different MSCs on retinal ganglion cell (RGC) injury induced by hydrostatic pressure.

Research methods

The RGC injury model was constructed by RGC damage under different hydrostatic pressures (40, 80, 120 mmHg). Then RGCs were cultured with ADSC-Exos and BMSC-Exos. Cell Counting Kit-8, transmission electron microscopy, flow cytometry, immunofluorescence, real-time quantitative polymerase chain reaction, and western blotting were performed to detect the ameliorating effect of exos on pressure-induced RGC injury.

Research results

ADSC-Exos and BMSC-Exos were successfully separated. RGCs were in the shape of irregular ellipsoids, and the addition of ADSC-Exos and BMSC-Exos significantly restored the morphology of RGCs. In addition, the proliferative activity of

RGCs was increased and cell apoptosis was inhibited. Increased levels of lactate dehydrogenase and apoptosis-related proteins, and increased concentrations of anti-apoptotic proteins and neurotrophic substances.

Research conclusions

The results indicate that ADSC-Exos and BMSC-Exos can improve hydrostatic pressure-induced RGC damage by inhibiting apoptosis and increasing neurotrophic factor secretion.

Research perspectives

The results of this study will be verified by the experiment *in vivo* in the future.

FOOTNOTES

Co-first authors: Zhi-Kun Zheng and Lei Kong.

Author contributions: Zheng ZK and Kong L designed the study; Zheng ZK, Kong L, and Dai M acquired and analyzed data; Dai M performed the experiments; Chen YD interpreted the data; Zheng ZK, Kong L, and Chen YH wrote the manuscript; and all authors approved the final version of the article.

Supported by Yunnan Provincial High-level Talent Cultivation Support Program for Young Top-notch Talents, No. YNWR-QNBJ-2020-237; Yunnan Natural Science Foundation, No. 202301AT070190; and Kunming Medical University Joint Special Fund, No. 202301AY070001-222.

Institutional animal care and use committee statement: The study was reviewed and approved by the Laboratory Animal Welfare Ethics Committee Yunnan University (Approval No. YNU20230463).

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

Country/Territory of origin: China

ORCID number: Min Dai 0000-0002-5203-5585.

S-Editor: Wang JJ
L-Editor: A
P-Editor: Zhang XD

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World Journal of Stem Cells

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World J Stem Cells 2023 December 26; 15(12): 1093-1103

DOI: 10.4252/wjsc.v15.i12.1093

ISSN 1948-0210 (online)

ORIGINAL ARTICLE

Basic Study

Human mesenchymal stem cells exhibit altered mitochondrial dynamics and poor survival in high glucose microenvironment

Ejlal Abu-El-Rub, Fatimah Almahasneh, Ramada R Khasawneh, Ayman Alzu'bi, Doaa Ghorab, Rawan Almazari, Huthaifa Magableh, Ahmad Sanajleh, Haitham Shlool, Mohammad Mazari, Noor S Bader, Joud Al-Momani

Specialty type: Cell and tissue engineering

Provenance and peer review: Invited article; Externally peer reviewed.

Peer-review model: Single blind

Peer-review report's scientific quality classification

Grade A (Excellent): 0
Grade B (Very good): B
Grade C (Good): C, C, C
Grade D (Fair): 0
Grade E (Poor): 0

P-Reviewer: Luo ZW, China; Shen Y, China; Tanabe S, Japan

Received: September 28, 2023
Peer-review started: September 28, 2023
First decision: October 23, 2023
Revised: November 11, 2023
Accepted: November 24, 2023
Article in press: November 24, 2023
Published online: December 26, 2023

Ejlal Abu-El-Rub, Fatimah Almahasneh, Ramada R Khasawneh, Ayman Alzu'bi, Rawan Almazari, Huthaifa Magableh, Ahmad Sanajleh, Haitham Shlool, Mohammad Mazari, Department of Basic Medical Sciences, Faculty of Medicine, Yarmouk University, Irbid 21163, Jordan

Doaa Ghorab, Department of Pathology, Faculty of Medicine, Mansoura University, Mansoura 35516, Egypt

Noor S Bader, Joud Al-Momani, Department of Basic Medical Sciences, Yarmouk University, Irbid 21163, Jordan

Corresponding author: Ejlal Abu-El-Rub, PhD, Assistant Professor, Department of Basic Medical Sciences, Faculty of Medicine, Yarmouk University, Irbid-Shafiq Irshidat Street, Irbid 21163, Jordan. ejlal.abuelrub@yu.edu.jo

Abstract

BACKGROUND

Mesenchymal stem cells (MSCs) are a type of stem cells that possess relevant regenerative abilities and can be used to treat many chronic diseases. Diabetes mellitus (DM) is a frequently diagnosed chronic disease characterized by hyperglycemia which initiates many multisystem complications in the long-run. DM patients can benefit from MSCs transplantation to curb down the pathological consequences associated with hyperglycemia persistence and restore the function of damaged tissues. MSCs therapeutic outcomes are found to last for short period of time and ultimately these regenerative cells are eradicated and died in DM disease model.

AIM

To investigate the impact of high glucose or hyperglycemia on the cellular and molecular characteristics of MSCs.

METHODS

Human adipose tissue-derived MSCs (hAD-MSCs) were seeded in low (5.6 mmol/L of glucose) and high glucose (25 mmol/L of glucose) for 7 d. Cytotoxicity, viability, mitochondrial dynamics, and apoptosis were deplored using specific kits. Western blotting was performed to measure the protein

expression of phosphatidylinositol 3-kinase (PI3K), TSC1, and mammalian target of rapamycin (mTOR) in these cells.

RESULTS

hAD-MSCs cultured in high glucose for 7 d demonstrated marked decrease in their viability, as shown by a significant increase in lactate dehydrogenase ($P < 0.01$) and a significant decrease in Trypan blue ($P < 0.05$) in these cells compared to low glucose control. Mitochondrial membrane potential, indicated by tetramethylrhodamine ethyl ester (TMRE) fluorescence intensity, and nicotinamide adenine dinucleotide (NAD+)/NADH ratio were significantly dropped ($P < 0.05$ for TMRE and $P < 0.01$ for NAD+/NADH) in high glucose exposed hAD-MSCs, indicating disturbed mitochondrial function. PI3K protein expression significantly decreased in high glucose culture MSCs ($P < 0.05$ compared to low glucose) and it was coupled with significant upregulation in TSC1 ($P < 0.05$) and downregulation in mTOR protein expression ($P < 0.05$). Mitochondrial complexes I, IV, and V were downregulated profoundly in high glucose ($P < 0.05$ compared to low glucose). Apoptosis was induced as a result of mitochondrial impairment and explained the poor survival of MSCs in high glucose.

CONCLUSION

High glucose impaired the mitochondrial dynamics and regulatory proteins in hAD-MSCs ensuing their poor survival and high apoptosis rate in hyperglycemic microenvironment.

Key Words: Mesenchymal stem cells; High glucose; Mitochondrial dynamics; Apoptosis; Poor survival; Phosphatidylinositol 3-kinase/mammalian target of rapamycin pathway

Core Tip: Mesenchymal stem cells (MSCs) have significant regenerative properties that make them a potential treatment for many chronic diseases. Among these, diabetes mellitus (DM) was found to benefit from MSCs transplantation in which they restored the damaged tissues and prevented hyperglycemia-related complications. However, these therapeutic are short-lived hindering the clinical use of MSCs in the treatment of DM. This study aims to elucidate the mechanisms of hyperglycemia-induced effects on MSCs which will help in improving the therapeutic functions of these cells in this stress environment.

Citation: Abu-El-Rub E, Almahasneh F, Khasawneh RR, Alzu'bi A, Ghorab D, Almazari R, Magableh H, Sanajleh A, Shlool H, Mazari M, Bader NS, Al-Momani J. Human mesenchymal stem cells exhibit altered mitochondrial dynamics and poor survival in high glucose microenvironment. *World J Stem Cells* 2023; 15(12): 1093-1103
URL: https://www.wjgnet.com/1948-0210/full/v15/i12/1093.htm
DOI: https://dx.doi.org/10.4252/wjsc.v15.i12.1093

INTRODUCTION

Mesenchymal stem cells (MSCs) are the most commonly studied forms of stem cells as they possess powerful regenerative and immunomodulatory abilities[1]. MSCs have unique molecular machinery that empowers them to modulate many cellular signaling pathways and restore the functionality of damaged tissues rendering them suitable to treat many acute and chronic diseases[1]. Despite their unprecedented abilities to adapt to many stress conditions, they can still behave unexpectedly under certain stress microenvironments. Many preclinical and clinical studies not only demonstrated the positive outcomes after transplanting MSCs to injured sites[2,3], but also reported the short-lived therapeutic results in hostile microenvironments which need to be figured out[4]. When MSCs are undifferentiated, they rely mainly on glycolysis to support their proliferation, expansion, and immunomodulation[5]. When MSCs are programmed to differentiate, they switch to oxidative phosphorylation (OxPhos)[6], thereby, intact mitochondria are also important to maintain their multi-lineage differentiation capacities[6].

In the last decades, diabetes mellitus (DM) incidence has increased rapidly, and it is deemed among top five chronic diseases[7]. DM is associated with serious multisystem complications that impair the life quality of many patients[8]. DM is characterized mainly by chronic hyperglycemic state that causes many devastating, long-term complications[8]. DM patients can benefit remarkably from MSCs therapy to repair and regenerate damaged tissues, restore their functionality, and reduce the severity of DM-related pathological complications[7]. Several studies reported a significant improvement in glycemic parameters and cardiovascular complications after transplanting MSCs in DM patients[7,9,10]; however, other studies reported that DM can accelerate the decline in MSCs quantity and therapeutic quality, and impair their regenerative capabilities[11]. When MSCs turn into a dysfunctioning cells, they may increase the severity of DM-related complications[11]. Although previous studies had addressed the effects of hyperglycemia on the molecular and cellular characteristics of transplanted MSCs, many aspects still need to be elucidated. Thus, it is important to investigate the effect of high glucose on MSCs biological dynamics, which are essential to understand their fate and performance in

diabetic microenvironment, and provide new avenues on possible molecular targets to modulate their survival and therapeutic outcomes in DM patients.

MATERIALS AND METHODS

Human MSCs

Human adipose tissue-derived MSCs (hAD-MSCs) were commercially purchased from Lonza (Cat# PT5006, Lot# 21TL138912) and expanded using Dulbecco's Modified Eagle's Medium Low Glucose (DMEM-Low Glucose, Euroclone) which contained 5.6 mmol/L glucose and were supplemented with 10% fetal bovine serum (FBS, Gibco)[12], 0.1 mg/mL streptomycin and 100 units/mL penicillin G in standard cell culture incubators (5% CO_2/95% air; 37 °C). Medium was changed every 72 h, and cells were sub-cultured when confluence exceeded 60%[13]. For high glucose conditions, cells were cultured in Dulbecco's Modified Eagle's Medium High Glucose (DMEM-High Glucose, Euroclone) which contained 25 mmol/L glucose[14] and were supplemented with 10% FBS, 0.1 mg/mL streptomycin and 100 units/mL penicillin G for 3, 7, and 14 d in standard cell culture incubators (5% CO_2/95% air; 37 °C). High glucose complete medium was changed every 72 h. MSCs that were cultured in low glucose were considered as our study control. MSCs of passage 5 and 6 were used to perform the experiments. AD-MSCs characterization markers can be found in (Table 1).

Cytotoxicity assays

To measure the level of cytotoxicity in human MSCs after being cultured in low and high glucose, we seeded 5 × 10^4 cells/well in 96 well plate and we measured the lactate dehydrogenase (LDH) which was released from the damaged MSCs (LDH Assay Kit, Abcam, Cat# ab102526) and the absorbance values were obtained using Cytation 5 (BioTek, United States). The viability of MSCs in low and high glucose were also assessed using 0.4% Trypan blue and the percentage of viable cells as well as representative fluorescent images were obtained using Corning® CytoSmart Cell Counter.

Tetramethylrhodamine ethyl ester (MtMP measurement)

Mitochondrial membrane potential was assessed using tetramethylrhodamine ethyl ester (TMRE)-Mitochondrial Membrane Potential Assay Kit (Abcam, Cat # ab113852). Briefly, human AD-MSCs were placed in 24-well plate at 1 × 10^4 cells per well and allowed to adhere overnight. After that, cells were cultured either in low glucose or high glucose for 7 d. Then, media were aspirated, and cells were stained using 400 nM TMRE in culture media for 30 min in the incubator, and then media were replaced with 200 µL phosphate buffered saline (PBS) per well. Fluorescence intensity was detected using Cytation 5 (BioTek, United States). (λex = 549 nm, λem = 575 nm), and TMRE fluorescent images were captured at Texas red filter using Cytation 5 (BioTek, United States).

Nicotinamide adenine dinucleotide /NADH assay

Nicotinamide adenine dinucleotide (NAD+)/NADH ratio in AD-MSCs that were cultured in low and high glucose media was assessed using NAD/NADH-Glo™ assay kit (Promega, Cat# G9071). Briefly, cells were cultured in low and high glucose media for 7 d. After that, cells were detached and placed in 96 well plate at 5 × 10^5 in 50 µL of either low or high glucose media and were allowed to adhere overnight. 50 µL of NAD/NADH-Glo™ Detection Reagent was added to each well and the plate was gently shaken to mix and lyse the cells and was incubated with the added reagent for 60 min at room temperature. The luminescence values were recorded using Cytation 5 (BioTek, United States) which were corresponding to the total amount of NAD+ produced from NADH.

Western blotting

The protein levels for phosphatidylinositol 3-kinase (PI3K) (Santa Cruz Biotechnology Cat # sc-1637), TSC1 (Santa Cruz Biotechnology Cat # sc-377386), and mammalian target of rapamycin (mTOR) (Santa Cruz Biotechnology Cat # sc-293133), NDUFB8 (Abcam, Cat # ab192878), SDHB (Santa Cruz Biotechnology Cat # sc-271548), UQCRC2 (Santa Cruz Biotechnology Cat # sc-390378), COX5a (Santa Cruz Biotechnology Cat # sc-376907), ATP5A (Santa Cruz Biotechnology Cat # sc-136178), and β-actin (Santa Cruz Biotechnology Cat # sc-47778 HRP) were measured by western blot. Briefly, total protein levels were measured using NanoDrop™ Lite Spectrophotometer, and 40 µg of protein was loaded onto sodium-dodecyl sulfate gel electrophoresis. Following electrophoresis, proteins were transferred to polyvinylidene fluoride membrane and were incubated with appropriate primary and secondary antibodies. The membranes were visualized using VILBER FUSION Gel Documentation System, and bands were quantified using ImageJ for densitometry.

Apoptosis assay

To detect apoptosis in hAD-MSCs after being cultured in low and high glucose, we used RealTime-Glo™ Annexin V Apoptosis live assay (Promega, Cat# JA1011, Lot# 0000400486 following the manufacturer's guidelines. Briefly, cells were seeded 1 × 10^4 cells/well in 24 well plate and then cultured in low and high glucose for 7 d. The media were aspirated and each well was washed with PBS followed by the addition of 100 µL of fresh medium having Annexin V-LgBiT, Annexin V-SmBiT, CaCl2, and Annexin V NanoBiT Substrate to each well. After 1 h incubation, the fluorescent images of green color which represented cells undergoing apoptosis were detected at GFP filter using Cytation 5 (BioTek, United States).

Table 1 Cell surface characterization of human adipose tissue-derived mesenchymal stem cells based on the company analysis report

Positive markers	Negative markers
CD13	CD14
CD29	CD31
CD44	CD45
CD73	HLA-DR
CD90	
CD105	
CD166	

Statistical analysis

Data were reported as mean ± SD. Comparison of data between multiple groups was performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test, and analysis between two groups was made using Student's *t*-test (two-tailed). Statistical significance is determined as $P < 0.05$. Each figure represents one of at least three independent quantifiable experiments.

RESULTS

Culturing MSCs in high glucose triggers massive cytotoxicity and poor survival

MSCs are known to survive well in low glucose medium which is a physiological requirement to preserve their stemness features. We want to determine the viability of these cells in high glucose microenvironment. hAD-MSCs were cultured in high glucose medium containing 25 mmol/L of glucose for 3, 7, and 14 d. The cytotoxicity was measured by determining the amount of LDH released and was found to be significantly greater ($P < 0.01$) in hAD-MSCs cultured in high glucose for 7 and 14 d compared with hAD-MSCs cultured in normal low glucose medium containing 5.6 mmol/L of glucose (Figure 1A). To confirm these findings, the viability of hAD-MSCs was measured using 0.4% Trypan blue and found to be remarkably low ($P < 0.05$) in hAD-MSCs that were cultured in high glucose for 7 d compared to hAD-MSCs cultured in a low glucose medium (Figure 1B).

The mitochondrial dynamics of MSCs impaired by high glucose

Mitochondria are integral intracellular organelles promoting cells survival and viability. The variance of mitochondrial dynamics depends on the type of microenvironment where cells exist. Previous reports demonstrated that high glucose can induce the fission and fragmentation of mitochondria in glomeruli podocytes[15], but the knowledge surrounding the impact of high glucose on the mitochondrial dynamics of MSCs is still limited. The mitochondrial membrane potential (ΔΨm) generated by proton pumps (complexes I, III and IV) is imperative to ensure proper ATP production by mitochondrial OxPhos. Abnormalities in the mitochondrial membrane potential (ΔΨm) can significantly elicit a decline in cells viability and trigger unwanted signaling pathways. We measured the mitochondrial membrane potential (ΔΨm) in hAD-MSCs after being grown in a high glucose medium for 7 d using TMRE which is sequestered by active mitochondria. Our results illustrated that the fluorescent intensity of TMRE was significantly low ($P < 0.05$) in hAD-MSCs cultured in a high glucose medium compared to hAD-MSCs cultured in a normal low glucose medium indicating an impairment in the mitochondrial membrane potential in these cells (Figures 2A and B). NAD+ and its reduced form (NADH) regulate many metabolic pathways, including mitochondrial OxPhos. Maintaining NAD+/NADH pool is required to ensure that the influx of electrons is coupled with the translocation of protons to the intermembrane space to generate the required membrane potential (ΔΨm) across the inner mitochondrial membrane to be harnessed by complex V to produce ATP[16]. To investigate if the decrease in the mitochondrial membrane potential (ΔΨm) is correlated with abnormalities in NAD+/NADH pool, we measure the NAD+/NADH ratio in hAD-MSCs cultured in low and high glucose media (Figure 3). The findings revealed a considerable drop ($P < 0.01$) in NAD+/NADH ratio when hAD-MSCs were cultured in high glucose. This may explain the disturbance in the inner mitochondrial potential detected by TMRE assay (Figures 2A and B).

Disturbance of MSCs mitochondria dynamics parameters in high glucose engenders their apoptosis

Apoptosis is a programmed cell death that can be physiological or pathological depending on the triggering factors that enkindle it. Mitochondrial dysfunction is a notable inciting factor which is able to fire up debilitating cellular apoptosis and impact the survival of numerous cells. The noticeable deterioration in the mitochondrial parameters detected in hAD-MSCs after being cultured in high glucose prompted us to examine the level of apoptosis in these cells. To achieve that, we used RealTime-Glo™ Annexin V Apoptosis kit to unveil the presence of apoptosis or not. The luminescence signal which is proportionally linked to apoptosis intensity was considerably higher in hAD-MSCs that were cultured in high glucose (Figure 4). Fluorescent microscopic images showed that apoptosis (marked by green stained cells) was more in

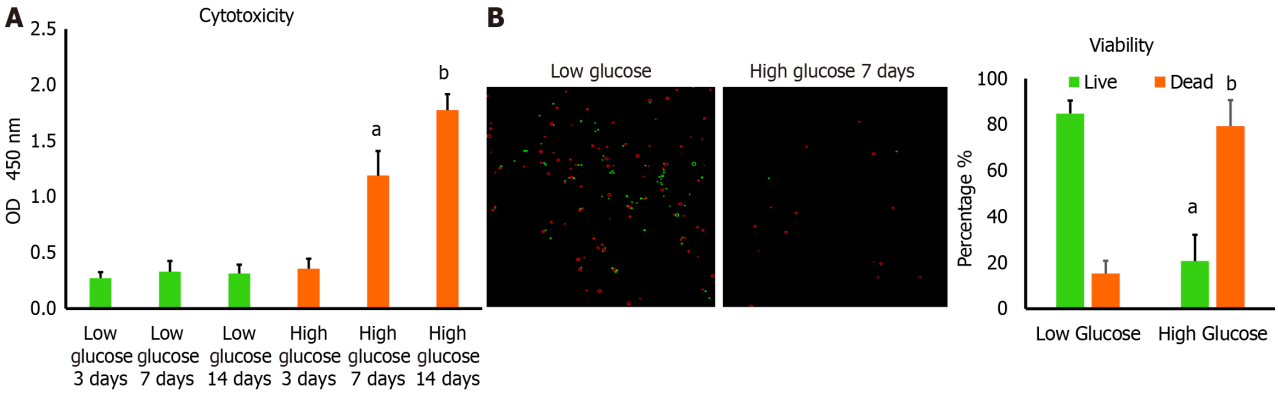

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Figure 1 Culturing human adipose tissue-derived mesenchymal stem cells in high glucose reduced their viability. A: Lactate dehydrogenase cytotoxicity assay revealed higher level of cytotoxicity in human adipose tissue-derived mesenchymal stem cells (hAD-MSCs) cultured in high glucose for 7 and 14 d compared to cells cultured in low glucose. n = 5, ^{a}P < 0.01 compared to low glucose 7 d, ^{b}P < 0.01 compared to low glucose 14 d; B: The percentage of cells viability detected by 0.4% Trypan blue showed significant reduction in the number of viable hAD-MSCs after being cultured in high glucose for 7 d. Fluorescent images of live and dead cells were obtained using Corning® CytoSmart Cell Counter. n = 5. n = 5, ^{a}P < 0.01 compared to live cells in low glucose 7 d, ^{b}P < 0.01 compared to dead cells in low glucose 7 d.

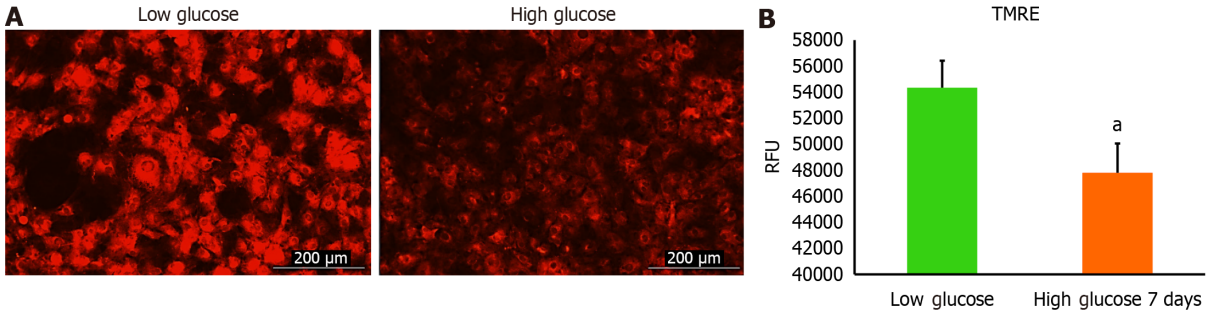

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Figure 2 High glucose decreased the mitochondrial membrane potential level of human adipose tissue-derived mesenchymal stem cells. The tetramethylrhodamine ethyl ester (TMRE) fluorescence intensity values of human adipose tissue-derived mesenchymal stem cells (hAD-MSCs) cultured in high glucose for 7 d were substantially declined compared to fluorescent intensity values of cells cultured in low glucose. Live fluorescent images showed a weak signal of TMRE stain in hAD-MSCs after being placed in high glucose for 7 d compared to strong TMRE signal in low glucose cultured cells. n = 4, ^{a}P < 0.05 compared to low glucose control. Fluroscnet images were captured at Texas Red filter using Cytation 5 (BioTek, United States). TMRE: Tetramethylrhodamine ethyl ester; RFU: Relative fluorescence units.

high glucose cultured hAD-MSCs (Figure 4).

High glucose affects the level of PI3K and mTOR in MSCs

PI3K orchestrates many vital signaling pathways and targets downstream factors that are required to maintain the vivacity of cells. PI3K controls integral proteins that are necessary to preserve mitochondrial dynamics[17,18]. PI3K is a known activator of mTOR which is a central regulator of cell survival and metabolism. It has been reported that mTOR is important to preserve mitochondrial dynamics and generate the required mitochondrial potential to produce ATP. PI3K is required to remove the inhibitory effect of tuberous sclerosis tumor suppressor (TSC1) which binds mTOR and inactivates it[19]. We performed western blotting analysis to measure the expression levels of PI3K, TSC1, and mTOR in high glucose treated hAD-MSCs. Western blotting results demonstrated the loss of PI3K and mTOR in high glucose treated hAD-MSCs (P < 0.05 compared to low glucose), while TSC1 is significantly increased in these cells (P < 0.05 compared to low glucose) (Figure 5). Those findings indicated impaired PI3K/mTOR axis which may explain the disruption in mitochondrial parameters in high glucose cultured MSCs. Taking together, our findings highlight a new mechanism that can be released by high glucose causing the poor survival of MSCs in diabetic microenvironment.

High glucose disturbs major mitochondrial complexes in MSCs

The reduction in mTOR which is essential for inducing the biogenesis of mitochondrial complexes, most importantly complex I and V[20] prompted us to investigate the expression of mitochondrial complexes in low and high glucose-cultured hAD-MSCs. The results demonstrated that hAD-MSCs that were cultured in high glucose exhibited significant downregulation (P < 0.05) in the level of complex I, IV, and V comparing to hAD-MSCs that were cultured in low glucose (Figure 6), while the expression of complex II and III showed no significant changes (P = 0.85) in both culturing

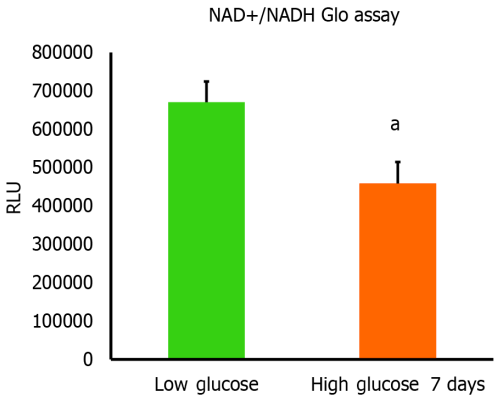

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Figure 3 Nicotinamide adenine dinucleotide and NADH amounts were declined in high glucose cultured human adipose tissue-derived mesenchymal stem cells. The amount of nicotinamide adenine dinucleotide (NAD+) produced from NADH was measured using NAD+/NADH Glo assay. Luminescence intensity was significantly less in human adipose tissue-derived mesenchymal stem cells cultured in high glucose for 7 d compared to cells cultured in low glucose. n = 4, ^{a}P < 0.01 compared to low glucose control. Luminescence values were measured using Cytation 5 (BioTek, United States). RFU: Relative fluorescence units.

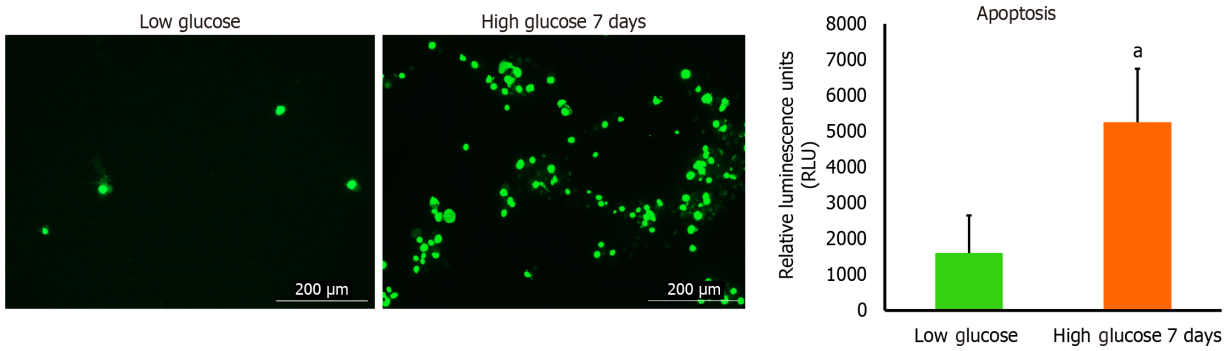

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Figure 4 The level of apoptosis was higher in human adipose tissue-derived mesenchymal stem cells cultured in high glucose. RealTime-Glo™ Annexin V Apoptosis live assay was used. The level of apoptosis was remarkably increased in human adipose tissue-derived mesenchymal stem cells cultured in high glucose for 7 d. Green fluorescence intensity is corresponding to the number of cells undergoing apoptosis. Fluorescent images were captured at GFP filter using Cytation 5 (BioTek, United States). n = 3, ^{a}P < 0.05 compared to low glucose control.

conditions (Figure 6). The disturbance in the level of these major complexes can impact the mitochondrial OxPhos and trigger the mitophagy of mitochondria[21].

DISCUSSION

MSCs are the most promising type of stem cells for regenerating damaged tissues and restoring their normal functions which can improve the quality of life of many patients who suffer from debilitating disorders[1]. However, the remarkable improvements in pathological markers reported after transplanting MSCs[22,23] were hindered by the short-lived outcomes and poor survival rate, which need extensive investigation[24,25]. Many pathologies are characterized by stress microenvironments, including hypoxia, inflammation, and nutritional imbalances, which can impact the expected outcomes of transplanting MSCs[26-29], by either inhibiting or activating cellular signaling pathways. DM is a frequent chronic disease that leads to significant multi-system complications[8]. The major hallmark of DM is glucotoxicity, which is featured by the presence of high glucose levels and impaired insulin secretion and function[8]. This glucotoxicity produces massive molecular changes in the residing cells and cause the appearance of DM-related complications, such as cardiovascular and neurological complications[8]. Both animal and clinical studies have demonstrated the great therapeutic efficacy of MSCs transplantation in alleviating chronic hyperglycemia by reversing insulin resistance, improving insulin sensitivity, controlling inflammation, relieving metabolic syndrome symptoms, and ameliorating β-cell destructive abilities[10,30]. Nevertheless, studies also revealed that MSCs exhibited short-lived outcomes due to their poor survival after being transplanted in DM disease model[4]. Several studies have asserted the importance of investigating the mechanisms leading to the poor survival of MSCs in hyperglycemia which is the major insult to DM patients [24,31]. Up to date little is known about the molecular and biological changes in MSCs under hyperglycemia. MSCs are

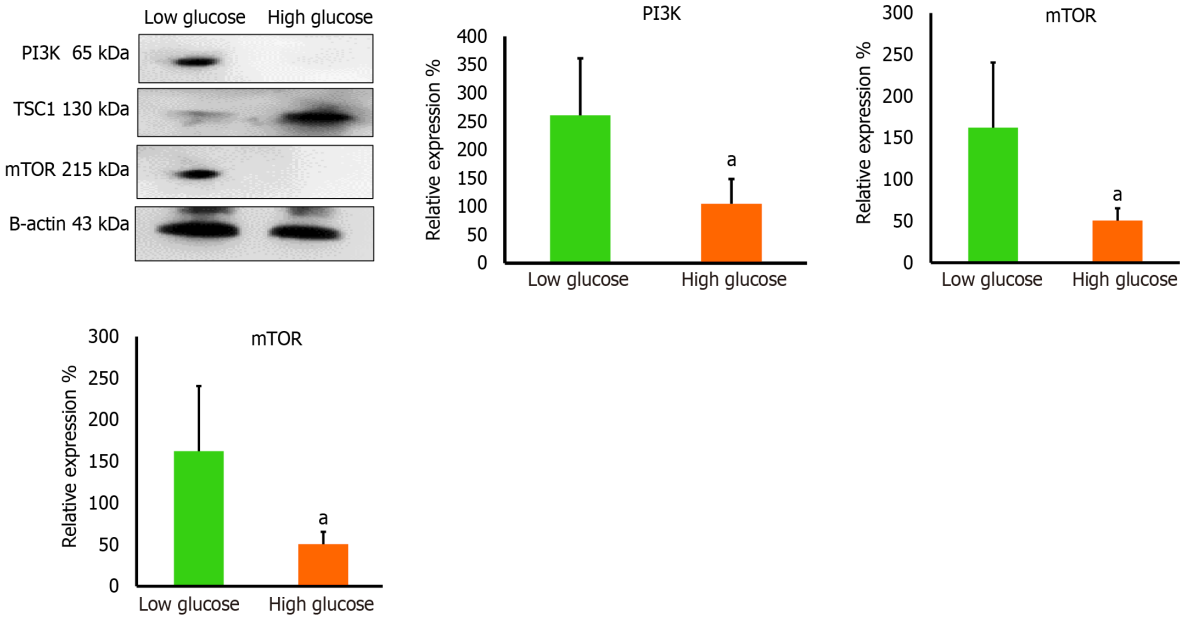

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Figure 5 The protein levels of mitochondrial regulators were reduced in high glucose cultured human adipose tissue-derived mesenchymal stem cells. Western blot protein analysis showed significant reduction in the protein levels of phosphatidylinositol 3-kinase and mammalian target of rapamycin (mTOR) in high glucose cultured cells, while the protein level of mTOR inhibitory protein; TSC1, was elevated in high glucose cultured cells. These proteins are important to maintain proper mitochondrial dynamics. $n = 3$, $^{a}P < 0.05$ compared to low glucose control. PI3K: Phosphatidylinositol 3-kinase; mTOR: Mammalian target of rapamycin.

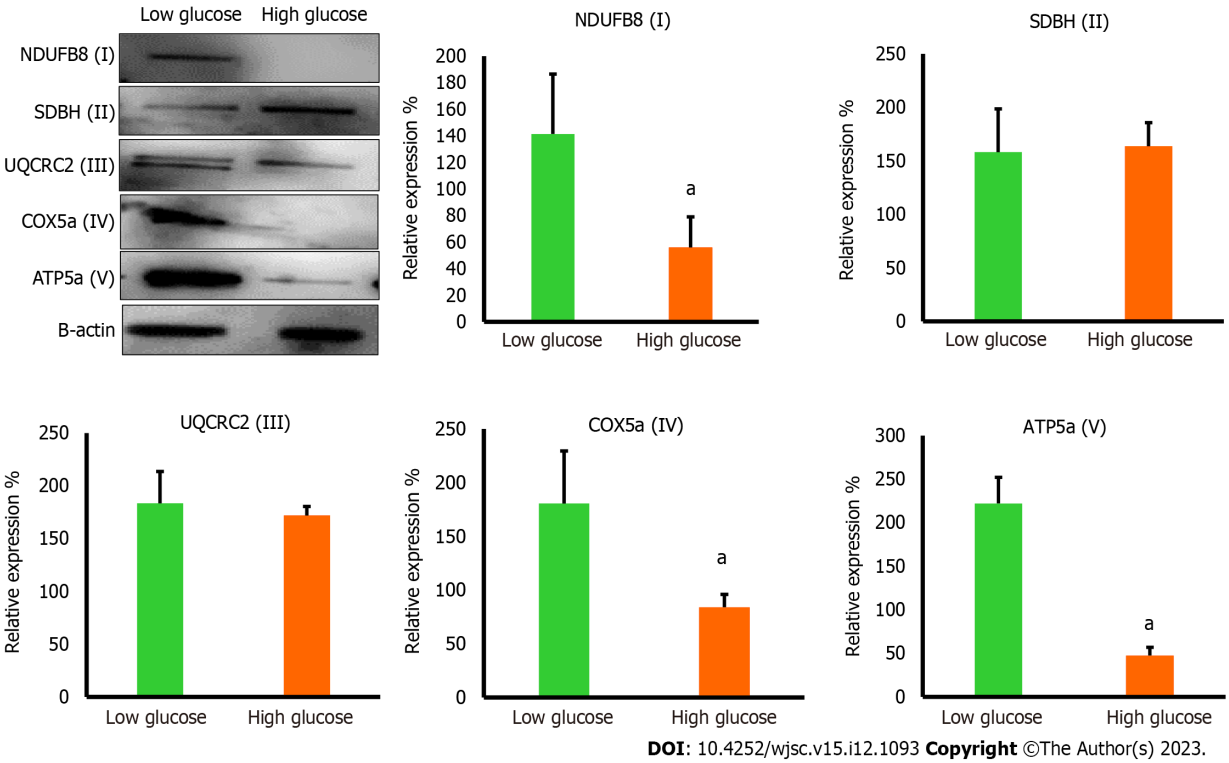

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Figure 6 The protein levels of mitochondrial complexes were reduced in high glucose cultured human adipose tissue-derived mesenchymal stem cells. Western blot protein analysis showed significant reduction in the protein levels of NDUF6B (complex I), COX5a (complex IV), and ATP5a (complex V) in high glucose cultured human adipose tissue-derived mesenchymal stem cells comparing to low glucose cultured cells, while the protein levels of SDBH (complex II) and UQCRC2 (complex III) were not significantly changed in both culturing conditions. $n = 3$, $^{a}P < 0.05$ compared to low glucose control.

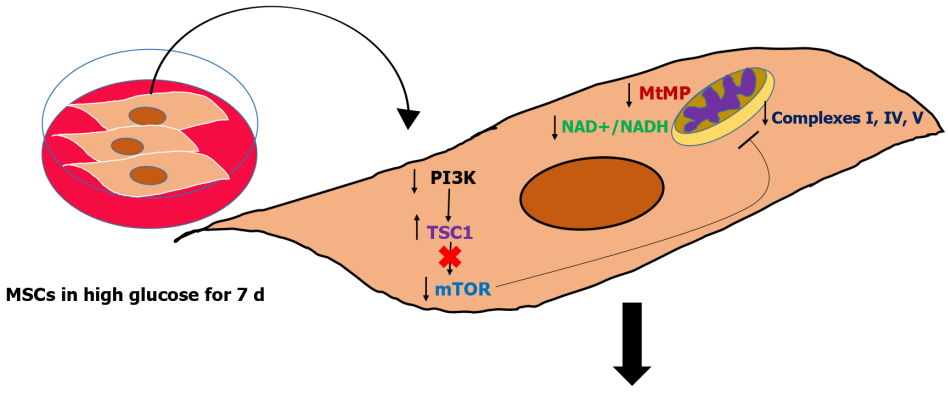

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Figure 7 Graphical summary of the study findings. High glucose impaired the mitochondrial function in mesenchymal stem cells (MSCs) by perturbing many mitochondrial regulatory dynamics and factors, particularly mitochondrial membrane potential, nicotinamide adenine dinucleotide (NAD+)/NADH pool, and mammalian target of rapamycin protein. This mitochondrial dysfunction is associated with triggering apoptosis in MSCs, which mechanistically explained the poor survival of MSCs in hyperglycemia. MSCs: Mesenchymal stem cells; PI3K: Phosphatidylinositol 3-kinase; mTOR: Mammalian target of rapamycin; NAD+: Nicotinamide adenine dinucleotide.

capable of switching their metabolism on to support their regenerative and immunomodulation abilities[32]. MSCs rely primarily on glycolysis when they are undifferentiated as it is important to preserve their stemness and proliferation[33]. Several reports illustrated that glycolysis is required to preserve the immunosuppressive characteristics of MSCs[34]. Upregulation in glycolysis has been found to increase the immunomodulatory abilities of MSCs and promote their capabilities to reprogram immune cells[35]. MSCs use mitochondrial OxPhos when they are induced to differentiate, thus the multi-lineage differentiation capacities of MSCs require intact mitochondria[6]. Maintaining intact mitochondrial dynamics is not only required to support the differentiation capacities of MSCs, but it is also crucial for redox regulation and prevention of excessive production of reactive oxygen species[36]. Recently, it has been proposed by many studies that MSCs are capable of transferring mitochondrial DNA to recipient cells and restoring the normal mitochondrial functions in these cells[37,38]. MSCs-mediated mitochondrial transfer further supports the necessity to conserve normal mitochondrial parameters[38]. Previous reports revealed that the MSCs proliferation decreased in high glucose medium [13,14]. Furthermore, other studies showed that MSCs differentiation capacities might vary when they are cultured in high glucose conditions[39,40]. Our study illustrated that high glucose can reduce the survival rate of MSCs. The reduction in MSCs viability reported in our study is correlated with an impairment in the inner mitochondrial membrane potential (ΔΨm) which is an important parameter and the energy generated from this potential gradient will be utilized by complex V to produce ATP molecules. The maintenance of mitochondrial membrane potential depends on the presence of a plentiful amount of NAD+ molecules[41]. NAD+ serves as an oxidoreductase cofactor that controls many vital energy production pathways. Maintaining normal NAD+/NADH ratio is integral to providing the required electron flux that will be used to generate proper mitochondrial membrane potential (ΔΨm)[41]. High glucose insult causing a substantial drop in NAD+/NADH ratio in hAD-MSCs indicates a needed disturbance in the oxidization power to produce NAD+ and donating electrons that will keep the functionality of mitochondrial complexes. Another important complex that is required to preserve the mitochondrial dynamics is mTOR complex[42]. mTOR is a known regulator of many cellular processes, including the biogenesis of mitochondrial complexes, most importantly complex I and V and physiological mitophagy[42]. The downregulation in the level of mTOR in MSCs cultured in high glucose was associated with significant disturbances in mitochondrial complexes I, IV, and V which can induce mitochondrial dysfunction, mitophagy and massive oxidative stress. The effect of high glucose on modulating mitochondrial functions by targeting mTOR is barely investigated and with controversial results[43]. While other studies found that hyperglycemia activate mTOR pathway in cardiomyocytes[44,45], other evidence showed that insulin can activate mTOR pathway in DM patients and protect cells from developing mitochondrial dysfunction[46], indicating that high glucose can deactivate mTOR and cause pathological consequences[46]. These discrepancies regarding the effect of high glucose on mTOR level and activity seem to differ depending on the type of cells and the upstream molecular regulators. Based on that, the role of high glucose in modulating the level of mTOR needs further investigation in different cell types. In our study, the upstream regulatory protein PI3K decreased remarkably in hAD-MSCs exposed to high glucose. A plentiful number of reports corroborated that PI3K-AKT pathway is required to activate mTOR by removing the inhibitory effect produced by TSC1 or Hamartin protein. We reported in this study that the reduction in PI3K protein level was coupled with marked elevation in TSC1 and downregulation in mTOR in hAD-MSCs cultured in high glucose. Our findings provide a mechanistic explanation of poor survival of MSCs in hyperglycemic microenvironment and suggests new targets that can be modified to enhance the survival rate of MSCs in DM patients. Moreover, our results highlighted the importance of maintaining the desirable pre-transplantation culturing conditions that preserves the salutary properties of MSCs by controlling the level of glucose in the culture media. Schematic summary of the study findings was illustrated in (Figure 7) which highlighted that high glucose microenvironment reduced the level of PI3K and removed its inhibitory effect on TSC1 and caused its upregulation. The increase in the level of TSC1 caused a reduction in mTOR which

disturbed many mitochondrial dynamics, mainly MtMB, NAD+/NADH ratio, and mitochondrial complexes that triggered the apoptosis of hAD-MSCs.

CONCLUSION

MSCs have the potential to regenerate damaged tissues and restore their functions. DM patients can hugely benefit from MSCs transplantation to abrogate the pathological consequences and improve their quality of life. The poor survival and short-lived positive outcomes following MSCs transplantation in DM patients are considered stumbling blocks. High glucose impaired mitochondrial function in MSCs by perturbing mitochondrial regulatory factors, particularly mitochondrial membrane potential, NAD+/NADH pool, and mTOR protein. This mitochondrial dysfunction is associated with triggering apoptosis in MSCs. Preserving these factors may help in improving the survival rate of MSCs in diabetic microenvironment and initiate long-lasting therapeutic outcomes. Future studies should focus on providing new strategies to overcome the poor survival of MSCs in high glucose using genetic modification or biomaterials and nanoparticles. Moreover, the impact of other stressors existed in DM patients other than hyperglycemia should be studied.

ARTICLE HIGHLIGHTS

Research background

Mesenchymal stem cells (MSCs) have the ability to cure many chronic diseases, including diabetes mellitus (DM) and its related multisystem complications.

Research motivation

The therapeutic outcomes of MSCs transplantation in DM are short-lived which require thorough investigation.

Research objectives

This study aimed to determine the effects of hyperglycemia microenvironment on various mitochondrial-related parameters in MSCs to better understand their fate in DM patients.

Research methods

Adipose tissue-derived MSCs were exposed to low and high glucose media and mitochondrial dynamics and regulators were measured and analyzed.

Research results

High glucose induces the apoptosis of adipose tissue-derived MSCs by disturbing many mitochondrial parameters, including mitochondrial membrane potential, nicotinamide adenine dinucleotide (NAD+)/NADH pool, mammalian target of rapamycin, and mitochondrial complexes I, IV, and V.

Research conclusions

Hyperglycemia decreases the survival of MSCs by triggering mitochondrial dysfunction in these cells causing their short-lived therapeutic outcomes.

Research perspectives

New strategies to improve the survival rate of MSCs in hyperglycemia should be the focus of future studies which can help in increasing the chances of using these cells clinically for the treatment of DM.

ACKNOWLEDGEMENTS

This work was conducted in the Applied and Basic Medical Research Lab in the Faculty of Medicine at Yarmouk University. We would like to thank Mr. Muhammad Abu-El-Rub for his efforts in proof-editing and correcting the manuscript.

FOOTNOTES

Author contributions: Abu-El-Rub E conceptualized the study; Abu-El-Rub E, Khasawneh RR, Almazari R, Sanajleh A, Magableh H, Shlool H, Mazari M, Bader NS, and Al-Momani J carried out the experiments; Abu-El-Rub E and Almahasneh F analyzed the data; Abu-El-Rub E, Almahasneh F, Alzu'bi A, and Ghorab D drafted and wrote the manuscript; Abu-El-Rub E and Almahasneh F revised and formatted the content of the manuscript and verified spelling, punctuation and grammatical errors; and all authors have read and

approve the final manuscript.

Institutional review board statement: The ethical approval was not needed in this study. The human cell lines used for this study were commercially purchased.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: No additional data are available.

Country/Territory of origin: Jordan

ORCID number: Ejlal Abu-El-Rub 0000-0001-9217-2560; Fatimah Almahasneh 0000-0002-8799-2721; Ramada R Khasawneh 0000-0003-3873-925X; Ayman Alzu'bi 0000-0001-7848-7070; Doaa Ghorab 0000-0002-2175-7163; Rawan Almazari 0009-0001-5133-0945; Huthaifa Magableh 0009-0006-2039-431X; Ahmad Sanajleh 0009-0004-4232-4270; Haitham Shlool 0009-0009-3861-7974; Mohammad Mazari 0009-0003-1551-1524.

S-Editor: Wang JJ
L-Editor: A
P-Editor: Zhao S

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