



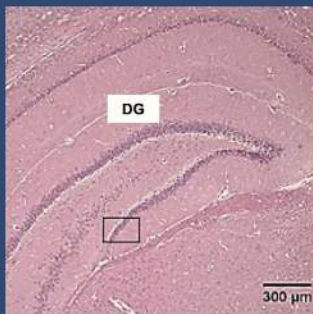
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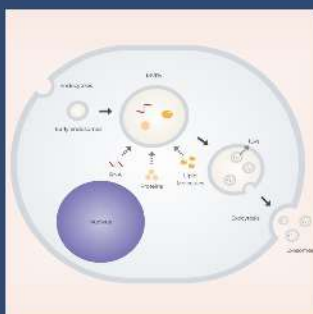
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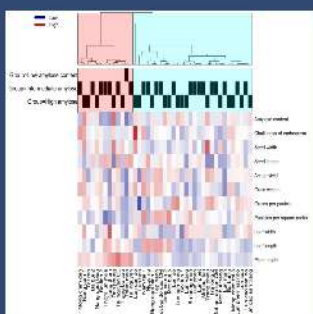
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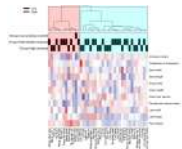
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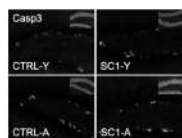
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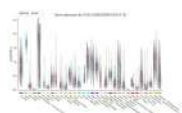
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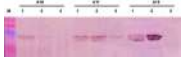
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The potential of mesenchymal stem-cell secretome for regeneration of intervertebral disc: A review article

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ABSTRACT Low back pain is a crucial public health problem that is commonly associated with intervertebral disc degeneration and has vast socio-economic impact worldwide. Current treatments for disc degeneration are conservative, non-surgical, or surgical interventions, and there is no current clinical therapy aimed at directly reversing the degeneration. Given the limited capacity of intervertebral disc (IVD) cells to self-repair, treatment aiming to regenerate IVDs is a topic of interest and mesenchymal stem cells (MSCs) have been identified as having potential in this regeneration. Recent studies have revealed that the benefits of MSC therapy could result from the molecules the cells secrete and that play principal roles in regulating essential biologic processes, rather than from the implanted cells themselves. Therefore, the objective of this study is to review the potential use of the MSC secretome to regenerate IVDs. Current evidence shows that the secretome may regenerate IVDs by modulating the gene expressions of nucleus pulposus cells (upregulation of keratin 19 and downregulation of matrix metalloproteinase 12 and matrix Gla protein) and stimulating IVD progenitor cells to repair the degenerated disc.

KEYWORDS Intervertebral disc degeneration; low back pain; mesenchymal stem cells; regenerative properties; secretome

1. Introduction

Low back pain (LBP) is a crucial public health problem (Driscoll et al. 2014). It has an enormous socio-economic impact worldwide and reduces the patient's quality of life remarkably (Katz 2006; Husky et al. 2018). About 577 million people are affected by LBP, and its cost exceeds 100 billion dollars per year in the United States alone (Katz 2006; Wu et al. 2020). Globally, LBP is the leading cause of disability (Wu et al. 2020).

Low back pain is commonly associated with intervertebral disc degeneration (IDD) (Clouet et al. 2019). Intervertebral disc degeneration may be present in more than 90% of people, but many of them have no signs of the disease (Cheung et al. 2009). Degeneration of the disc often

begins in the second decade of life, earlier than other connective tissues in the human body, and is viewed as one of the inevitable consequences of aging (Siemionow et al. 2011; Kepler et al. 2013). As degeneration occurs, this disturbs the disc's ability to perform its mechanical functions (Roberts et al. 2006). At the cellular level, degeneration of the disc is characterized by increased degradative enzyme production, increased apoptosis, increased inflammatory cytokines expression, decreased extracellular matrix production, and neurovascular ingrowth (Kepler et al. 2013).

Intervertebral disc (IVD) has a limited ability to repair itself following injury and degeneration (Vadalà et al. 2016). Current treatments for disc degeneration are con-

servative treatments, non-surgical, or surgical interventions aimed for muscular stabilization and symptomatic relief with no clinical therapy targeting to reverse the degenerated disc itself (Wei et al. 2014). However, the clinical results of surgical interventions such as spinal fusion and total disc replacement remain suboptimal (Van Den Eerenbeemt et al. 2010).

Considering the limited capability of the IVD to repair itself, clinical therapy aimed at the regeneration of intervertebral disc has become an appealing research topic. Disc degeneration results in changes in the biochemical microenvironment in IVD that challenge the successful application of some potential biological therapies, such as decreased nutrient and oxygen supplies, declined pH, and increased cellular apoptosis (Kepler et al. 2013; Loibl et al. 2019). One of those potential treatments is using mesenchymal stem cells (MSCs) for IVD regeneration. Some studies conducted in animal models have shown its potential regenerative effect in IVD degeneration (Bach et al. 2014; Freeman et al. 2016; Vadalà et al. 2016). However, recent studies have revealed that MSCs therapy benefit could be due to their vast secreted molecules, which play a principal role in regulating many essential biologic processes, rather than from the implanted cells themselves (Vizoso et al. 2017). Therefore, this study aims to review the potential use of MSCs secretome to regenerate the intervertebral disc.

2. Pathophysiology of IDD

The intervertebral disc is a cartilaginous structure located between the vertebral body of the spinal column (Urban and Roberts 2003). They provide flexibility for motion and act as a shock absorber. The IVD is composed of three parts; the nucleus pulposus (NP) located centrally, the annulus fibrosus (AF) that surrounded the NP, and the cartilaginous end plates (EP) that separates IVD from the adjacent vertebral bodies (Kepler et al. 2013).

The NP is an avascular and immune-privileged structure that consisted of three different cell types: NP stem/progenitor cells (NPPCs), notochordal cells (NCs), and chondrocyte-like cells (NP) (Hunter et al. 2003; Erwin 2010; Erwin and Hood 2014). The NP's extracellular matrix (ECM) is mainly composed of type II collagen and has a considerably higher proteoglycan concentration than AF (Kepler et al. 2013). Matrix component of the NP consists of proteoglycan aggrecan and type II collagen in a ratio of approximately 27:1 (Mwale et al. 2004). Nucleus pulposus contains a large amount of proteoglycan, up to its 50% dry weight (Walker and Anderson 2004). Since proteoglycans are negatively charged and highly polar, they attract water into the ECM, thus maintain the high-water content in the IVD (Kepler et al. 2013).

The AF consists of concentrically arranged type I collagen fibers that serve as a border containing the inner NP (Kepler et al. 2013). The AF can be divided into the outer annulus and inner annulus (Walker and Anderson 2004). The collagen fibers in the outer annulus are not oriented

uniformly instead, they are aligned at approximately 30° to the longitudinal axis of the spine and alternate their direction with each lamella (Walker and Anderson 2004). This characteristic gives optimal tensile strength to maintain the NP in place during spine movement (Freemont 2009). Initially, there is a transition zone between the NP and the inner annulus, but this distinction disappears as the disc degeneration begins (Roberts et al. 2006).

The cartilaginous end plate separates NP from the vertebral bone and gives resilience to prevent the load transmitted through the IVD from fracturing the vertebral bone (Freemont 2009). The EP is an avascular organ, but there are capillary networks near the central portion of the EP that are directly connected with the vertebral body vasculature (Erwin and Hood 2014).

The IVD is an avascular organ because they lose their blood supply in the first decade of life (Rodriguez et al. 2011). The IVD cells have adapted to function in this condition by relying on diffusion and convection for nutrient and metabolite exchange (Mokhbi Soukane et al. 2007). Nutrients and metabolites are transported to and from the IVD by diffusion from the blood vessels at the outer NP peripherally and the cartilaginous EP centrally (Holm et al. 1981). Hence, NP cells congregate near the cartilaginous EP where specialized capillary layers between the bony and cartilaginous EP provide nutritional supply (Urban et al. 1978).

In the second decade of life, the blood supply to the cartilaginous EP is diminished, consequently, their diffusional capacity is decreased (Boos et al. 2002). This condition leads to a change in the microenvironment of the IVD which becomes acidic because of lactic acid's buildup (Kepler et al. 2013). Exposure to this acidic condition has a profound effect on the cell-matrix turnover because it decreases the IVD cells ability to produce ECM (i.e., sulfated glycosaminoglycan and tissue inhibitors of metalloproteinases-1). However, it does not inhibit degradative enzyme production, such as matrix metalloproteinases (MMPs) (Razaq et al. 2003). These microenvironment changes will lead to ECM breakdown and therefore the IVD degeneration. Other conditions that could compromise the cartilaginous EP's vascular supply are vasoconstriction (e.g., from nicotine or vibration exposure) (Deyo and Bass 1989; Wilder and Pope 1996), vasoocclusive process (e.g., atherosclerosis and arterial stenosis) (Kauppila 2009), and end plate sclerosis (Roberts et al. 1996).

Usually, innervation of the IVD is limited to the outer AF, but during the degeneration process, the nociceptive nerve endings grow deeper into the disc and play a role in pain transmission from the IVD (Bogduk et al. 1988; Freemont et al. 2002). This neuronal ingrowth is induced by nerve growth factor (NGF) secreted by vascular tissue accompanying them (Freemont et al. 2002). Some studies also found that brain-derived neurotrophic factor (BDNF), a substance secreted by the IVD cells, especially during the degenerative process also appears to encourage neuronal ingrowth (Gruber et al. 2008; Kepler et al. 2013). An-

other possible source of pain in patients with IVD degeneration is the upregulation of proinflammatory cytokines, especially tumor necrosis factor alpha (TNF- α) (Takahashi et al. 1996; Bachmeier et al. 2007).

Another molecular basis of IVD degeneration is cellular senescence (Kepler et al. 2013). Cellular senescence is an irreversible and progressive loss of replicative capability of the cells (Hayflick and Moorhead 1961). Based on the underlying cause, cell senescence is divided into two groups, stress-induced premature senescence (SIPS) and replicative senescence (Kepler et al. 2013). Replicative senescence is caused by the loss of telomeres, the tip of the chromosome which serves as protection from genomic instability (Vitorelli and Passos 2017). The telomeres shorten in each replication, when they run out, it will lead to permanent cell cycle arrest (Vitorelli and Passos 2017). The cellular mechanism underlying the pathophysiology of SIPS is the accumulation of unreparable DNA damage caused by reactive oxygen species (ROS) from mechanical injury or inflammatory cytokines release (Toussaint et al. 2000). Although senescence is a physiological process, studies found that this process is accelerated during disc degeneration (Le Maitre et al. 2007; Kim et al. 2009).

3. Mesenchymal Stem Cells (MSCs)

Stem cells are cells with the ability to renew themselves and differentiate into various specialized cell types (Wei et al. 2013). Stem cells can be categorized into embryonic stem cells (ESCs), adult stem cells, and induced pluripotent stem cells (iPSCs) (Ullah et al. 2015). ESCs are pluripotent stem cells that have distinctive self-renewal ability, genomic stability, can differentiate to most lineages, and hold promise for regenerative medicine (Ullah et al. 2015). However, their use is restricted for ethical reasons and tissue rejection problems following transplantation in patients (Takahashi and Yamanaka 2006). iPSCs are made from adult cells by introducing four transcription factors, c-Myc (avian myelocytomatosis virus oncogene cellular homologue), Oct3/4 (octamer-binding transcription factor 3/4), Klf4 (kruppel-like factor 4), and Sox2 (sex-determining region Y) (Takahashi and Yamanaka 2006; Ullah et al. 2015). iPSCs share many properties with ESCs, but their genomic stability is still questionable (Ullah et al. 2015). Due to the limitation of ESCs and iPSCs, great attention has come to MSCs, which are free from both ethical reasons and genomic stability problems (Wei et al. 2013).

MSCs are adult stem cell which possesses the ability to differentiate into connective tissue cells' lineages including bone, IVD, ligament, muscle, and fat (Richardson et al. 2010). In accordance with the International Society for Cellular Therapy, MSCs can be identified using three criteria: they must adhere to the plastic surface; express CD73, CD 90, and CD105 and does not express CD14, CD34, CD45, or CD11b, CD79 α or CD19 and HLA Class II; and finally, they must be able to dif-

ferentiate into osteoblasts, chondroblasts, and adipocytes (Dominici et al. 2006). However, these criteria still produce a relatively heterogeneous progenitor cell population (Loibl et al. 2019). Therefore, many researchers are trying to solve this problem by pre-selecting particular MSCs populations; for example, CD271-MSCs have a higher potential to differentiate into nucleus pulposus than their CD271+ counterparts (Jeziarska-Wozniak et al. 2017). Furthermore, another study found that MSCs subpopulations expressing CD146 or CD271 markers performed better in repairing cartilage (Pérez-Silos et al. 2016).

Mesenchymal stem cells exist in almost all tissues, but the most common source tissues for human MSCs are bone marrow and the adipose tissue because these tissues are considered renewable (bone marrow) or undesirable (adipose tissue) (Pittenger et al. 2019). MSCs also have been harvested from other sources, such as synovial fluid (Morito et al. 2008), articular cartilage (Alsalamah et al. 2004), brain (Appaix 2014), dental tissue (Huang et al. 2009), peripheral blood (Ab Kadir et al. 2012), skin and foreskin (Riekstina et al. 2008), menstrual blood (Allickson 2011), endometrium (Schüring et al. 2011), placenta and fetal membrane (Raynaud et al. 2012), amniotic fluid (In 't Anker et al. 2003), amniotic membrane (Cai et al. 2010), Wharton's jelly (Hou et al. 2009), and umbilical cord tissue (Wagner et al. 2005).

Mesenchymal stem cells therapy for tissue repair depends not only on the ability of MSCs to differentiate into specific cell types but also on their immunomodulatory and trophic effects (Wei et al. 2014). MSCs' therapeutic effect in IVD degeneration can occur in various ways. First, interactions between nucleus pulposus cells (NPCs) and implanted MSCs induce differentiation of MSCs towards a more chondrogenic change (Vadalà et al. 2008). Second, MSCs pose a trophic effect by secreting various growth factors and cytokines that promote angiogenesis, stimulate differentiation and proliferation of progenitor host cells, and inhibit fibrosis formation (Caplan and Dennis 2006). This trophic effect also has been reported by another study showing direct cell-to-cell contact between MSCs and NPCs increases NPCs viability in a co-culture system (Yamamoto et al. 2004). Third, MSCs have immunomodulatory effects, they can exhibit pro-inflammatory or anti-inflammatory phenotype depending on the balance between the cytokines released into the surrounding microenvironment (Keating 2012). MSCs have been shown to be indirectly having stimulatory and inhibitory effects on B-cell differentiation, proliferation, and antibody production (Fierabracci et al. 2015). These effects seem to be mediated by other cell types and depend on the inflammatory environment (Comoli et al. 2008).

MSCs based therapy is generally safe (Comella et al. 2017) and also appears to be able to avoid allogeneic rejection due to their lack of MHC-II, CD 40, CD86, and CD80 expression on their cell surface, thus they can escape from T-cell recognition (Ryan et al. 2005). However, the complication arising from the implantation procedure may also occur such as osteocyte formation due to MSCs migra-

tion (Vadalà et al. 2012). Another challenge is determining which patients will benefit from the disc regeneration, as patients seek medical help for pain, not for degeneration (Bendtsen et al. 2016).

For MSCs to effectively exert their proposed regeneration effect, they must withstand the IVD microenvironment. MSCs depend on glycolysis for their energy source, and if glucose is removed, they will die rapidly (Moya et al. 2017). Furthermore, an acidic condition also has a detrimental effect on cellular activity and viability of the disc cells and MSCs (Wuertz et al. 2009). The IVD has a harsh microenvironment even in its healthy state due to low oxygen and nutritional supply, hyperosmolarity, and high mechanical loading which pose a challenge in clinical trials (Wuertz et al. 2009; Loibl et al. 2019). These conditions become heavier in the degeneration process because of inflammation and increased acidity (Kepler et al. 2013).

Due to the poor nutrient supply within the IVD, ECM proteins production by MSCs or the stimulation of ECM synthesis by the host cells is likely restricted and may not be sufficient to promote full IVD regeneration (Loibl et al. 2019). It has been reported that MSCs could survive and able to differentiate after administration to the IVD (Sakai et al. 2005; Henriksson et al. 2009), but the problem lies in the nutrient supply (Loibl et al. 2019). Because of the IVD's avascular nature, they can only support limited cell numbers (Smith et al. 2011). An additional number of cells from the MSCs will interfere with the nutritional balance in the IVD because increased cell number results in increased nutrient demand (Loibl et al. 2019). Furthermore, because of the diminished nutrient supply, the implanted MSCs will eventually die (Loibl et al. 2019). Another problem arises from the slow capacity of the MSCs to produce ECM. A study found that MSCs' ability to produce glycosaminoglycans (GAG), a component responsible for maintaining disc hydration and height, ranges from 0.017 to 0.086 mg GAG/million cells/month (Allon et al. 2010; McCorry et al. 2016). As GAG's concentration in the normal disc nucleus is about 70 mg/mL, it would take decades to restore 25% of disc tissues through implanting stimulated MSCs (Bendtsen et al. 2016; Loibl et al. 2019).

The problem with nutritional supply that resulted in cell death suggests that the principal effects of MSCs may be mediated by paracrine mechanisms (Maguire 2013). In contrast to the original paradigm that the MSCs' mechanism of action was based on their capability to replace cells, recent studies have shown that MSCs' secreted molecules are responsible for their therapeutic effects (Madrigal et al. 2014). Therefore, MSCs secretome has gained much interest for its potential use in regeneration and tissue repair (Baglio et al. 2012; Maguire 2013).

4. The Secretome of MSCs

The secretome is a set of molecules released by the stem cells, including cytokines, chemokines, anti-inflammatory factors, growth factors, and even proteins delivered by extracellular vesicles (EVs) (Maguire 2013; Eleuteri and

Fierabracci 2019). The latter can be classified according to their origin, size, density, and surface marker into exosomes, microparticles, and apoptotic bodies (Beer et al. 2017). The composition of the secretome can vary depending on the change in its microenvironment (Vizoso et al. 2017). MSCs secretome has multiple mechanisms of action such as immunomodulation and anti-inflammatory activity (Kyurkchiev 2014), neurotrophic and neuroprotective effects (Caseiro et al. 2016), anti-apoptotic activity (Li et al. 2015a), angiogenesis regulation (Kagiyada et al. 2008), and regenerative capacity (Osugi et al. 2012; Di et al. 2017). Table 1 lists some of the molecules secreted by the MSCs and their functions.

MSCs secretome provides some advantages over cell-based therapy in the regenerative medicine field: (1) secretome resolves safety and risk problem related to MSCs implantation such as immune compatibility (Herberts et al. 2011), migration of the cells outside the implantation site which can result in osteophyte (Vadalà et al. 2012) or emboli formation (Tatsumi et al. 2013), and tumorigenicity (Herberts et al. 2011); (2) secretome can be evaluated for dosage, safety and potency like other conventional pharmaceutical agents (Vizoso et al. 2017); (3) The use of MSCs-cultured conditioned media (CM) can reduce several problems that are encountered in clinical applications of stem cells, such as safety, time, and expense (Osugi et al. 2012), therefore increase the possibility of mass-production; (4) and lastly, the biologic products in the secretome can be modified to desired specific effects (Vizoso et al. 2017).

5. MSCs Secretome Mechanisms of Actions

5.1. Immunomodulation and anti-inflammatory activity

MSCs have been reported to have an immunomodulatory property and anti-inflammatory effect on both innate and adaptive immune systems through various mechanisms, notably via cytokine and chemokine secretions (Abumaree et al. 2012). Transforming growth factor beta (TGF- β) is a cytokine produced and constitutively secreted by the MSCs (Kyurkchiev 2014). TGF- β has an essential role for the immunomodulatory property of the MSCs, including inhibition of effector T-cell function and proliferation; attenuation cytokine production and cytolytic activity of natural killer (NK) cells; conversion of naive T cells into T-reg; and suppression of dendritic cells (DCs), B cells, and macrophages (Yoshimura and Muto 2011). Besides TGF- β , MSCs also secrete Galectin-1 (Gal-1) constitutively and Galectin-9 (Gal-9) when induced by pro-inflammatory stimuli (e.g. IL-1 β and IFN- γ) (Gieseke et al. 2013). Both Gal-1 and Gal-9 share immunomodulatory property via inhibition of Th1 and Th17 cells proliferation, but Gal-9 is more potent to induce T cells death (Gieseke et al. 2010, 2013). Prostaglandin E2 (PGE2) is another main effector for the anti-inflammatory effect of MSCs, with its cellular target mainly are monocytes, macrophages, pe-

ipheral blood mononuclear cells (PBMCs), NK cells, and transitional processes of monocyte differentiation into mature DCs (Van Elssen et al. 2011). PGE2 exerts its anti-inflammatory effect by reducing IL-6, TNF- α , and vascular permeability in mice models of sepsis (Németh et al. 2009).

5.2. Neuroprotective and neurotrophic effects

Numerous studies have reported neuroprotective and neurotrophic effects of MSCs secretome (Eleuteri and Fierabracci 2019). A group of growth factors such as brain-derived neurotrophic factor (BDNF), nerve growth

factor (NGF), basic fibroblast growth factor (bFGF), ciliary neurotrophic factor (CNTF), erythropoietin (EPO), neurotrophin- (NT-) 3, and NT-4/5 have been shown to have neuroprotection and neuroregeneration effects on the central nervous system (CNS) (Salgado et al. 2010). Whereas neurotrophic factors can be classified according to their receptors into three groups: neurotrophins (BDNF, NGF, NT-3, and NT-4/5), neurokinins (CNTF and leukemia inhibitory factor (LIF)); and the transforming growth factor β family (TGF- β 1, TGF- β 2, TGF- β 3; and glial cell-derived neurotrophic factor (GDNF)) (Fornaro et al. 2020). Studies found that some of these growth factors are also secreted by MSCs (e.g., NGF, bFGF, GDNF, NT-3, and NT-4), hence MSCs secretome also possesses neurotrophic and neuroprotective effects (Balasubramanian et al. 2013; Caseiro et al. 2016). Moreover, these growth factors are not only secreted in their soluble forms but also carried by the EVs (Caseiro et al. 2016).

5.3. Anti-apoptotic activity

MSCs prevent cell death by secreting molecules such as monocyte chemoattractant protein-1 (CCL2/MCP-1) and hepatocyte growth factor-1 (HGF-1) which have anti-apoptotic activity (Kyurkchiev 2014; Madrigal et al. 2014). However, CCL2/MCP-1 is also reported to have pro-apoptotic activity, and the balance between these two effects depends on the microenvironment and cytokine profile (Rafei et al. 2009). A study also reported that MSCs increase the anti-apoptotic Bcl-2 levels and decrease the pro-apoptotic Bax levels in rat models of myocardial infarction treated with MSCs (Yao et al. 2005). Furthermore, another study found that MSCs secretome from the normal human uterine cervix (hUCESCs) promoted apoptosis in cancer cells both in vitro and in vivo (Eiró et al. 2014).

5.4. Angiogenesis regulation

Angiogenesis is the formation of new blood vessels from the existing ones, which is necessary for the wound healing process (Vizoso et al. 2017). Recent studies reported that the beneficial angiogenic effects of MSCs are mediated by their secretome (Maacha et al. 2020). This angiogenic property is attributable to the secretion of growth factors such as vascular endothelial growth factor (VEGF) and HGF-1 (Madrigal et al. 2014). However, a study reported that MSCs also secrete tissue inhibitor of metalloproteinase-1 (TIMP-1) that carries an anti-angiogenic effect (Zanotti et al. 2016). The data indicate that the balance of these pro and anti-angiogenic factors may be modified by hypoxic conditions and chemokine (Vizoso et al. 2017). Moreover, a study indicated that these bioactive molecules are carried by the EVs to perform their angiogenic modulation (Maacha et al. 2020).

5.5. Regenerative capacity

Previously, the regenerative properties of MSCs are attributed to their ability to differentiate into specialized cell types, but recent evidence showed that it is due to their

TABLE 1 The molecules secreted by the MSCs and their functions.

Molecules	Function
TGF- β	T-reg activation, therefore promote systemic immune tolerance Inhibition effector T-cell proliferation and function Reduction of cytolytic activity and cytokine production of NK cells Suppression of DCs, B cells, and macrophages
PGE2	Immunosuppressive factor, its cellular target particularly are NK cells, PBMCs, monocytes, macrophages and transitional processes of monocyte differentiation into mature DCs
VEGF	Stimulates angiogenesis Pro-inflammatory function by recruiting mononuclear cells, upregulate Th1 and Th17 Immunosuppressive function by down-regulate the transcription factor NF- κ B, resulting in an inhibition of DCs maturation
IDO	Immunosuppressive and anti-microbial effects
CCL2/MCP-1	Regulating recruitment and migration of monocyte Inhibition of Th17 Pro-apoptotic or anti-apoptotic activity depends on the microenvironment and cytokine profile
IL-6	Neutrophil apoptosis suppression and induce CD8+FoxP3+ cells' formation
TSG6	Inhibit disease progression and induce corneal regeneration
HGF-1	Angiogenesis, immunomodulation, and anti-apoptotic activity
NGF	Neuroprotective effect
GDNF	Neurotrophic effect
Gal-1	Inhibits proliferation of CD4+ and CD8+ T cells
Gal-9	Induces T cells death

Abbreviations: Transforming growth factor beta (TGF- β), natural killer cells (NK), dendritic cells (DCs), prostaglandin E2 (PGE2), peripheral blood mononuclear cells (PBMCs), vascular endothelial growth factor (VEGF), T helper 1 cells (Th1), T helper 17 cells (Th17), Indoleamine-2,3-dioxygenase (IDO), monocyte chemoattractant protein-1 (CCL2/MCP-1), interleukin-6 (IL-6), tumor necrosis factor-inducible gene 6 protein (TSG6), hepatocyte growth factor-1 (HGF-1), nerve growth factor (NGF), glial cell-derived neurotrophic factor (GDNF), galectin-1 (Gal-1), galectin-9 (Gal-9) (Kim et al. 2009; Rafei et al. 2009; Gieseke et al. 2010; Ben-Ami et al. 2011; Van Elssen et al. 2011; Yoshimura and Muto 2011; Fisher-Shoval et al. 2012; Gieseke et al. 2013; Kyurkchiev 2014; Madrigal et al. 2014; Marti et al. 2014; Di et al. 2017; Fornaro et al. 2020).

secreted byproducts acting at a distance that mediates regenerative outcomes (Basu and Ludlow 2016). A study reported that MSCs secrete tumor necrosis factor-inducible gene 6 protein (TSG6) that promotes corneal epithelial wound healing in diabetic mice models by stimulating mitogenic activity of endogenous corneal progenitor cells and enhancing the colony-forming efficiency (Di et al. 2017). This regenerative property was also demonstrated in a study conducted by Osugi et al. that used conditioned media to accumulate paracrine factors of the MSCs to regenerate bone defect in rat models (Osugi et al. 2012). They showed that MSCs-conditioned media (MSCs-CM) could stimulate the migration of endogenous MSCs and accelerate new bone formation (Osugi et al. 2012). This is caused by a cooperative effect between VEGF and insulin-like growth factor-1 (IGF-1) that promotes angiogenesis and osteogenesis (Osugi et al. 2012). Hingert et al. (2020) have reported that conditioned media from human MSCs (hMSCs) has the potential to regenerate the IVD by increasing disc cell viability and ECM production. These effects are probably caused by the presence of growth factors in the CM (Hingert et al. 2020). Apart from being an immunomodulator, TGF- β may also have a role in the regenerative capacity of MSCs. In a study conducted by Matta et al. (2017), they reported that TGF- β 1 was able to promote cell proliferation, increase healthy ECM protein synthesis, and decrease cell apoptosis in NP cells from degenerated human disc, therefore promote its regeneration.

6. Extracellular Vesicles (EVs) of MSCs

Besides growth factors and cytokines, most of the MSCs also secrete a large amount of micro and nanovesicles as components of their secretome, either constitutively or upon activation signals (Baglio et al. 2012). The biological properties of these vesicles have not been understood completely, but their potential as mediators for cell communication has gained much attention, especially for exosomes (Vizoso et al. 2017). The exosomes are a subclass of extracellular nanovesicles with a diameter of 40-150 nm and a density of 1.09–1.18 g/mL that are derived from specialized intracellular compartments known as multi-vesicular bodies (MVBs) or late endosomes (Baglio et al. 2012; Vizoso et al. 2017) (Figure 1).

They are able to transfer proteins, lipid molecules, and functional genetic material such as microRNAs (miRNAs) and messenger RNA (mRNAs) to the other cells (Valadi et al. 2007). The presence of RNA in the exosomes opens up its potential use as drug and gene carrier in the field of regenerative medicine and tissue engineering (Lamichhane et al. 2015). A study showed that there is a control mechanism for the sorting of miRNAs into the vesicles (Collino et al. 2010). However, this loading mechanism of miRNAs into exosomes may be modified selectively by engineering an extra-seed sequence (hEXO motif) (Santangelo et al. 2016). Therefore, by selectively modifying exosomes cargo, miRNAs may be specifically transported into the target cells which lack these miRNAs for special-

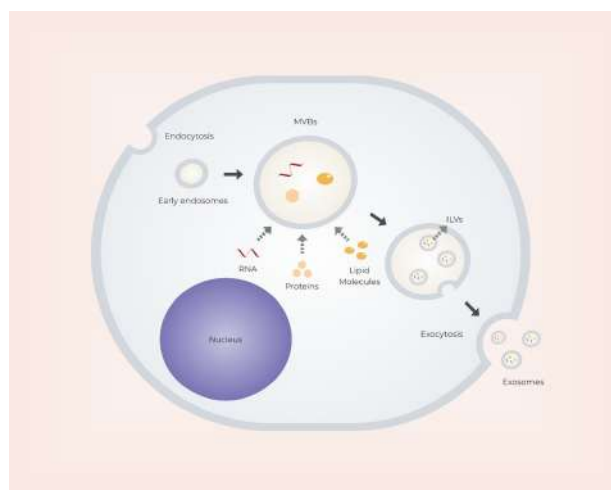


FIGURE 1 Schematic view of exosomes formation. The exosome formation begins with inward budding of the MVBs' membrane to create intraluminal vesicles (ILVs). The ILVs can contain molecules such as proteins, lipids, and RNA. It is then released from the cells and then taken up by cell targets to perform their action (adapted from (Baglio et al. 2012).

ized function (Rader and Parmacek 2012).

Recent evidence found that regenerative properties previously credited for stem cells are actually mediated by their secreted exosomes (Basu and Ludlow 2016). Some pre-clinical studies have been done to demonstrate these effects, such as a study conducted by Nakamura et al., which demonstrated that MSCs-derived exosomes, primarily due to their miRNA content promote muscle regeneration by enhancing angiogenesis and myogenesis process in a cardiotoxin muscle injury model (Nakamura et al. 2015). Another study conducted by Zhang et al. also demonstrates that MSCs exosomes improve functional recovery of rats with traumatic brain injury (TBI) model by promoting neurogenesis, angiogenesis, and reducing neuroinflammation (Zhang et al. 2015). MSCs-derived EVs administration showed similar effects as MSCs in the treatment of focal brain ischemia in C57BL6 mice, improving neurological impairment, neuroregeneration, and inducing long-term neuroprotection (Doepfner et al. 2015).

Even with all these beneficial and protective effects of MSCs-EVs, it is mandatory to be cautious when using engineered MSCs-EVs in clinical therapy. A study conducted by Zhu et al. showed that exosome from bone marrow MSCs promoted angiogenesis and tumor growth in a mouse xenograft model of gastric carcinoma, this effect may be mediated by increasing tumor cells VEGF expression through activation of extracellular signal-regulated kinase1/2 (ERK1/2) pathway (Zhu et al. 2012). This finding is not entirely unexpected because MSCs have been reported to contribute to tumor growth (Roorda et al. 2009).

Other challenges that emerge are how to avoid artifacts and ensure the reproducibility of studies since there are no available methods to ensure absolute purification and characterization of the EVs (Eleuteri and Fierabracci 2019). Minimal Information for Studies of Extracellular Vesicles

(MISEV) guidelines has summarized recommendation of how to characterize the EVs properly that depends on the presence of at least one protein of these three categories (Théry et al. 2018): (1) GPI-anchored or transmembrane proteins associated with the endosome and/or plasma membrane, e.g., MHC class I, tetraspanins, or integrins; (2) cytosolic proteins, e.g., caveolins (CAV*), flotillins-1 and 2 (FLOT1/2), or heat shock proteins HSC70 (HSPA8); and (3) non-EVs co-isolated proteins, e.g., lipoprotein, albumin (ALB), or Tamm-Horsfall protein.

Furthermore, other two categories recommended to be analyzed for studies that focused on one or more EVs subtype: (1) proteins associated with intracellular compartments other than endosomes and plasma membrane, e.g., histones (HIST1H**), or cytochrome C (CYC1); and (2) proteins that can bind to specific EVs surface receptors, e.g., collagen (COL**), or fibronectin (FN1).

7. Modification of MSCs secretome

The MSCs' secretome contents appear to match the IVD tissue requirements (Wangler et al. 2021). Wangler et al. (2021) have demonstrated that by exposing MSCs with healthy IVD, MSCs respond with releasing secretome that induces immunomodulation; and when exposed to traumatic and degenerative IVD, MSCs respond with releasing secretome that stabilizes ECM turnover. Recent evidence suggests that pre-conditioning MSCs could affect their secretory profile, hence improve the therapeutic effects of their secretome (Vizoso et al. 2017). These in vitro pre-conditioning included hypoxia (Ejtehadifar et al. 2015), pro-inflammatory stimuli (Croitoru-Lamoury et al. 2011), tri-dimensional culture (Bartosh et al. 2010), and pharmacological compounds (Ferreira et al. 2018).

7.1. Hypoxia

In the context of cell culture, hypoxia refers to oxygen tension of less than 10% (Das et al. 2010). Generally, hypoxic pre-conditioning of MSCs increases the cytoprotective and regenerative effects of MSCs (Ferreira et al. 2018). Effects of hypoxia are mediated by hypoxia-inducible factors (HIF-1 α), which induce the expression of angiogenic factors such as VEGF and interleukin-6 (IL-6) (Ejtehadifar et al. 2015). Since neovascularization is the first step in the regenerative process of damaged tissues, this may result in a better therapeutic effect of pre-conditioned-MSCs with hypoxia (Ferreira et al. 2018). Furthermore, MSCs' proliferation rate and viability are increased under hypoxic conditions (Ejtehadifar et al. 2015).

7.2. Pro-inflammatory stimuli

Exposure to the pro-inflammatory stimuli, particularly IFN- γ induces MSCs to release indoleamine 2,3 dioxygenase (IDO) enzyme, which has an immunosuppressive effect (Croitoru-Lamoury et al. 2011; Kyurkchiev 2014). IDO exerts this effect by decreasing tryptophan and/or the accumulation of kynurenine, which then decreases cytotoxic T cells activity (Soliman et al. 2010). Other

cytokines (e.g. IL-1, TNF- α , IFN- β , and IFN- γ), and lipopolysaccharides are also able to induce production of IDO enzyme, although to a lesser degree (Croitoru-Lamoury et al. 2011). Another study also reported that pre-conditioning of MSCs with TNF- α enhances proliferation, osteogenic differentiation, and mobilization of MSCs through upregulation of bone morphogenetic protein-2 (BMP-2) (Lu et al. 2013). Moreover, toll-like receptors (TLR) 2/6, receptors of the innate immune response, are also reported to be able to stimulate MSCs' angiogenic activity (Grote et al. 2013).

7.3. Tri-dimensional (3D) culture configuration

Typically, MSCs are cultured in vitro in monolayered systems however a new approach via tri-dimensional configuration such as spheroid culture has been reported to stimulate a higher level of trophic factors secretion than with monolayer culture (Madrigal et al. 2014). It is worth noting that cells located at the center of spheroid configuration will be exposed to a hypoxic condition hence increasing their viability and proliferation rate as mentioned above. Conditioned media from MSCs spheroids inhibit the production of IL6, IL23, IL12p40, CXCL2, and TNF- α from LPS-stimulated macrophages and encourage higher production of prostaglandin E2 (PGE2) (Vizoso et al. 2017). A study conducted by Bartosh et al. showed that hMSCs cultured in a spheroid culture expressed and secreted higher levels of anti-inflammatory molecule TSG-6 compared with hMSCs cultured in a monolayered structure (Bartosh et al. 2010). Moreover, when they administered hMSCs to the zymosan-induced peritonitis mouse model, they showed that spheroid hMSCs culture have more effective anti-inflammatory effects than monolayered hMSCs culture (Bartosh et al. 2010).

7.4. Pharmacological compounds

In some specific cases, pre-conditioning MSCs with pharmacological compounds may be considered as an alternative approach (Ferreira et al. 2018). A study reported that MSCs pre-conditioned with atorvastatin seemed to increase migration of MSCs and improves cardiac performance due to upregulation of CXCR4 expression in rats with acute myocardial infarction models (Li et al. 2015b). In concordance with this finding, another study found that preconditioned-MSCs with oxytocin improved cardiac function in ischemia/reperfusion injury rat models (Kim et al. 2012). Moreover, Liu et al. demonstrated that pre-conditioning MSCs with curcumin resulted in better heart function, smaller infarct size, higher cells retention, decreased myocardial apoptosis, promoted neovascularization, and enhanced VEGF secretion in myocardial ischemia-reperfusion injury (IRI) rat models (Liu et al. 2015).

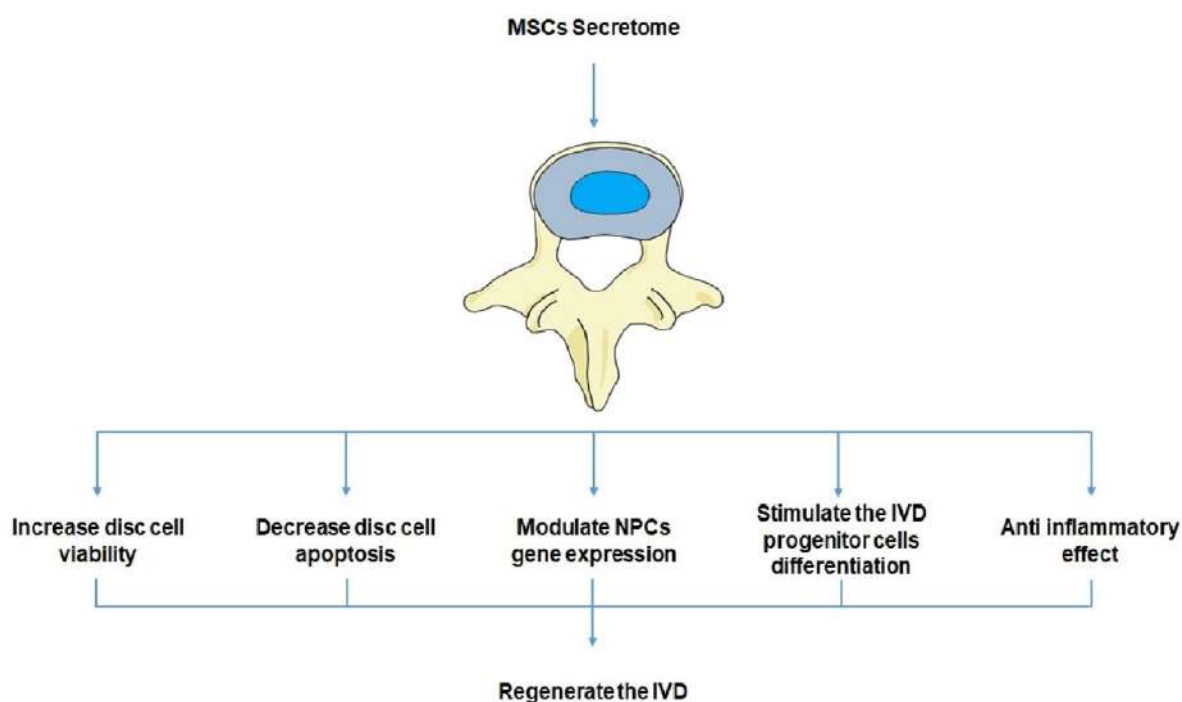


FIGURE 2 Possible mechanisms of MSCs secretome to tackle the IVD degeneration. The degenerated disc is characterized by increased ECM breakdown, neurovascular ingrowth, cellular senescence, and upregulation of proinflammatory cytokines (Freemont et al. 2002; Kepler et al. 2013). The MSCs could tackle these changes via enhancing disc cell viability, decrease disc cell apoptosis, modulate NPCs gene expression, stimulate the IVD progenitor cells differentiation, and give an anti-inflammatory effect (Brisby et al. 2013; Kyurkchiev 2014; Lv et al. 2014; Matta et al. 2017).

8. MSCs Secretome: Pre-clinical Evidence of Potential Use in the IVD Regeneration

In the animal models, MSCs have been demonstrated to be able to regenerate the disc (Bach et al. 2014; Freeman et al. 2016; Vadalà et al. 2016). MSCs and degenerated NP cells mainly communicate through extensive bidirectional membrane components exchange and microvesicles (Strassburg et al. 2012). An experimental study conducted in the bovine proinflammatory/degenerative disc models showed that MSCs have an immunomodulatory paracrine effect via their secretome products (Teixeira et al. 2018). Another study conducted by Lv et al. showed that NP-like cells that were treated with MSCs conditioned media (MSCs-CM) showed upregulation of keratine 19 (KRT19) and downregulation of matrix metalloproteinase 12 (MMP12) and matrix gla protein (MGP) (Lv et al. 2014). Since KRT19, MMP12, and MGP have been associated with IVD degeneration, it is suggested that MSCs-CM could regenerate healthy NP cells (Ferreira et al. 2018). It was further proposed that the IVD progenitor cell populations present in the degenerated IVD may be stimulated by MSCs secretome and take part in repair attempts (Brisby et al. 2013) (Figure 2).

9. Conclusions

The MSCs secretome is better than MSCs in some aspects such as safety, production, storage, product shelf life, and their potential as a readily available biological therapeutic agent. The potential use of MSCs secretome for IVD regeneration is mediated by their ability to modulate NP cells' gene expressions (upregulation of KRT19 and downregulation of MMP12 and MGP) and stimulate the IVD progenitor cells to repair the degenerated disc. This review still lacks evidence from clinical trials regarding the use of MSCs secretome to regenerate the degenerated disc.

Authors' contributions

R devised the study. R, CRSP, DT, DNU, HS, F collected the main literature related to the study. R, DNU, HS, F, FAR, HBN analyzed and interpreted the data. R, CRSP, DT, DNU, HS, F wrote the manuscript. FAR, HBN made the illustration for the manuscript. CRSP, DT provided critical revision of the article. All authors read and approved the final version of the manuscript.

Competing interests

The author declare that they have no competing interest.

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