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Mesenchymal stromal/stem cells and their exosomes application in the treatment of intervertebral disc disease: A promising frontier

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ABSTRACT

Today, the application of mesenchymal stromal/stem cells (MSCs) and their exosomes to treat degenerative diseases has received attention. Due to the characteristics of these cells, such as self-renewability, differentiative and immunomodulatory effects, their use in laboratory and clinical studies shows promising results. However, the allogeneic transplantation problems of MSCs limit the use of these cells in the clinic. Scientists propose the application of exosomes to use from the therapeutic effect of MSCs and overcome their defects. These vesicles change the target cell behaviour and transcription profile by transferring various cargo such as proteins, miRNAs, and lipids. One of the degenerative tissue diseases in which MSCs and their exosomes are used in their treatment is intervertebral disc disease (IDD). Different factors such as genetics, nutrition, ageing, and environmental factors play a significant role in the onset and progression of this disease. These factors affect the cellular and molecular properties of the disc, leading to tissue destruction. Nucleus pulposus cells (NPCs) are among the most important cells involved in the pathogenesis of disc degeneration. MSCs exert their therapeutic effects by differentiating, reducing apoptosis, increasing proliferation, and decreasing senescence in NPCs. In addition, the use of MSCs and their exosomes also affects the annulus fibrosus and cartilaginous endplate cells in disc tissue and prevents disc degeneration progression.

Abbreviations: MSCs, mesenchymal stromal/stem cells; IVD, intervertebral disc; IDD, Intervertebral disc disease; NP, nucleus pulposus; AF, annulus fibrosus; CEP, cartilage endplates; LBP, low back pain; ECM, extracellular matrix; NCs, notochordal cells; MMPs, matrix metalloproteinases; PGs, Proteoglycans; GAGs, glycosaminoglycans; PGE2, Prostaglandin E_2; NO, nitric oxide; TGF-B, Transforming growth factor-beta; TLR, Toll-like receptor; MAPK, Mitogen-activated protein kinase; WJ-MSC, Wharton's Jelly-Derived Mesenchymal Stromal/Stem Cells; PCR, Polymerase chain reaction; BM-MSC, Bone marrow-derived mesenchymal stromal/stem cell; NF-κB, Nuclear Factor Kappa B; BMP, bone morphogenic protein; PI3K, Phosphoinositide 3-kinase; PTEN, Phosphatase and tensin homolog.

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1. Introduction

The intervertebral disc (IVD) is a complex tissue that contributes to the health of the vertebrae and spine by being placed between the vertebras and have abilities such as bending and distributing mechanical load between the vertebrae [1]. IVD comprises three components: inner soft nucleus pulposus (NP) cells, annulus fibrosus (AF), and cartilage endplates (CEP) [2]. Due to NP's hydrated and gelatinous nature, this tissue plays a significant role in counteracting mechanical stimuli, and its main resident cells are the pulpous nucleus cells (NPC), which are responsible for the synthesis and maintenance of the extracellular matrix [3]. The AF is divided into two distinct areas, the inner and the outer AF. This tissue consists of ligament fibres rich in type 1 collagen and elongated fibro-chondrocytes surrounding the NP and connects the spinal vertebrae above and below the disc [4]. The CEP is the nutrition channel, and usually, its thicknesses are less than 1 mm [5] and help nutrient transport in the endplates [6].

Intervertebral disc disease (IDD) occurs as a consequence of excessive mechanical loading, genetic disorders and environmental factors [7]. Apoptosis and a decrease in the cell number of each of the three components of the IVD lead to IDD. IDD is one of the leading causes of low back pain (LBP), and its incidence increases with age [8]. LBP poses a significant threat to human quality of life and has engulfed diverse communities [9]. Various conservative treatments for IDD include surgical discectomy, spinal fusion surgery and intervertebral disc displacement [10]. These treatments show many side effects, such as recurrent disc herniation and an urgent need to find safer treatments in the long term.

Mesenchymal stromal/stem cells (MSCs), which are present in most stromal tissues, are a heterogeneous population composed of different cell populations such as multipotent stem cells, precursors, and differentiated cells [11]. Mesenchymal stromal/stem cells can differentiate into cells derived from various tissues such as osteocytes, chondrocytes, adipocytes, muscle cells, and NP-like cells [12]. Until 2006, there were no homogeneous criteria for mesenchymal stem cell isolation and culture, creating a reliable and reproducible application in preclinical and clinical contexts, and this led to the International Association for Cell Therapy (ISCT) necessary and objective criteria. Recommend that MSCs are useful in describing the unique population.

(1) MSCs must adhere to plastic under standard conditions. (2) These cells should be positive for CD105, CD73 and CD90 markers and express low MHC class I levels. This is negative for MHC class II, CD11b, CD34, CD14, CD45 and CD31. (3) MSCs must be able to differentiate in vitro in different tissues of mesoderm origin - such as osteocytes, fat cells and chondrocytes - under appropriate growth conditions [13].

MSCs are multipotent cells that have the ability to differentiate into different cells. These cells are self-renewable and can be isolated from various embryonic and adult sources [14]. Due to the characteristics of these cells they are widely used in cell therapy. The use of stem cells as biological therapies has shown many advances in IDD treatment [15]. During this biological treatment exogenous MSCs differentiate into IVD-related cells after transplantation by migrating to the injury site and also stimulates proliferation in IVD cells [16]. This action of MSCs leads to a quantitative increase in IVD cells at the injury site and helps in IDD treatment. These MSCs also help improve and regenerate IVD by preventing cell apoptosis and modulating the immune microenvironment at the injury site [17]. Although many studies have used MSCs as a treatment for various tissue disorders problems such as tumorigenesis functional erosion fibrosis injection toxicity cell rejection and the restricted ability of MSCs to differentiate are the barriers that can significantly affect the therapeutic efficacy of these cells [18]. Therefore today MSC produced soluble mediators such as extracellular vesicles (EVs) [19] and their supernatant [19] which is used for therapeutic applications [20] Apoptotic body, microvesicles (MVs), and exosomes are three types of EVs divided based on their size, content, and formation [21]. Apoptotic bodies are typically produced n the last stage of apoptosis from apoptotic cells and are 50–4000 nm in size. Apoptotic bodies are heterogeneous and contain membrane contents, cellular organelles and nuclear-derived molecules [22]. Unlike apoptotic bodies, microcycles are shedding directly from the healthy cells membrane. These EVs sizes range from 100 nm to 1000 nm and have a heterogeneous morphology [23]. Exosomes are the other type of EVs whose size range from 30 to 150 nm are the smallest EVs produced during late endosome membrane inward invagination and the multiple vesicular bodies (MVBs) formation [24]. MSCs-derived exosomes have a therapeutic effect in many degenerative tissue diseases [25]. Exosome injection for IDD treatment is a cell-free procedure that does not have the disadvantages of stem cell therapy. In addition, according to the properties of exosomes, they can be used as drug carriers. The biocompatibility of these vesicles, their small size, their ability to migrate and carry various substances to damaged tissue have made exosomes a promising therapeutic agent [26].

2. Etiology of IDD

Various factors such as aging, genetic factors, environmental factors, and nutritional factors play a role in the etiology of IVD [27]. Environmental factors include lack of exercise, smoking, unhealthy lifestyle, severe trauma, and constant vibration exposure, leading to IVD degeneration [28]. Polymorphisms in genes encoding extracellular matrix (ECM) function, Proteoglycans, and catabolic genes are also risk factors for IDD etiology [29]. Some mononucleotide polymorphisms, such as those found in Transforming Gross Factor-beta (TGF-B), can also trigger IDD [30]. Other reasons for the onset and development of IDD include changes in the nutrient supply of intervertebral disc cells that lead to hypoxia and changes in pH [31]. These factors affect the ability of IVD cells to synthesize and support ECM, leading to disc degeneration (Fig. 1).

3. Cellular and molecular biology of IDD

NP development is mediated by MSCs and notochordal cells (NCs). During embryogenesis, NP tissue is mainly composed of NCs [32]; however, in late puberty, NCs are replaced by chondrocyte-like cells called NPCs [33]. Reports indicate that in healthy IVD, some progenitor and stem cells play a role in the homeostasis and maintenance of the disc cells number [34]. These progenitor cells are also present in degraded IVD but are differentiated into adipogenic, osteogenic and chondrogenic lines [35]. IDD-derived cells have a reduced ability to proliferate and differentiate and therefore cannot regenerate damaged tissue [35]. In addition, in vitro studies show that NPCs isolated from the damaged disc undergo accelerated cell senescence, which affects the production of factors involved in ECM formation and contributes to tissue destruction [36,37]. Cells appear in clusters in the internal NP and AF in the damaged disc. Many of the cells present in clusters have the increased ability to produce matrix-degrading enzymes such as matrix metalloproteinases (MMPs) [38,39].

As mentioned, IVD comprises three types of tissues called NP, AF, and CEP, and changes in the composition of the components and the number of cells in these tissues contribute to the onset and progression of the IDD. The normal NP structure in young people comprises 2–3% cells and the rest of the water and extracellular matrix [40]. With increasing age and also the influence of the mentioned factors, this ratio changes and leads to the loss of normal IVD function [41]. In AF, most fibres are type 1 collagen due to their supporting role, while the main fibre in NP is type 2 collagen [42]. Due to NP's hydrated and ECM-rich nature, the complex structure of type 2 collagen leads to its interaction with water and ECM components and is required to maintain the normal function of NP. During IDD, a part of the type 2 collagen in NP is replaced by type 1 collagen, and this leads to a change in the structure of NP tissue [43].

Low pH, low glucose levels, and hypoxic conditions form the NP tissue niche, in which resident cells supply energy through anaerobic

glycolysis [44]. The change in favourable microenvironment regulated by CEP in IVD can also contribute to IDD progression by altering ECM components and affecting resident cells [45].

Proteoglycans (PGs) and glycosaminoglycans (GAGs) in the ECM of IVD play a significant role in maintaining health. Due to the negative charge of Sulfated GAGs attached to PGs, their side chains bind to water molecules to preserve tissue hydration [36]. In addition, due to the binding of growth factors, cytokines, and various chemokines to the PGs in the ECM, these structures play a role in the signaling, proliferation, and migration of the IVD resident cells [46]. Different types of proteases cleave the binding sites between PG and GAG and lead to type 2 collagen degradation [45].

In addition to the above, immune system responses also play a role in disease progression. T cells, macrophages, and neutrophils produce pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-a), interleukins (such as IL 1-beta, IL 6, IL 17, IL 2, IL 4), and interferon-gamma (IFN-γ) in NP and AF tissues and induce autophagy, senescence, and apoptosis in their resident cells [47]. IL-6, PGE2, and nitric oxide (NO) [48] seem to have an inhibitory effect on proteoglycan synthesis. These cytokines also help intervertebral disc degeneration by decreasing the production of proteoglycans and increasing the production of MMPs [3].

4. IDD conservative, interventional and biological treatments

In IDD treatment, therapeutic interventions vary based on the severity, degree, persistence, and chronic or acute pain. If LBP is low or acute, medicines such as rest, physiotherapy [49], steroidal and non-steroidal anti-inflammatory drugs, muscle relaxants, and analgesics are used [50]. These treatments use different mechanisms to reduce acute pain in the patient but can not prevent disc destruction [51]. For this reason, in some patients, the pain becomes chronic and makes the mentioned drugs useless. In these cases, intervention therapies such as surgery (discectomy, fusion, and complete disc replacement) and epidural steroid injections can be used [52].

But in surgery, due to the lack of attention to the physiological pathology of the degeneration process, it usually has limited and short-term effectiveness [53]. That's why researchers are looking for treatments to prevent and improve the physiological and pathological damage of the IVD [54,55]. Biological methods such as the use of growth factors, gene therapy, MSC transplantation, and use from MSCs-derived exosomes have received much attention [27].

The use of growth factors in IDD treatment leads to reduced inflammation, increased extracellular matrix synthesis, proliferation, and differentiation in damaged cells [56,57]. Many different in vitro and in vivo studies show that exogenous administration of TGF-B1 [31], bone morphogenic proteins (BMP-2,7,13,14) [58,59], insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), And basal fibroblast growth factor (bFGF) stimulates the synthesis of ECM components in NPCs [60].

IDD treatment by gene therapy can be used in two ways [61]. First, in vivo injections of viral and non-viral vectors can transfer appropriate and healthy genes to the patient. In the second method, with ex vivo gene therapy, after extracting the defective cells and changing the genetics (at the site of the defective gene that causes the destruction of the disc) and culturing in the lab, they are then transplanted back to the patient and can perform the proper function in them [62]. Studies have shown that TGF-B gene transfer through adenovirus vector leads to increased production of proteoglycans by NPCs [63]. Transfection of the SOX-9 gene by an adenoviral vector has also been shown to increase the production of type 2 collagen in IVD [64]. In addition, in recent years, the use of non-coding RNAs that inhibit gene expressions, such as siRNAs, miRNAs, and long non-coding RNAs (Lnc-RNA), has been proposed for the biological IDD treatment [27]. But in the meantime, the use of mesenchymal stromal/stem cells has shown promising results in IDD treatment [65]. These cells directly and through their exosomes play an important role in treating this disease [10], which we will discuss below.

5. MSCs application in IDD treatment

The use of MSCs helps treat IDD by modulating the immune system, increasing ECM production, and producing soluble factors [53]. Interestingly MSCs isolated from different tissues and individuals show different abilities to differentiate under the same In vitro conditions [50]. In general, there are two ways to use MSCs in tissue repair.

First, MSCs are injected naturally and without manipulation into the injury site and differentiate into different cells in the tissue microenvironment [66]. In this method, the injected cells may differentiate into undesired cells and reduce the effectiveness of the treatment. In the second method, MSCs, before transplantation, differentiate into desired cells in vitro by growth factors, differentiation factors, and different gene therapy techniques [67]. In this type of treatment, differentiated cells show a stable phenotype that is resistant to trans conditions of body tissues [68]. However, as mentioned at the beginning of the section, pre-injection differentiation of MSCs into specific cell lines can affect their immunomodulatory properties and reduce treatment efficacy [69]. Of course, it should be noted that in both types of treatment, the number of cells isolated MSCs from different sources is usually low and must be

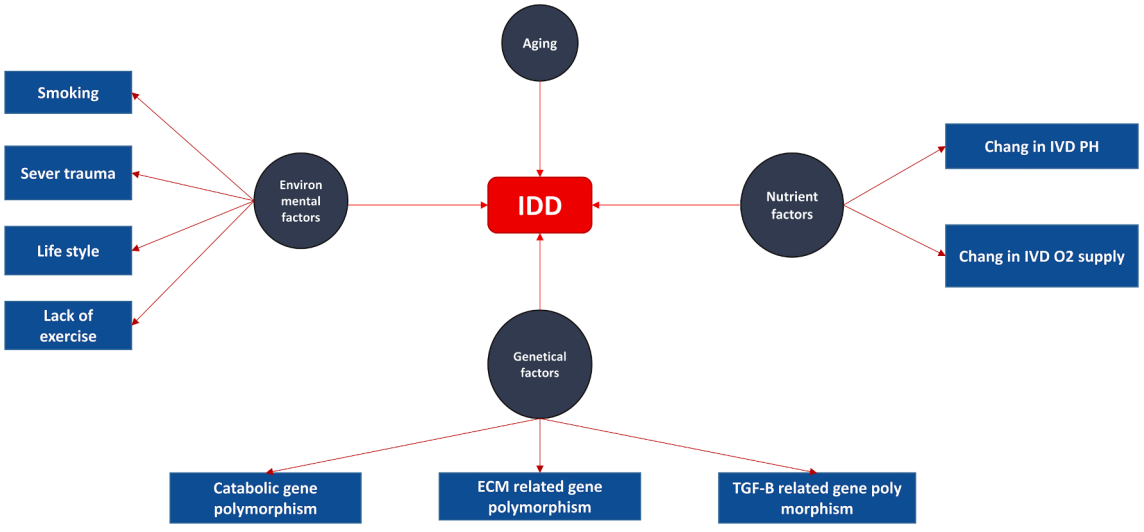

Fig. 1. Factors involved in IDD etiology.

cultured before transplantation to increase their number and be suitable for treatment [70]. Intravenous injection of cells in cell therapy is the main method of administration, but it has been shown that the number of cells in the damaged tissue is much less than the number of injected cells. To prevent this phenomenon, intra-disc injection of MSCs is recommended. However, intra-disc injection is not without its disadvantages and can lead to the removal of MSCs from the disc tissue by puncturing the IVD. Also, cells reflux out of IVD can induce the adverse formation of osteophytes by altering the CEP tissue and affecting its function [71]. Although the survival rate of MSCs after transplantation is unknown, it is believed that most of these cells do not survive in IVD [72]. As a result, this leads to the accumulation of necrotic cell debris and apoptosis and, therefore, may have detrimental effects on IVD homeostasis and therapeutic outcomes. A study that evaluates MSCs survival showed that these cells disappear regardless of the injection method after seven days of injection. Within one day after transplantation, due to the physiology of various tissues, especially IVD hypoxic conditions, MSCs activate hypoxic pathways, followed by caspase-3-mediated apoptosis [73]. Macrophages in the tissue remove these apoptotic MSCs. There is ample evidence in various studies that MSC apoptosis modulates both innate and adaptive immune responses that affect the therapeutic effects of MSC [74]. How the complex microenvironment of IVD is regulated, the survival and function of BM-MSCs after injection is not clearly understood, and most current studies have focused on MSC transplant results [75]. However, the results of preclinical and clinical studies on the use of MSCs have been very promising.

5.1. *In vitro evidence of the MSCs therapeutic efficacy*

Studies have shown that the addition of growth factors such as TGF-B to the culture medium of MSCs stimulates their differentiation into NP-like cells and dramatically increases the expression of type II collagen and aggrecan in them [76]. It has also been shown that due to the hypoxic environment in IVD tissue, if MSCs cultured in hypoxic conditions with TGF-B, the degree of stability of their differentiation into NP-like cells will increase [77]. So it can be concluded that the IVD hypoxic environment can stimulate differentiation of MSCs in the in vivo conditions [78,79]. In addition, coculture of adipose tissue derived mesenchymal stem cells (AD-MSCs) with NP and AF-derived cells increases their proliferation [80]. This coculture also increases the MSCs differentiation to NPCs [81]. It has been shown that the main factor in the differentiation of MSCs in coculture with NPCs is intercellular communication, soluble factors, and exosomes produced from NPCs [82].

A study by ZHAO et al. showed that co-incubation of Wharton jelly derived mesenchymal stem cells (WJ-MSCs) with NPCs reduces apoptosis in these cells by inhibiting the Wnt/B-catenin signaling pathway [83]. Studies show that Wnt signaling inhibits the aquaporin 3 channel protein expression, and this water channel has a protective role in preventing apoptosis and regulating ECM degradation [84]. Wnt signaling inhibits the proliferation of NPCs and stimulates senescence in these cells [85]. In addition, it promotes the development of IDD by stimulating the production of TNF-α and initiating inflammatory responses [86]. Also, due to the lack of cell–cell interactions in this study, MSCs act through paracrine signaling transduction to decrease apoptosis in NPCs [83].

Different ligands can activate signaling pathways related to toll-like receptors (TLRs) in inflammatory conditions of disc degeneration [87]. TLR-2 is one of these receptors at the surfaces of NPCs, which activates the transcription factor NF-κB through the Myd88 adaptor protein [88]. This transcription factor plays an important role in producing inflammatory factors such as cytokines and chemokines [89]. Various studies have shown that inhibition of NF-κB activation can delay the process of disc degradation [90]. MSCs inhibit the activation of the transcription factor NF-κB by producing TNF-α-stimulated protein 6 (TSG-6), a 30-kDa glycoprotein, and play an important role in IDD treatment [91]. Because the ability of MSCs treated with TSG-6 expression inhibitory siRNA is less than that of intact MSCs, it is suggested that TSG-6 performs part of the therapeutic function of MSCs [91]. The application of recombinant TSG-6 decreases the expression of MMP-3 and MMP-13 and also increases the production of ECM components such as collagen II and aggrecan.

On the other hand, a study by Eun-Kyung Shim shows that the coculture of isolated cells from NP and AF with MSCs increases proliferation in all three of these cells compared to monocultures. The analysis showed that the mRNA expression of growth factors such as IGF-1, OP-1, and growth and differentiation factor 7 (GDF-7) in both NP and AP cells significantly increased in coculture with mesenchymal stromal/stem cells. Also, EGF and TGF-b expression levels increase only in NPCs [92].

According to this study, in the coculture of MSCs with NPCs, the mRNA expression of genes involved in ECM formation such as SOX9, VCAN, Aggrecan, COL 2, and COL 6 increases in both MSCs and NPCs. However, the coculture of MSCs with AF cells leads to increased COL 5 gene expression only in MSCs [93,94]. This difference in gene expression in cultured MSCs with cells isolated from NP and AF tissues seems quite reasonable given their different roles. In this study, the analysis of proinflammatory cytokines expressions such as IL-1a, IL-1b, IL-6, and TNF-a showed a significant decrease in NPCs and AP cells derived from a degenerative disc in co-culture of MSCs [92].

Mitogen-activated protein kinase (MAPK) plays an important role in many cellular processes, including response to inflammation, injury or stress, and cell differentiation [95]. The MAPK/p38 related signaling pathway is associated with increased PGE2, IL-6, and MMPs [96]. Various studies show that this signal pathway plays a pivotal role in promoting disc degradation in NPCs [97]. When isolated NPCs from the disc treated with TNF-α, they exhibit features similar to those seen in IDD [98]. SB-203580 is one of the inhibitors of the MAPK signaling pathway and inhibits the inflammatory response in NPCs leading to disc damage. According to various analyzes, it was shown that the coculture of NPCs with Wharton's Jelly-derived mesenchymal stromal/stem cells (WJ-MSC) exerts similar effects using SB-203580 on the gene expression and inflammatory profile of NPCs. Therefore, it was demonstrated that culturing NPCs with WJ-MSC inhibits the inflammatory responses of NPCs by inhibiting the MAPK/p38 pathway [98].

The culture of AF-isolated cells in IL-1β-containing medium is a way to mimic IDD inflammatory conditions to study their effects on these cells [99]. Indirect culture of BM-MSC with these cells shows promising results from the impact of mesenchymal stromal/stem cells [100]. Western blot and PCR analyzes of IDD conditions mimicking AF cells with BM-MSCs showed a decrease in the expression of MMP-3 and MMP-13 and an increase in the production of aggrecan, type 1 collagen, and TIMP-1 in them. In addition, ELISA results indicate a reduction in inflammatory factors such as PGE-2, cyclooxygenase-2 (COX-2), and IL-6 [100]. Studies to evaluate the apoptosis rate in IL-1β-stimulated AF cells show that treatment of these cells with MSCs reduces apoptosis in them. Overall, the results of this study suggest that treatment of IDD-related AF cells with BM-MSCs can help treat IDD by relative inhibition of Nuclear Factor Kappa B (NF-κB) transcription factor activation and reducing mitochondrial apoptosis in them [100].

Li et al. show that culturing BM-MSC with NPC can reduce their apoptosis rate by regulating autophagy [101]. Methylation of the amino group (N6) in adenosine base is one of the most common post-transcriptional changes in eukaryotic mRNAs and plays an important role in various cellular processes. Multiple studies suggest that N6 modification of methyladenosine (m6A) on mRNA regulates autophagy and cell fate [102]. The complex consisting of METTL3, METTL14, and WTAP is responsible for this methylation [103]. ALKBH5 and FTO also reverse the function of the methylating complex by demethylating adenosine in mRNA [104]. ULK1 is an autophagy-related gene, and post-transcriptional changes play an important role in regulating its expression [105]. ULK1 and FIP200 are part of the ULK1 complex and

upregulate NPCs in coculturing with BM-MSC [106]. FIP200 expression is reduced in NPCs under compression and in the degenerated disc environment [106]. But when NPCs are cultured with BM-MSCs, the FIP200 mRNA is hypomethylated through the function of ALKBH5 and FTO and is protected from degradation by YTHDF2 [101]. This study shows that BMSC stimulates autophagy in NPC by regulating the ULK1-FIP200-Atg13 complex.

The use of cultured BMSCs in hypoxia conditions increases their tolerance to subsequent injuries and their therapeutic potential [107]. Hypoxia affects cells by regulating intracellular signaling, regulating migration, increasing growth factor secretion, and regulating cell migration [108,109]. CoCl2 is one of the classic hypoxia simulators, which has many applications due to its easy and accurate use and control [110,111]. The study by Weiheng Wang shows that hypoxia increases cell migration through the signaling pathway of HIF-1α and CXCR4 in BMSC and decreases apoptosis by regulating the expression of caspase-3 and Bcl-2 pathways [112].

5.2. *In vivo evidence of the MSCs therapeutic efficacy*

Numerous studies have demonstrated the efficacy of MSC transplantation in treating IDD in vivo, and promising results have been obtained. The use of autograft MSCs immunologically is the best source for MSCs transplanting [113]. However, since a person's genetic predisposition to disc degeneration can affect the performance of MSCs (having disruptive genetic problems) for treatment, the use of allograft resources is recommended [114]. Allograft MSCs are more accessible than collecting and culturing specific autograft MSCs and reduce costs. Many studies since 2004 have examined the efficacy of MSCs in the treatment of IDD in animals. In summary, these studies suggest that mesenchymal stromal/stem cell transplantation in IVD leads to their differentiation into NPCs [115], increases ECM production [116], increases survival, and decreases apoptosis in resident NPCs [117], modulates the immune microenvironment [114], and decreases ECM degrading enzymes [118] in vivo. These results approve the in vitro studies' result and present a new regenerative treatment for IDD.

A study by En-Rung Chiang in 2019 shows that in rabbit degenerated discs, injection of hypoxic MSCs increases BMP-7 expression more than normoxic MSCs. Therefore, due to the pivotal role of BMP-7 in the regeneration of IVD tissue, hypoxic mesenchymal stromal/stem cells have more therapeutic potential than normoxic [119]. (Table 2)

A short report published on five patients in 2016 assessed the safety and feasibility of using autologous, hypoxic MSCs to treat LBP. Intradiscal injection of these MSCs improves symptoms and reduces pain in patients. Follow-ups performed during 4–6 days after MSC injection, such as physical examination, completion of a quality of life questionnaire, and lumbar MRI, indicate the feasibility of this type of treatment. In addition, no side effects were observed during this study for up to 6 years after injection [120].

In a study performed on 33 patients, injection of autologous MSCs expanded with platelet lysate in vitro was used. Extended MSCs were examined in vitro before injection into patients' discs for karyotype, and if any genetic abnormalities were observed, the injection would be cancelled according to safety protocols. Also, no neoplasms were observed during the imaging of the injected area of the cell, and no new neoplastic events occurred in any of the patients after surgery. Follow-up over 7 years after cell injection improves patients' symptoms, reduces pain, and increases IVD function [121].

In another study, autologous bone marrow Good Manufacturing Practice (GMP)-compliant MSCs was used to treat IDD in chronic LBP patients [65]. In this study, ten patients were followed for one year after injection. Immunophenotyping of cells and injected cell viability have not changed due to syringe injection into the disc and have been stable over time. The reported karyotype results were also satisfactory. This study shows that during the one year after the intervention, the height of the disc did not change in patients. Still, the fluid content of the

Table 1
The five types of discogram and their stages of disc degeneration.

Stage	Discogram type	Stage of disc degeneration
1	Cottonball	No signs of degeneration
2	Lobular	Mature disc with nucleus starting to coalesce into fibrous lumps
3	Irregular	Degenerated disc with fissures and clefts in the soft nucleus pulposus and annulus fibrosus
4	Fissured	Degenerated disc with radial fissure leading to the outer edge of the annulus
5	Ruptured	Complete radial fissure in discs

IVD: intervertebral disc, BM: bone marrow, MSCs: mesenchymal stem cell.

Table 2
New studies using MSCs to treat IDD in animals.

study	Animal model	MSC sources	Number of injected cells	reference
Combined Hydrogel and Mesenchymal Stem Cell Therapy for Moderate-Severity Disc Degeneration in Goats	Goats	bone marrow	N.A	[165]
Treatment of Intervertebral Dis Degeneration in Wistar Rats with Mesenchymal Stem Cells	Rat	bone marrow	10^5	[166]
Transplantation of Hypoxic-Preconditioned Bone Mesenchymal Stem Cells Retards Intervertebral Disc Degeneration via Enhancing Implanted Cell Survival and Migration in Rats	Rat	bone marrow	2×10^4	[167]
Efficacy of matriline-3-primed adipose-derived mesenchymal stem cell spheroids in a rabbit model of disc degeneration	rabbit	Adipose tissue	2×10^6	[168]
Mesenchymal stem cells reduce intervertebral disc fibrosis and facilitate repair	Rabbit	bone marrow	5×10^3–1.5×10^5	[169]
Injectable kartogenin and apocynin loaded micelle enhances the alleviation of intervertebral disc degeneration by adipose-derived stem cell	Rat	Human Adipose tissue	N.A	[170]
Sox9 Gene Transfer Enhanced Regenerative Effect of Bone Marrow Mesenchymal Stem Cells on the Degenerated Intervertebral Disc in a Rabbit Model	Rabbit	bone marrow	10^6	[171]
Evaluation of regenerative processes in the pig model of intervertebral disc degeneration after transplantation of bone marrow-derived mesenchymal stem cells	pig	bone marrow	10^6	[172]
Injectable Hydrogel Combined with Nucleus Pulposus-Derived Mesenchymal Stem Cells for the Treatment of Degenerative Intervertebral Disc in Rats	Rat	Nucleus Pulposus	N.A	[173]

damaged discs increased significantly, and its analgesic effects were 71% effective [65]. These observations are consistent with the results obtained in animals, where MSC could prevent the development of disc dehydration and regenerate disc tissue [122,123]. However, with the fact that the height of the discs has not changed and the pain has been greatly reduced in patients, it can be concluded that trophic effects occur faster than regenerative effects in patients [65]. (Fig. 2) The researchers of this study demanded more studies to reveal better the mechanisms involved and evaluate the subsequent improvements in this disease. Tables 3 and 4 summarize some clinical studies and clinical trials, respectively.

5.3. Immunomodulation by MSCs in IDD

In IVD, the presence of T and B lymphocytes in human and experimental models of pigs herniated discs has already been demonstrated, and no inflammatory cells other than macrophages have been observed in the damaged disc. Monocyte-derived macrophages migrate to the hernia, and during the process of phagocytosis and exocytosis, lysosomal enzymes contribute to the regression of the hernia and IVD. MSCs can inhibit the proliferation and differentiation of T lymphocytes [45,46], but MSC activities and therapeutic outcomes are highly dependent on disease-related tissue microenvironments. Systemic transplantation of MSCs leads to an increase in $TCD4^+$ cells in the spleen, including $CD4^+$ T helper and Treg cells. Elevated IL-2 levels in the rat receiving MSCs group lead to increased differentiation of $TCD4^+$ cells into Treg cells, suppressing inflammation. Transplantation of MSCs also leads to a decrease in the $MHCII^+$ cell population in IDD model rats. MSCs, through the production of IL-6, can keep DCs immature or even force DCs to acquire a tolerogenic phenotype with less expression of MHCII molecules. Thus, in addition to the direct effect, MSCs also indirectly affect the differentiation of $TD4^+$ cells and reduce their inflammatory responses, which play a role in disc damage. Tolerogenic DCs stimulate the differentiation of $TCD4^+$ into Th2 and Treg, increase serum levels of IL-4 and Il-10, and provide a regenerative immune environment for disc tissue improvement. MSCs can interact directly with B cells present at the injury site and reduce their differentiation into antibody-producing plasmablasts, leading to the induction of regulatory phenotype in these cells (Breg) [124]. In addition to suppressing T cell activity, MSCs induce macrophage polarization to the M2 phenotype by producing the interleukin-1 receptor antagonist (IL1-RA). These macrophages help reduce inflammation and increase the function of cells involved in tissue repair by producing immunosuppressive factors [125]. Thus, MSCs can help improve function and increase tissue repair by affecting the responses of the three types of inflammatory cells present in the damaged disc.

5.4. Limitations of MSCs use in IDD treatment

Many studies suggest the use of Pufferman [126], Adams [127], and Modic's [128] scores to determine inclusion and exclusion criteria for IDD treatment with MSCs. Different types of discograms were identified based on continuously identifiable features in the shape and density of the radio-opaque shadow. According to the discogram, disk destruction is divided into five stages [126]. Table 1 summarizes the specifications of different levels of disk destruction. Based on these scores, the overall condition of the destroyed disk and the effectiveness of using MSCs is determined. Since the use of mesenchymal stromal/stem cells can not induce treatment in a completely destroyed and necrotic disc [129], these cells should be used in the early stages of LBP and low damage to disc tissue [130,131]. The therapeutic potential of MSCs is exerted by signaling patterns and mutual interaction between resident cells. This potential therapeutic reduced due to the loss of resident cells in the wholly destroyed disc [92]. The distinction between NP and AF is unclear in the early stages of degeneration, and the discs' height has not changed much [132]. MSCs cannot be used in damaged discs with full radial fissure. This type of damage can lead to the escape of transplanted MSCs into the disc and significantly reduce the effectiveness of treatment [132].

6. MSCs-derived exosomes

According to various studies, the use of MSCs for IDD treatment shows many promising results. But as mentioned, cell therapy has some limitations. In order to use the therapeutic potential of MSCs and reduce their limits, the use of their exosomes was proposed [133]. Exosomes are nanosized vesicles released from a wide range of cells and play an important role in intercellular and paracrine communication [134].

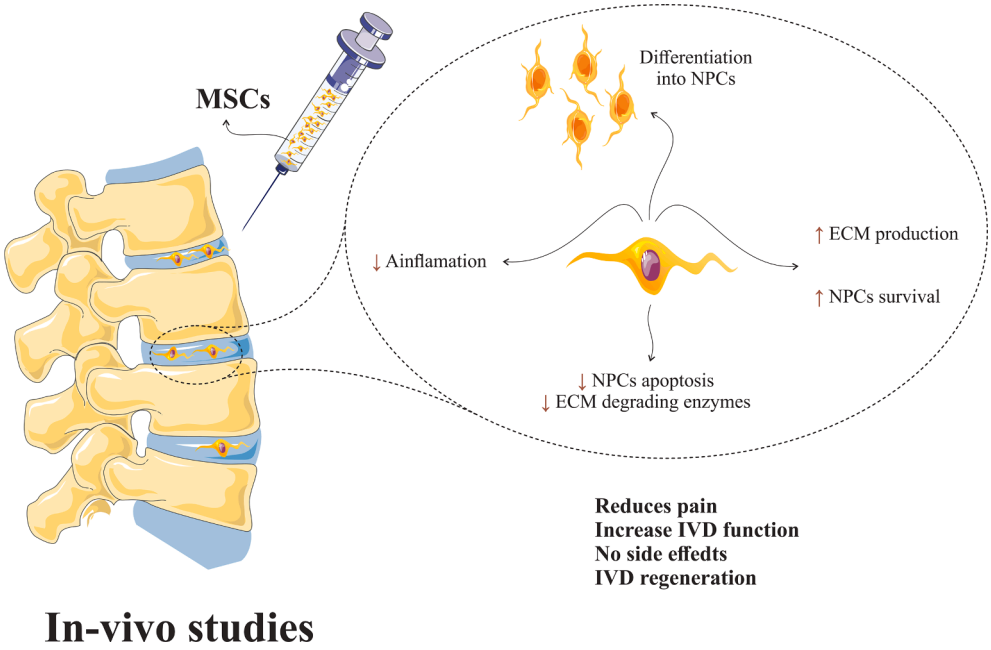

Fig. 2. Injection of mesenchymal stromal/stem cells into damaged discs through various mechanisms can help improve the disease. These mechanisms include reducing pain, increasing the differentiation and survival of NPCs, and increasing extracellular matrix production.

Table 3
Intradiscal transplantation of MSCs in clinical studies.

Study	Results	Year of publication	Number of Patients	Number of Injected Cells	Follow Up time	Reference
Injection of autologous BM-MSCs into the IVD	85% of patients showed improvement in IVD properties	2017	33	N/A	6 year	[158]
Injection of the autologous stromal vascular fraction containing adipose tissue-derived MSCs together with platelet-rich plasma	Significant improvement in flexion, VAS, PPI, and pain	2017	15	$30–60 \times 10^6$	6–12 month	[159]
Injection of autologous BM-derived MSCs into the IVD	Improvement in pain and disability. Elevated water content in IVDs. No change in disk height.	2011	10	$10 \pm 5 \times 10^6$ cells per disc	12 month	[160]
Injection of adipose tissue-derived MSCs combined with hyaluronic acid derivates	Improvement in VAS and ODI And Elevated IVD water in 3 patient	2017	10	$20–40 \times 10^6$	12 month	[161]
Injection of autologous BM-derived MSCs into the IVD	Improvement in VAS and OD.also 40% of patients showed improvement on Pfirrmann's grade	2017	26	5426 CFU-F	3 year	[162]
The Traceability of MSCs After Injection Into Degenerated Discs in Patients with Low Back Pain	MSCs differentiate into chondrocyte-like cells	2019	4	10^6	8–28 month	[163]
Injection of autologous BM-derived MSCs into the IVD	Improved functional indices and Pfirrmann's grade	2017	24	25×10^6	12 month	[164]

Table 4
MSC based clinical trials in IDD treatment.

Study title	Sorce of MSC	Status	Intervention Model	Phase	NCT number
Mesenchymal Stem Cells for Lumbar Degenerative Disc Disease	Bone marrow	Not yet recruiting	Parallel Assignment	Early Phase 1	NCT03692221
Human Umbilical Cord Mesenchymal Stem Cells For the Treatment of Lumbar Disc Degeneration Disease	Umbilical Cord	Recruiting	Single Group Assignment	Not Applicable	NCT04414592
Treatment of Degenerative Disc Disease With Allogenic Mesenchymal Stem Cells	Bone marrow	Completed	Parallel Assignment	Phase 2	NCT01860417
Autologous Adipose Derived Stem Cell Therapy for Intervertebral Disc Degeneration	Adipose	Unknown	Single Group Assignment	Phase 1	NCT02338271
Clinical Trial Based on the Use of Mesenchymal Stem Cells From Autologous Bone Marrow in Patients With Lumbar Intervertebral Degenerative Disc Disease	Bone marrow	Completed	Single Group Assignment	Phase 2	NCT01513694
Effectiveness and Safety of Mesenchymal Stem Cell (MSC) Implantation on Degenerative Discus Disease Patients	umbilical cord	Recruiting	Single Group Assignment	Phase 2	NCT04499105
Human Autograft Mesenchymal Stem Cell Mediated Stabilization of The Degenerative Lumbar Spine	Not Applicable	Unknown	Cohort	Observational	NCT02529566
Utilization of Autologous Mesenchymal Cells in Posterolateral Spinal Fusion in Degenerative Spine Disease	Adipose	Terminated	Single Group Assignment	Phase 2	NCT03827096
Efficacy of Intradiscal Injection of Autologous BM-MSC in Worker Patients Affected by Chronic LBP Due to Multilevel IDD	Bone marrow	Recruiting	Parallel Assignment	Phase 2	NCT04759105

These vesicles contain various cargos such as lipids, proteins, and a variety of nucleic acids. Transfer of these cargos to the target cell leads to changes in molecular mechanisms and changes the behavior of these cells [135]. Mesenchymal stromal/stem cells produce more exosomes than other cells and are more useful for therapeutic use [133]. In addition, when MSCs starved by decreasing the amount of FBS in their culture medium, the production of exosomes increases [136]. Methods such as ultracentrifugation, differential centrifugation, Microfluidics-Based methods [137], pegylation-based methods, and kits are used for exosome separation [138]. After separating, there are various methods for characterizing them, and such as tests such as Western blotting, scanning electron microscopy (SEM), transient electron microscopy (TEM), dynamic light scanning (DLS), and zeta potential analysis [139]. After characterizing, exosomes can be used in in-vivo and in-vitro studies. At this stage, using methods such as electroporation, sonication, freeze–thaw, and incubation, various substances can be loaded into the exosomes and used as drug carriers [140,141]. Drug loading increases the therapeutic efficacy of exosomes and enhances their clinical use [142]. MSCs exosomes help treat IDD through a variety of mechanisms.

In vitro studies show that culturing NPCs isolated from degraded discs with exosomes isolated from BM-MSCs increases their proliferation [10]. MSCs Exosomes not only induce proliferation in NPCs but also reduce apoptosis in them. A study by Cheng has shown that BM-MSCs-derived exosomes inhibit TNF-α-induced apoptosis in NPCs in vitro [143]. In addition, the in vivo study confirms the in vitro findings based on Pfirrmann scores, histological grade, and apoptosis rate. Another study showed that culturing NPCs with MSCs exosomes reduced caspase-3 and caspase-12 in NPCs [144]. Microarray analysis of hybridization in TNF-α-treated NPCs shows that the expression of miR-18a, miR-21, miR-106b, miR-217, and miR-26a in these cells is significantly reduced [143]. Studies on mesenchymal stromal/stem cell exosomes show the presence of miR-21 in them [145]. Also, the level of this miR increases in the exosomes of TNF-a-treated MSCs [143]. The culture of NPCs with TNF-a-treated MSCs derived exosomes reduces the rate of apoptosis in them. Phosphatase and tensin homolog (PTEN) prevents survival responses by inhibiting the PI3K/Akt signaling pathway [146]. miR-21 inhibits act by binding to 3′UTR in PTEN mRNA and reducing the apoptosis of these cells [143].

Another study showed that endoplasmic reticulum stress markers such as GRP78 and CHOP increased in NPCs isolated from IDD. The culture of these cells in the presence of MSCs exosomes reduces the expression of these markers and apoptosis in them. In fact, it can be said that the exosomes of MSCs reduce the level of CHOP (the main stress molecule of the endoplasmic reticulum network) through AKT and ERK signaling pathways and inhibit endoplasmic reticulum stress-mediated apoptosis [144]. (Fig. 3)

Oxidative-induced cellular stress at the injury site leads to increased

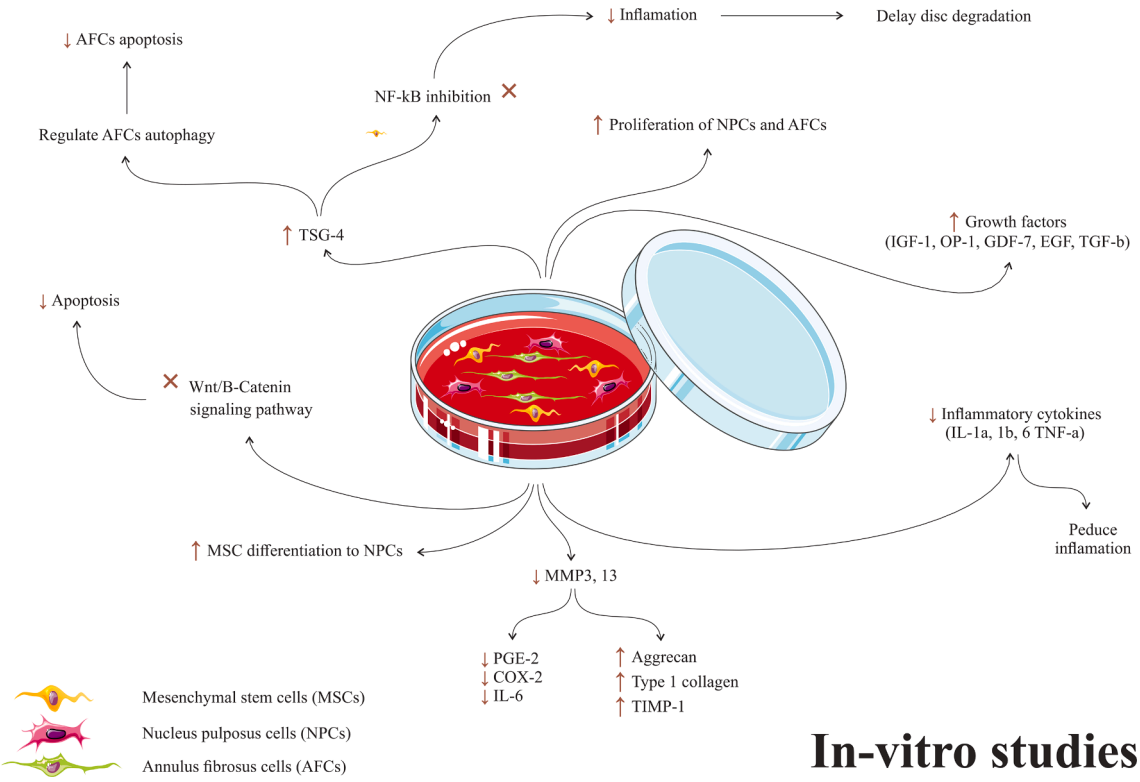

Fig. 3. Numerous studies have examined the effects of mesenchymal stromal/stem cell co-culture with cells derived from damaged discs. In fact, this figure summarizes the studies performed in vitro.

apoptosis and calcification of endplate chondrocytes (EPCs) by increasing endoplasmic reticulum stress [147]. ATF6 stimulates the migration of transcription factors through interaction with endoplasmic reticulum stress elements, leading to the upregulation of genes related to unfolded proteins such as XBP1, CHOP, and GRP78 [148]. The in vivo and in vitro studies of MSCs exosomes therapeutic effect show a reduction in apoptosis and calcification in EPCs [149]. The expression levels of active caspase-3, caspase-7, and caspase-9 are reduced in EPCs treated with MSCs exosomes. The use of these exosomes inhibits endoplasmic reticulum stress-induced by oxidative stress by decreasing the expression of ATF6, XBP1, CHOP, and GRP78 in EPCs. In addition, because inhibition of miR-31-5p function leads to reduced control of apoptosis and calcification in ECPs, this therapeutic property of MSCs exosomes is attributed to miR-31-5p / ATF6 Axis [149].

Microanalysis of NPCs in IDD patients shows that miR-4450 levels increase in them. miR-4450 binds to ZNF121 mRNA, reducing its level in NPCs [150]. ZNF121 plays an important role in regulating cell proliferation and apoptosis in breast cancer development [151]. Human placenta mesenchymal stromal/stem cells (hPLMSC) derived Exosomes transfer antagomiR-4450 and inhibited miR-4450 function result in increased ZNF121 levels in TNF-α-treated NPCs [150]. Thereby hPLMSC exosomes decrease apoptosis and inflammation and increase cell migration and proliferation in vivo and in vitro in the NPCs.

In addition to NPCs and CEPs, the effect of MSCs exosomes on AF cells has also been investigated. In a study by Zhong-qi Li, AF cells were treated with IL-1B to mimic the inflammatory microenvironment of IDD. This study shows that BM-MSC exosomes inhibit inflammation and apoptosis in AF cells [152]. Since the effect of exosomes on these cells is inhibited by rapamycin, it is proven that this action is performed by regulating the PI3K/AKT/mTOR signaling pathway [152].

Oxidative stress plays a significant role in the apoptosis of IVD cells and IDD progression [153]. In a study by Xia et al. [154], H2O2 has been used to induce a laboratory oxidative stress model in NPCs. Examination of ROS levels in the H2O2 treated NPCs indicates an increase in them. This intracellular increase in ROS leads to increased expression of MMP3 and MMP13 and leads to ECM degradation. The expression of cellular stress-related proteins such as NLRP3 and TXNIP also increases in these cells. In this study, MSCs exosomes were used to evaluate the therapeutic effects and improve the function of H2O2 treated NPCs. This study shows that the levels of ROS, caspase 3, caspase 9, NLRP3, TXNIP, MMP3, and MMP13 in NPCs treated with BM-MSCs exosomes are significantly reduced. Therefore, it can be concluded that the use of BM-MSCs exosomes helps to improve IDD by inhibiting inflammasome-mediated inflammation, apoptosis, ECM degradation, and ultimately inhibiting oxidative stress in NPCs [154]. In vivo studies on the rabbit model of IDD confirm the in vitro study results [154] (Fig. 4).

7. Conclusion

With modern lifestyles, IVD related problems rise and become a public health concern. IVD degeneration is associated with low back pain, and there is currently no definitive cure for the disease, and current treatments focus on pain relief. In addition, treatments such as surgery have many side effects. None of the current therapies for IDD has focused on regenerating this tissue, while regenerative therapies can significantly improve disease conditions. MSCs are one of the candidates for use in IDD due to their high differentiative and immunomodulatory potential. In addition, these cells produce exosomes, and their application in various tissues regeneration showed favourable therapeutic properties. These cells and their exosomes increase proliferation, synthesize ECM-related substances, and reduce apoptosis, ECM degradation, and cell senescence in NPCs. Also, they reduce the inflammatory environment, increase the migration of repair-involved cells to the injury site, and increase the differentiation of MSCs into NPCs. All of these mechanisms play an important role in the regeneration of IVD tissue. The dose dependence of MSCs and exosomes are complex problems in clinical applications. At this stage, the main route of administration is the intravenous injection, and most pre-clinical studies and

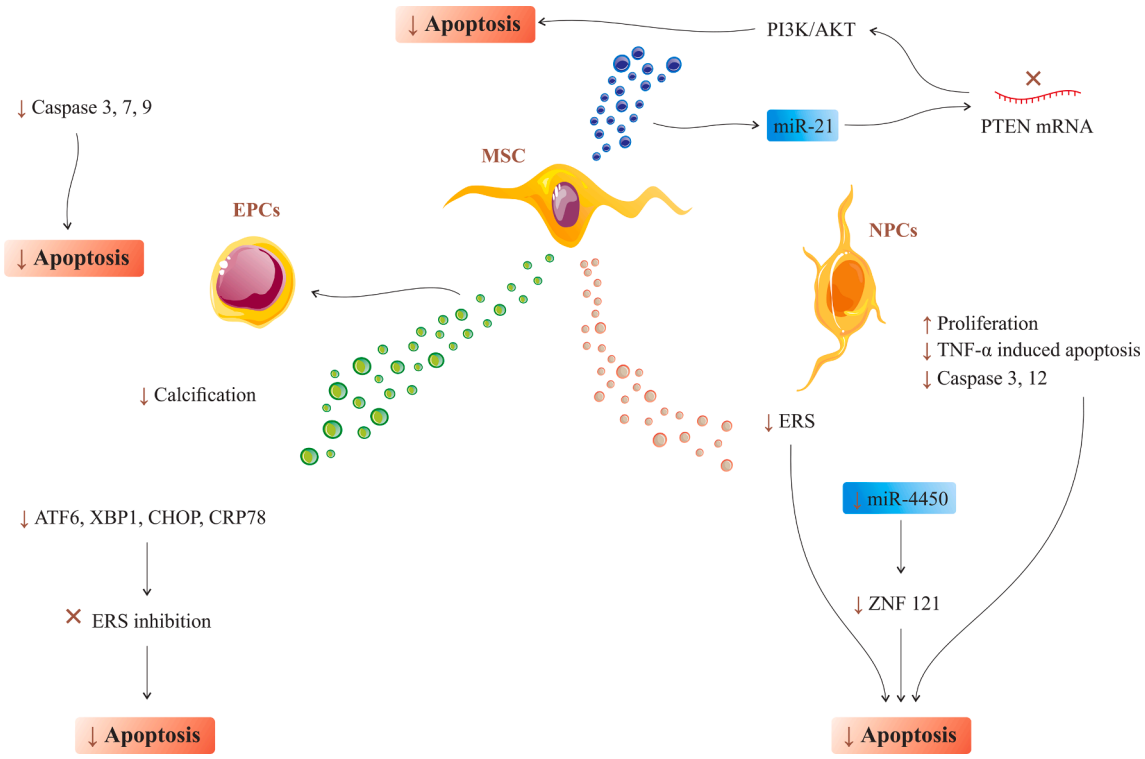

Fig. 4. Mesenchymal stromal/stem cells derived Exosomes perform many immune-modulating functions and prevent apoptosis and cell ageing. These vesicles can also increase the proliferation and efficiency of NPCs. Since NPCs play an essential role in preserving disc tissue, improving their performance is particularly important.

clinical trials have used different doses [155]. However, the biological role of exosomes is not yet fully understood, and we still need to pay close attention to their side effects. MSC-derived exosomes certainly play an immunomodulatory role, and we are confident that the research of MSC-derived exosomes will make a significant breakthrough in the future. For clinical safety, the production of MSCs for therapeutic purposes must comply with good manufacturing practices (GMPs) to ensure the provision of safe, repeatable and efficient products. Some factors should be considered during the production process to produce safe and reliable MSCs sources for clinical application, including the following. (1) Identical tissue source for mesenchymal stromal/stem cell isolation. (2) Donor age and age of MSCs. (3) Donor-to-donor diversity and previous pathological conditions. (4) Allogeneic source versus autologous sources. (5) The same MSC separation steps. (6) Heterogeneity in MSC culture [156]. These MSCs produced under GMPs processes can be used for EVs production. For this purpose, EVs, including exosomes, should be standardized for clinical use. Different exosome isolation methods are the criteria that can affect the results [157]. MSC-EVs' potential criteria include the ratio of MSC to non-MSC surface antigens, the percentage of specific lipids, the ratio of membrane lipids to proteins, vesicle integrity and biological activity and the concentration of membrane lipid vesicles [156]. The next step will be to determine the quantity and validation of each criterion, which requires further study and research. The results of many in vivo and in vitro studies have confirmed the therapeutic and supportive role of MSCs and their exosomes in IDD treatment. Therefore, it is believed that in the future, with the expansion of stem cell therapeutic application and overcoming cell therapy deficiencies, the use of MSCs will become one of the main options in the treatment of degenerative diseases such as IDD.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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None.

Authors' contributions

Gunawan Widjaja and Abduladheem Turki Jalil wrote the manuscript, Rauza Sukma Rita and Andri Praja Satria designed the review, Surendar Aravindhan and Marwan Mahmood Saleh edited the manuscript, Mohammed Nader Shalaby and Alexei Valerievich Yumashev In reviewing studies and data searching, Walid Kamal Abdelbasset and Wanich Suksatan draw the schematic figure and tables, Hendrik Setia Budi and Syahril Efendi designed and supervised the study, and correspondence during the paper submission.

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